Comparative proteome analysis of lung tissue from patients with idiopathic pulmonary fibrosis (IPF), non-specific interstitial pneumonia (NSIP) and organ donors☆

Martina Korfeia, Daniel von der Becka, Ingrid Hennekea, Philipp Markarta, g, Clemens Rupperta, Poornima Mahavadia, Bahil Ghanimb, Walter Klepetkob, h, Ludger Finha, g, Silke Meinersc, g, Oliver Holger Krämerd, Werner Seegera, g, Carlo Vancherie, h, Andreas Guenthera, f, g, h, *

aUniversities of Giessen and Marburg Lung Center (UGMLC), D-35392 Giessen, Germany
bDepartment of Thoracic Surgery, Vienna General Hospital, A-1090 Vienna, Austria
cCPC-Comprehensive Pneumology Center Großhadern, D-81377 Munich, Germany
dInstitute of Toxicology, Medical Center of the University Mainz, D-55131 Mainz, Germany
eDepartment of Clinical and Molecular Biomedicine, University of Catania, I-95123 Catania, Italy
fAgaplesion Lung Clinic Waldhof Elgershausen, D-35753 Greifenstein, Germany
gGerman Center for Lung Research, Germany
hEuropean IPF Network and European IPF Registry, Germany

ARTICLE INFO

Article history:
Received 4 February 2013
Accepted 26 April 2013
Available online 6 May 2013

Keywords:
Idiopathic pulmonary fibrosis (IPF)
Non-specific interstitial pneumonia (NSIP)
DIGE technique
Type-II alveolar epithelial cell (AECII)
Oxidative stress
Antioxidant

ABSTRACT

Among the idiopathic interstitial pneumonias (IIP), the two entities IPF and NSIP seem to be clinically related, but NSIP has a better outcome. The proteomic signatures which distinguish NSIP from IPF remain still elusive. We therefore performed comparative proteomic analysis of peripheral lung tissue from patients with sporadic IPF (n = 14) and fibrotic NSIP (fNSIP, n = 8) and organ donors (Controls, n = 10), by using the 2-dimensional DIGE technique and MALDI-TOF-MS. The study revealed that the proteomic profiles of IPF and fNSIP were quite similar. Among the upregulated proteins in IPF and fNSIP were stress-induced genes involved in the ER stress-pathway, whereas downregulated proteins in IPF and fNSIP included antiapoptotic factors and antifibrotic molecules. The comparison fNSIP versus IPF indicated upregulation of subunits of the proteasome activator complex and antioxidant enzymes of the peroxiredoxin family. We conclude, that only few protein expression changes exist between IPF and fNSIP, and that epithelial ER- and oxidative stress play a major role in the pathogenesis of both diseases. In contrast to IPF, intracellular clearance of ROS and misfolded protein carbonyls seem to be enhanced in fNSIP due to enhanced expression of antioxidant acting proteins, and may explain the better outcome and survival in patients with fNSIP.

☆ This is an open-access article distributed under the terms of the Creative Commons Attribution-NonCommercial-No Derivative Works License, which permits non-commercial use, distribution, and reproduction in any medium, provided the original author and source are credited.

* Corresponding author at: Universities of Giessen and Marburg Lung Center (UGMLC), Department of Internal Medicine II, Klinikstrasse 36, 35392 Giessen, Germany. Tel.: +49 641 99 42515; fax: +49 641 99 42508.
E-mail address: Andreas.Guenther@innere.med.uni-giessen.de (A. Guenther).

1874-3919/$ – see front matter © 2013 The Authors. Published by Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.jprot.2013.04.033
1. Introduction

The idiopathic interstitial pneumonias (IIPs) are a heterogeneous group of diffuse parenchymal lung diseases of unknown etiology, which are characterized by distortion of distal lung architecture by variable inflammation and fibrosis [1]. Of the IIPs, idiopathic pulmonary fibrosis (IPF) and nonspecific interstitial pneumonia (NSIP) have provoked most attention and also debate. Although IPF and NSIP can present in a clinically similar manner, it is currently accepted that they are different diseases, especially with respect to prognosis [1,2]. IPF, histologically defined by the usual interstitial pneumonia (UIP) pattern, is a disease in individuals aged 50 to 60 years and occurs somewhat more frequently in men [3–5]. UIP is characterized by dense fibrosis that causes destruction of the alveolar architecture with frequent honeycombing and bronchiolization of the alveoli, scattered fibroblast foci, and patchy lung involvement. At low magnification, the lung characteristically has a heterogeneous appearance with alternating areas of normal parenchyma, fibrosis, and (bronchiolized) honeycomb cysts [1,3,4].

NSIP patients tend to be approximatively 10 years younger than IPF subjects, are more frequently female and may be sub-differentiated into cellular (cNSIP) and fibrotic NSIP (fNSIP) [1,2,5–7]. The histology of NSIP is temporally uniform, without the spatial and temporal heterogeneity of UIP. When NSIP is predominantly cellular, chronic interstitial inflammation involves the alveolar walls. In fNSIP, fibrosis consists of uniform collagen accumulation, resulting in expansion of alveolar septa and peribroncholar interstitium without the patchwork distribution of UIP. Honeycombing and fibroblast foci are rare [2,5].

Regarding prognosis, the average survival time in IPF from time of diagnosis is 2–3 years, due to the quite aggressive course of this disease [1,3,4]. In marked contrast, cNSIP appears as a rather benign disease with slow progression. Patients with cNSIP usually show a good response to steroid treatment, and an only modest limitation in life expectancy [8,9]. The prognosis of patients with fNSIP seems to be between IPF and cNSIP [5]. It has also been suggested that IPF/UIP simply represents a late presentation stage of untreated (or poorly responsive) NSIP [10,11]. With regard to therapy, pirfenidone has been authorized for treatment of mild to moderate IPF only [12], and steroid/immunosuppressive therapy has recently found to be inefficient in this disease [13]. In contrast, steroids/immunosuppressants seem to work in cNSIP, but not in fNSIP [8].

In summary, despite the prognostic and therapeutic implications, it is often clinically difficult to distinguish IPF from fNSIP, and, in absence of a definite UIP pattern in HRCT, surgical lung biopsy is still considered as diagnostic gold standard for both disorders [1–4]. Therefore, there is a great need for surrogates for diagnosis of both fatal diseases.

The molecular pathomechanisms underlying IPF and NSIP are incompletely understood, with most studies being done in the field of IPF. According to these, IPF is thought to be the result of a chronic injury to the alveolar epithelium, with continuous activation of aberrant repair processes and consecutive replacement of alveolar architecture by fibrotic scars and cysts lined by abnormal bronchiolar epithelium [14–17]. In line with this, apoptosis of type-II alveolar epithelial cells (AECII) is a prominent finding in IPF [18–22], but has also been observed in patients with exogen allergic alveolitis (EAA), cNSIP and fNSIP [23,24]. Such observation is supported by the finding of SP-C gene mutations in familial forms of IIP (mostly IPF and NSIP), which have been shown to result in the production of abundant misfolded proproteins, causing proteasome inhibition and endoplasmic reticulum (ER) stress, and finally, death of the AECII [25–27]. Moreover, proapoptotic ER stress in AECII is also a prominent feature in patients with sporadic IPF in the absence of any gene mutation [28]—and does not seem to differ in extent from familial IPF cases [29]. With regard to NSIP, however, data are much more scarce. According to our own preliminary studies, the extent of the proapoptotic ER stress response in AECII may almost be identical in fibrotic NSIP as compared to IPF [30]. However, the precise trigger mechanisms that culminate in ER stress, and AECII cell death are still unknown and molecular signatures and pathways that distinguish (fibrotic) NSIP from UIP remain elusive. To this end, comparative gene expression profiling in patients with UIP and NSIP (both: cNSIP and fNSIP) showed that there are consistent, but only few significant differences between the two types of IIP at the transcriptional level [10,11,31]. However, the lung proteomic patterns of the different types of IIP have yet not received attention. We hypothesized that IPF and fNSIP may exhibit different proteome signatures that may be useful to distinguish both entities, hence allowing development of new, disease-specific biomarkers and detection of molecular pathomechanisms/signatures that underlie the distinct lung phenotypes in IPF and NSIP. We therefore performed a comparative proteome analysis of subpleural lung tissue from patients with sporadic IPF and fNSIP, with explanted donor lungs serving as controls. We employed the 2-Dimensional Difference in Gel Electrophoresis (2D-DIGE) approach, which allows the simultaneous co-separation of multiple, fluorescently labelled
samples on a single 2D-gel, thus representing an effective way to remove gel-to-gel variation, thereby significantly increasing accuracy and reproducibility. The present study reports the separation and comparison of the IPF-, fNSIP- and healthy control lung proteome on one and the same 2D-gel, and reports the validation and cellular localization of specific proteomic signatures in all categories. To our surprise, the proteomic profiles of IPF and fNSIP were quite similar and only differed with regard to subunits of the proteasome activator complex and antioxidant enzymes, and cytokeratin-19.

2. Material and methods

2.1. Human lung tissue

Lung tissue samples were obtained from 14 patients with IPF (UIP pattern; mean age ± SD: 54.29 ± 14.40 years; 4 females, 10 males), 8 patients with fibrotic NSIP (fNSIP; mean age ± SD: 51.25 ± 8.52 years; 5 females, 3 males), and 10 control subjects (organ donors; mean age ± SD: 46.20 ± 18.25 years; 5 females, 5 males). Explanted lungs or lobes were obtained from the Dept. of Thoracic Surgery, Vienna (W. Klepetko). Already at the surgical theatre, peripheral lung tissue samples were snap-frozen or placed in 4% (w/v) paraformaldehyde immediately after explantation. Thereafter, the remaining lung lobes were placed on ice, and shipped (together with the other samples) to our institute immediately. Upon arrival, lung lobes were sectioned under the hood on ice according to a predefined algorithm; and additional lung tissue samples from subpleural and hilar regions were placed in 4% (w/v) PFA or snap-frozen in liquid nitrogen. The latter samples were stored at –80 °C until used.

Retrospectively, the diagnosis of IPF or fNSIP was reviewed and validated by A. Guenther and an expert pathologist (L. Fink) using current American Thoracic Society/European Respiratory Society (ATS/ERS) guidelines [1–4], and patients were included only when current ATS/ERS criteria were met. The study protocol was approved by the Ethics Committee of the Justus-Liebig-University School of Medicine (No. 31/93, 84/93, 29/01). Informed consent was obtained in written form from each subject for the study protocol. Demographic and clinical data (lung function test parameters) on donor subjects or patients receiving transplantation in Vienna are summarized in Table 1 (upper panel).

2.2. Human bronchoalveolar lavage fluid (BALF)

For the proteome analysis, peripheral lung tissue samples from the lower lobe, from the subpleural region of the lung was used. Frozen lung tissue samples (size 1 cm3) from IPF-, fNSIP patients and controls were homogenized in extraction buffer, as described previously [33]. Crude lung extracts were centrifuged at 10,000 × g for 10 min at 4 °C to remove cell debris. The protein concentration in each supernatant lung homogenate was determined according to the BCA protein assay from Perbio Science. Thereafter, equal masses of total protein (0.5 mg) of each individual IPF-, fNSIP- or control homogenate were pooled, respectively.

2.3. Sample preparation for two-dimensional gel analysis/DIGE technique

For the proteome analysis, peripheral lung tissue samples from the lower lobe, from the subpleural region of the lung was used. Frozen lung tissue samples (size 1 cm3) from IPF-, fNSIP patients and controls were homogenized in extraction buffer, as described previously [33]. Crude lung extracts were centrifuged at 10,000 × g for 10 min at 4 °C to remove cell debris. The protein concentration in each supernatant lung homogenate was determined according to the BCA protein assay from Perbio Science. Thereafter, equal masses of total protein (0.5 mg) of each individual IPF-, fNSIP- or control homogenate were pooled, respectively.

For isolectric focusing (IEF), proteins were then precipitated with acetone (80% final concentration). After brief air drying in the hood, precipitated proteins of the IPF-, fNSIP- and control pool were resolubilized using a rehydration buffer containing 7 mol/L urea, 2 mol/L thiourea, 0.5% (v/v) pharmalyte buffer for commercial pH 4–7 and pH 6–11 linear IPG strips (GE Healthcare, Uppsala, Sweden), 4% (w/v) CHAPS and 20 mmol/L Tris. The concentration was adjusted for all pools to approx. 1.5 μg/μL. The resulting protein samples (in a volume of 2 mL) were frozen at −80 °C. The consecutive DIGE-proteome analysis was then undertaken with the help of TOPLAB GmbH, a proteomics company ( Martinsried, Germany). In brief, the protein concentration in each pool was again determined by a commercially available kit. Thereafter, 50 μg proteins of each pool were labelled with 400 pmol of different CyDye fluorophors (IPF: Cy3, fNSIP: Cy5, control lungs: Cy2). The Cy2-, Cy3- and Cy5-labelled samples were subsequently combined and separated by using Two-dimensional gel electrophoresis (2D-PAGE). The separation was carried out on Multiphor™ II (GE Healthcare) in the first dimension (IEF electrophoresis, pH 4–7), followed by equilibration and reduction of IEF-strips according to standard protocols. Focused IPG gel strips were then loaded on top of SDS-polyacrylamid gels (13% total acrylamide, 3% bisacrylamide), and the second dimension electrophoresis was carried out using a Hoefer-ISO-DALT vertical gel electrophoresis system (GE Healthcare) according to standard procedures. The resulting 2D-DIGE-gel representing three different proteomes was performed in duplicate. It has to be noted that we did not perform the classical DIGE procedure by preparing an internal standard (pool of all samples) labelled with Cy2; instead, the control lung protein pool was labelled with Cy2 and served as the healthy, normal reference lung.
proteome. Because of the intention to analyze three different proteomes on one 2D-gel we chose this strategy.

2.4. Image analysis of two-dimensional-DIGE-gels

After 2D-PAGE, gels were scanned with 100 μm resolution using the Ettan™ DIGE Image. Excitation wavelengths and emission filters were chosen specifically for each of the CyDyes, and images with DIGE readouts Cy2, Cy3 and Cy5 were recorded (Supplementary Fig. E1).

Computer-assisted analysis of imaged 2D-gels/DIGE readouts was performed with Proteom Weaver Software (v 3.0.0.3, BioRad, Munich, Germany). In brief, spot detection, matching of protein/peptide spots between gels and different readouts (‘in-gel spot-codetection’), as well as background subtraction and normalization etc. were carried out by the software using standard settings. Individual spot intensities were calculated by the software. For the comparative analysis of the different CyDye labelled lung proteomes, three distinct overlays of the EttanTM DIGE Imager were compared (IPF-Cy3 versus Control-Cy2, fNSIP-Cy5 versus Control-Cy2 and fNSIP-Cy5 versus IPF-Cy3) [see Supplementary Figs. E2 and E3] using the "default setting". The scatterblots for the comparisons IPF-Cy3 versus Control-Cy2 [Suppl. Fig. E2A] and fNSIP-Cy5 versus Control-Cy2 [Suppl. Fig. E2B] indicated evidently similar amounts of differentially regulated proteins in both entities with regard to the control proteome. Interestingly, in the comparison fNSIP versus IPF, only a sparse amount of protein spots revealed differential regulation (Suppl. Fig. E3). The spot quantities and regulation factors for the comparison IPF and fNSIP versus control group are summarized in Supplementary Table E1, and these for the comparison fNSIP versus IPF in Supplementary Table E2.

Due to low abundance of differential regulated protein spots in 2D-DIGE-PAGES, micropreparative 2D-PAGE’s using 600–1000 μg lung proteins (from the fNSIP pool or from a 1:1 mixture of the fNSIP pool with the control lung pool, both “mixtures” contained wholly all differential regulated protein spots identified by DIGE-image analysis) were performed and stained with coomassie, in order to guarantee enough protein content for the identification by MALDI-TOF-MS.

2.5. Protein digestion and MALDI-TOF-MS analysis

In brief, the selected spots were excised and trypsin digested according to standard protocols. Prior to digestion, the proteins were destained using 50 mM (NH4)HCO3 in 30% acetonitrile. In-gel digestion was performed overnight with 0.05–0.15 μg trypsin sequencing grade (Roche Diagnostics, Mannheim, Germany 2006, Serva 2011) in 10 mM (NH4)HCO3. The resulting peptide mixture was desalted using μ C18 ZipTips (Millipore,
Bedford, MA) according to the manufacturer’s instructions. Then the desalted peptide mixture was spotted onto a stainless steel target (AB Sciex) using the dried droplet method with the matrix alpha-cyano-4-hydroxy cinnamic acid. The peptides on the target were measured with a MALDI mass spectrometer (4800 Proteomics Analyzer, AB SCIEX, Foster City, CA, USA). The spectrum was acquired in the mass range of 700–4500 m/z (2006: 600–4000 m/z) with the instrument operating in reflection mode.

The raw spectra were processed with the Data explorer (version 4.3.; AB Sciex) software. All spectra were externally calibrated using a peptide calibration standard (AB Sciex 2006, Bruker 2011). Database queries of the monoisotopic masses were performed with the search engine ProFound (prowl.rockefeller.edu), which uses the Bayesian probability to identify unknown sequences against a protein sequence database. ProFound takes into account individual properties of each protein in the database as well as other information relevant to the experiment such as the mass range in which the protein is expected to lie (this information can be taken from 2D-gel).

The sequence database to be searched was the NCBI non-redundant database (the version released in Sep. and Nov. 2007, and Feb. 2012). The taxonomic category was “Homo sapiens (human)” (119 130 sequences). The other search parameters were Mr, ranges within ±25% of measured values, pI range from 0.0 to 14.0, monoisotopic peptide masses, one missed cleavage by trypsin, complete modifications: iodoacetamide-C, partial modifications: oxidation (M), charge state: MH+, and mass accuracy: (1:100, Santa Cruz B. I.), rabbit polyclonal for human PSME1/PA28α (1:500, Calbiochem), rat monoclonal for human clara-cell protein 10 [CC10] (1:75, R&D Systems), mouse monoclonal for human Foxg1/HFH4 (1:75, Abcam), rabbit polyclonal for CD68 (1:200, Abcam), rabbit polyclonal for human Prdx1 (1:800, Abcam), rabbit polyclonal for human ATF6/p50ATF6 (1:100, Abcam), rabbit polyclonal for human XBP1 (1:50, Santa Cruz B. I.), mouse monoclonal for human Prdx6 (1:1000, Abcam) and rabbit polyclonal for cytokeratin-19 [KRT19] (1:100, Abcam). Control sections were treated with PBS or with rabbit or mouse primary antibody isotype control (Acris Antibodies GmbH, Hiddenhausen, Germany) to determine the specificity of the staining.

Lung tissue sections were scanned with a Mirax Desk slide scanning device (Mirax Desk, Zeiss, Göttingen, Germany), and examined histopathologically at 100×, 200× and 400× original magnification. IHC for mentioned antibodies was undertaken in 10 IPF-, 5 fNSIP- and 8 control lung samples.

2.7. Quantitative Western Blot analysis of human BALF and statistics

The protein concentration in BALF was determined according to the BCA protein assay from Perbio Science, followed by concentration of BALF samples to 5 or 10 μg lavage protein per case using a SpeedVac concentrator. Samples were then each dissolved in 20 μL of SDS-sample buffer (final concentration 2% (w/v) SDS, 2.5% (v/v) β-mercaptoethanol, 10% (v/v) glycerol, 12.5 mmol/L Tris-HCl [pH 6.8], 0.1% (w/v) bromophenol blue) and immersed in boiling water for 10 min, followed by separation on 10%, 12%- or 15% SDS-PAGEs. Thereafter, separated proteins were transferred in a semi-dry blotting chamber to PVDF membranes (GE Healthcare), followed by immunostaining for LAP3 (diluted 1:250), Prdx1 (diluted 1:1000), Prdx6 (diluted 1:500), serum amyloid P component [SAP] (rabbit polyclonal, abcam, diluted 1:40,000) and KRT19 (diluted 1:200) with use of respective horseradish peroxidase-conjugated secondary antibodies (DakoCytomation, Hamburg, Germany; rabbit anti-mouse-IgG and swine anti-rabbit IgG, both diluted 1:2000). Blot membranes were developed with the ECL Plus chemiluminescent detection system (GE Healthcare), and emitted signals were detected with a chemiluminescence imager (Intas Chemostar, Intas, Göttingen, Germany). Thereafter, band intensities in acquired TIFF/JPEG-images were analyzed by densitometric scanning and quantified using ImageJ software (Version 1.45 s, NIH).

For the statistical comparison of differences between two groups (IPF vs. HV, fNSIP vs. HV and fNSIP vs. IPF) the non-parametric Mann–Whitney U-test was applied using the software GraphPad Prism version 4.0 (GraphPad Software Inc., La Jolla, CA). Data are presented as mean ± SEM of the individual values of different subjects. A p-value < 0.05 was considered statistically significant.

3. Results

3.1. Comparative proteomic analysis of IPF and fibrotic NSIP versus Control lung tissues

For comparative proteomic profiling of IPF- (n = 14), fNSIP- (n = 8) and control lungs (n = 10), equal protein concentrations of lung
homogenates of each patient group and the control group were pooled in order to identify reproducible and robust differences. Differential labelling with three distinct fluorescent dyes was carried out resulting in a Cy3-labelled IPF-, a Cy5-labelled NSIP- and a Cy2-labelled control lung proteome. The subsequent comparison of the NSIP proteome with regard to the control and a Cy2-labelled control lung proteome. The subsequent differential labelling with three distinct fluorescent dyes was pooled in order to identify reproducible and robust differences. The accumulation of the Unfolded Protein Response (UPR) was validated by immunohistochemistry (IHC) in order to identify the cellular distribution in NSIP-, IPF- and control lung tissues. In addition, we performed staining for prosurfactant protein C (proSP-C), a protein specific to AECII, of parallel sections, in order to designate alveolar structures in these lung tissues (Fig. 2A–D, and O). A very strong overexpression of PPIA was observed in AECII in areas of thickened alveolar septae in NSIP (Fig. 2E, F) as well as in AECII overlying dense zones of fibrotic remodelling in IPF/UIP lungs (Fig. 2G, H), which was in contrast to AECII in control lungs with normal histological appearance (Fig. 2P) indicating no or only a faint PPIA expression. Interestingly, bronchiolar basal cells (which were characterized by cytoplasmic cytokeratin-5/KRT5 staining) in abnormal bronchiolar structures such as basal cell hyperplasia (Fig. 2) or hyperplastic bronchioles (Fig. 2) also revealed a pronounced expression of PPIA (Fig. 2I, M) which was only weak or barely detectable in the basal proportion of normal lung tissues (Fig. 2K, N). Basal cells in bronchioles of NSIP lungs, which have a less abnormal bronchiolar tree as compared to IPF/UIP also showed a prominent expression of PPIA (not shown). Fibroblastic cells revealed no or minimal immunostaining for PPIA, whereas interstitial inflammatory cells (mast cells, lymphocytes) in both—fibrotic and normal lung—also showed considerable expression of PPIA (Fig. 2E–H, L–N). Next, we went on to characterize the localization of expression of LAP3, a cytosolic leucine aminopeptidase involved in processing of antigenic proteins [34], in the fibrotic—and normal lung. Similar to PPIA and in line with its observed upregulation in NSIP and IPF (Table 2), a robust LAP3 expression was predominantly found to co-localize with the proSP-C expression in the AECII in areas of thickened alveolar septae in NSIP (Fig. 3A, B, E, F) as well as in AECII near dense zones of fibrosis in IPF lungs (Fig. 3C, D, G, H), whereas AECII of control lungs with normal alveolar architecture indicated a basal, lower level of expression of LAP3 (Fig. 3M–P). No notable staining was observed in fibrotic tissue itself, but inflammatory cells in the interstitium as well as macrophages in fibrotic and normal lungs revealed

### Table 2 – Proteins significantly upregulated in IPF and fNSIP lungs relative to control lung tissue (RF ≥ 1.5).

<table>
<thead>
<tr>
<th>Spot no:</th>
<th>Accession number (NCBI)/Protein name</th>
<th>Score/Est’d Z (Z-score)</th>
<th>Seq. cov. (%)</th>
<th>Matched peptides</th>
<th>Theor. MW [kDa]</th>
<th>RF_IPF/ donor</th>
<th>RF_NSIP/ donor</th>
</tr>
</thead>
<tbody>
<tr>
<td>3158</td>
<td>gi</td>
<td>11493459: PRO2619, serum albumin (ALB)</td>
<td>2.06</td>
<td>32</td>
<td>18</td>
<td>58.53</td>
<td>5.7869</td>
</tr>
<tr>
<td>3012</td>
<td>gi</td>
<td>37588925: LAP3 protein, Leucine aminopeptidase</td>
<td>2.42</td>
<td>54</td>
<td>22</td>
<td>54.77</td>
<td>3.3611</td>
</tr>
<tr>
<td>3104</td>
<td>gi</td>
<td>15277503: ACTB protein, Procapase</td>
<td>2.24</td>
<td>32</td>
<td>10</td>
<td>40.54</td>
<td>2.1605</td>
</tr>
<tr>
<td>3064</td>
<td>gi</td>
<td>9999892: Tissuephosphate isomerase (TPIS)</td>
<td>2.37</td>
<td>90</td>
<td>21</td>
<td>26.81</td>
<td>3.2599</td>
</tr>
<tr>
<td>2981</td>
<td>gi</td>
<td>178777: Proopioniprotein A-1 preprotein (AOPA1BP)</td>
<td>2.32</td>
<td>43</td>
<td>12</td>
<td>30.75</td>
<td>2.3927</td>
</tr>
<tr>
<td>3032</td>
<td>gi</td>
<td>55595887: peroxiredoxin 1 (PRDX1)</td>
<td>2.26</td>
<td>51</td>
<td>8</td>
<td>19.13</td>
<td>2.8129</td>
</tr>
<tr>
<td>2780</td>
<td>gi</td>
<td>20149498: ferritin, light polypeptide</td>
<td>2.37</td>
<td>63</td>
<td>14</td>
<td>20.03</td>
<td>2.3630</td>
</tr>
<tr>
<td>3550</td>
<td>gi</td>
<td>H503571: enolase 1 (ENO1)</td>
<td>2.27</td>
<td>61</td>
<td>22</td>
<td>47.49</td>
<td>n.a.</td>
</tr>
<tr>
<td>2881</td>
<td>gi</td>
<td>24234696: keratin 19 (KRT19)</td>
<td>2.36</td>
<td>41</td>
<td>17</td>
<td>44.09</td>
<td>6.2736</td>
</tr>
<tr>
<td>3089</td>
<td>gi</td>
<td>H501883: alpha 2 actin (ACTA2)</td>
<td>2.25</td>
<td>50</td>
<td>12</td>
<td>42.39</td>
<td>1.6950</td>
</tr>
<tr>
<td>2922</td>
<td>gi</td>
<td>9999892: Tissuephosphate isomerase (TPIS)</td>
<td>2.31</td>
<td>79</td>
<td>18</td>
<td>26.81</td>
<td>2.8328</td>
</tr>
<tr>
<td>3219</td>
<td>gi</td>
<td>1431788: Cyclophilin A, peptidylprolyl isomerase A (PPIA)</td>
<td>2.37</td>
<td>55</td>
<td>17</td>
<td>18.09</td>
<td>2.3102</td>
</tr>
<tr>
<td>2719</td>
<td>No significant identification</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>3178</td>
<td>No significant identification</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

**Definition of abbreviations:** RF = regulation factor; Est’d Z = Z-score/Profound score; a Z-score of ≥1.65 is considered significant at the 5% level (p < 0.05); Seq. cov. (%) = sequence coverage in %; Theor. MW [kDa] = theoretical molecular weight in kDa; n. a. = not applicable.
Table 3 – Proteins significantly downregulated in IPF and fNSIP lungs relative to control lung tissue (RF ≤ 0.75).

<table>
<thead>
<tr>
<th>Spot no.</th>
<th>Accession number (NCBI)/Protein name</th>
<th>Score/Est’d Z (Z-score)</th>
<th>Seq. cov. (%)</th>
<th>Matched peptides</th>
<th>Theor. MW [kDa]</th>
<th>RF_IPF/donor</th>
<th>RF_NSIP/donor</th>
</tr>
</thead>
<tbody>
<tr>
<td>2799</td>
<td>gi</td>
<td>809185: Annexin 5, ANXA5, Lipocortin V, Placental anticoagulant protein 1 (PAP-I)</td>
<td>2.30 64 23</td>
<td>35.84 0.5296</td>
<td>0.5556</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3170†</td>
<td>gi</td>
<td>4503143: Cathepsin D preproprotein (CTSD)</td>
<td>2.32 34 12</td>
<td>45.05 0.5674</td>
<td>0.5717</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2949</td>
<td>gi</td>
<td>576259: Serum amyloid P component (SAP)</td>
<td>2.30 40 8</td>
<td>23.35 0.5318</td>
<td>0.5829</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3077</td>
<td>gi</td>
<td>87564: glutathione transferase (EC 2.5.1.18) (GST)</td>
<td>1.43 56 7</td>
<td>23.44 0.4891</td>
<td>0.5420</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2854</td>
<td>gi</td>
<td>306880: prothromboplastin, haptoglobin (HTP)</td>
<td>2.11 33 8</td>
<td>38.95 0.6025</td>
<td>0.4849</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2959</td>
<td>gi</td>
<td>13787109: Alpha-1-antitrypsin (A1AT)</td>
<td>2.18 29 11</td>
<td>44.32 0.8358</td>
<td>0.5253</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Definition of abbreviations: RF = regulation factor; Est’d Z: Z-score/Profound score: a Z-score of ≥ 1.65 is considered significant at the 5% level (p < 0.05); Seq. cov. (%) = sequence coverage in %; Theor. MW [kDa] = theoretical molecular weight in kDa.
† = spot migrates with MW ~ 31 kDa in 2-DE, Cathepsin D heavy chain identified; * = only identified using up to 30 kDa range; # = identified with Mascot/Swiss-Prot database version 20110609.

Fig. 1 – Representative reference 2D-gels of 1000 µg lung proteins mapping differentially regulated proteins in (A) the comparison fNSIP/IPF- versus Control lung proteome and (B) the comparison fNSIP- versus IPF proteome. (A) Separation of proteins from a 1:1 mixture of the fNSIP—Control lung proteome pool (which contained wholly all differentially protein spots identified by precedent DIGE-image analysis) was performed on linear strips with a pH range of 4–7 followed by 16% SDS-PAGE. The 2D-gel was stained with Coomassie. Proteins upregulated in fNSIP and IPF versus Control are indicated in italic font, downregulated proteins are underlined. Differentially expressed protein spots which could not be successfully identified, are indicated with n. i. (= not identified). (B) The same 2D-separation setting as shown in (A) was performed, followed by staining of the 2D-gel with Coomassie. Proteins upregulated in fNSIP versus IPF are indicated in italic font, downregulated proteins are underlined. Both 2D-gels were used as reference proteomes, for excising and MS identification of the differentially regulated protein spots.
also considerable expression of LAP3 (Fig. 3G, H, and N). Furthermore, ciliated bronchial cells (as indicated by dashed arrows in Fig. 3I–L and parallel immunostaining with the marker FoxJ1 in Fig. 3K), but not non-ciliated Clara cells (indicated by CC10 staining in Fig. 3J), revealed a strong apical staining pattern of LAP3 in IPF (Fig. 3I), fNSIP- (not shown) and control lungs (Supplementary Fig. E4), supporting secretion of this aminopeptidase. Quantitative immunoblot analysis of bronchoalveolar lavage fluids for LAP3 indicated evident extracellular appearance of this enzyme in fNSIP-, IPF- and normal lungs, but no statistical significant differences between the three categories in secretion of LAP3 (Supplementary Fig. E5).

Another stress-induced gene found to be upregulated in fNSIP and IPF was the antioxidant protein peroxiredoxin 1 (PRDX1). By IHC, PRDX1 expression was predominantly found in ciliated bronchial cells in IPF lungs (Fig. 4B, brown staining), which were clearly marked by nuclear FoxJ1 staining in a parallel section (Fig. 4D), whereas non-ciliated Clara cells (indicated by CC10 staining in Fig. 4C) did not express this enzyme (Fig. 4B). Furthermore, AECII near regions of dense fibrosis of IPF lungs (indicated by proSP-C staining in Fig. 4A, F) revealed also no significant expression of PRDX1 (Fig. 4B, G), even not after overstaining of lung tissues sections (Supplementary Fig. E6A + B). Instead, a strong PRDX1 overexpression was observed in alveolar macrophages (AM) of IPF—(Fig. 4G, AM are indicated by CD68 staining in Fig. 4H) and fNSIP lungs (Supplementary Figs. E6G + H), whereas AM in control lung tissues revealed no or faint expression of PRDX1 (Fig. 4J, K). Similarly, AECII in control lungs indicated no notable or only weak expression of PRDX1 (Fig. 4F, J), and only the ciliated bronchial epithelium expressed PRDX1 in basal amounts in normal lungs (Supplementary Fig. E6T).

Interestingly and much to our surprise, AECII in areas of fibrotic, thickened alveolar septae of fNSIP lungs (Fig. 4L–N) indicated a pronounced induction and thus upregulation of PRDX1 expression (Fig. 4O–Q, indicated by arrows), and which was—similar to our IHC results in IPF—not observed in AECII near areas of dense uniform fibrosis in fNSIP lungs (not shown). Importantly, fibroblastic cells or fibroblast foci did not reveal a notable PRDX1 expression (not shown).

Due to reported appearance of PRDX1 and other peroxiredoxins in BALF of IIP patients [35–37], we next performed a quantitative immunoblot analysis of BALF samples
from fNSIP- and IPF patients, as well as healthy volunteers (HV) for PRDX1. Representative immunoblot (Fig. 4R) and densitometric quantification (Fig. 4S) indicated an inconsistent and insignificant upregulation of extracellular PRDX1 levels in BALF of fNSIP- and IPF patients when compared to HV.

3.3. Down-regulated proteins in IPF and fibrotic NSIP versus Control lung tissues

Among the downregulated proteins in IPF and fNSIP were genes crucial for cell healthiness and survival, namely antioxidants (glutathione transferase, haptoglobin [HPT]) and anticoagulant proteins, such as annexin A5 (ANXA5), the latter being also involved in autophagy [38] (Table 3). Another interesting protein found to be downregulated in IPF and fNSIP was serum amyloid P component (SAP) which has been previously described as an inhibitor of fibrocyte differentiation [39,40] (Table 3). We therefore wanted to investigate the localization of biosynthesis of this very interesting enzyme in IPF-, fNSIP- and control lung tissues by IHC, but failed due to the nature of commercially available antibodies against SAP, which turned out to be not suitable for IHC. Due to its reported secretion, we performed comparative immunoblot analysis of BALF samples from IPF-,
fNSIP patients and HV for SAP. SAP protein levels were significantly reduced in BALF from IPF patients in comparison to HV (p < 0.05, Fig. 5A and B), and were also evidently lower in comparison to fNSIP (without statistical significance, p = 0.1014). Interestingly, BALF contents of SAP did not differ significantly between the fNSIP— and HV group (Fig. 5A and B).

3.4. Differentially regulated proteins in fibrotic NSIP versus IPF lungs and their localization in epithelium

The subsequent comparison of the fNSIP- to the IPF lung proteome revealed 10 differentially regulated proteins which differed more than or equal to 1.5-fold, and of which nine
were upregulated (RF ≥ 1.5, Table 4) and one downregulated in fNSIP (RF ≤ 0.75, Table 4). Table 4 provides information about the identification of these differently expressed protein spots, as well as the magnitude of difference with the IPF group (indicated as RF). As additional information, the RF values for the relations fNSIP vs. controls as well as IPF vs. controls are given for these spots in Table 4, because these “differentially regulated in fNSIP vs. IPF spots” were often upregulated in both entities relative to control lungs. For an overview, all differentially regulated proteins for the comparison fNSIP-
versus IPF proteome are depicted in the proteome map in Fig. 1B.

Most of the proteins we identified as being upregulated in fNSIP versus IPF appeared to be involved in defense mechanisms against oxidative stress, namely antioxidants (PRDX6, thioredoxin peroxidase B) or were activators of the proteasome (PSME1). Genes involved in energy generation such as glycolytic enzymes (PGAM1) were also found to be upregulated in fNSIP relative to IPF (Table 4).

First, we investigated the expression of proteasome activator complex subunit 1 (PSME1), which appeared to be upregulated in fNSIP relative to IPF- and control lungs, and the expression of which did not seem to differ between IPF and controls according to the DIGE analysis (Table 4), by IHC in respective lung tissues. AECII of thickened alveolar septae in fNSIP lungs (Fig. 6A–D) revealed a strong induction of PSME1 (indicated by arrows in Fig. 6E–H), and could also be encountered in AECII near more dense fibrotic regions in fNSIP lungs (Suppl. Fig. E7), whereas IPF-AECII near zones of dense fibrosis (Fig. 6I, J) or near areas of active fibrotic remodelling (Fig. 6K) indicated no or weak expression of this proteasomal subunit (indicated by arrows in Fig. 6M, N, O). In some cases, a pronounced PSME1 staining was also encountered in IPF-AECII near dense fibrotic regions (not shown), but was nevertheless generally weaker as compared to fNSIP lungs. Furthermore, PSME1 expression was absent in fibroblast foci of IPF lungs (Fig. 6O). With regard to control lung tissues, “normal” AECII indicated no significant expression of PSME1 (see arrows in Fig. 6L, P). Finally, considerable expression of PSME1 was generally observed in interstitial inflammatory cells (especially lymphocytes) of fNSIP-, IPF- and control lungs (Fig. 6M, N; Suppl. Fig. E7).

We next went on to characterize the localization of expression of peroxiredoxin 6 (PRDX6) in fNSIP-, IPF- and control lungs. Abundant PRDX6 expression was observed in AECII of thickened dense fibrotic regions in fNSIP lungs (Suppl. Fig. E7), whereas IPF-AECII near zones of dense fibrosis (Fig. 6I, J) or near areas of active fibrotic remodelling (Fig. 6K) indicated no or weak expression of this proteasomal subunit (indicated by arrows in Fig. 6M, N, O). In some cases, a pronounced PSME1 staining was also encountered in IPF-AECII near dense fibrotic regions (not shown), but was nevertheless generally weaker as compared to fNSIP lungs. Furthermore, PSME1 expression was absent in fibroblast foci of IPF lungs (Fig. 6O). With regard to control lung tissues, “normal” AECII indicated no significant expression of PSME1 (see arrows in Fig. 6L, P). Finally, considerable expression of PSME1 was generally observed in interstitial inflammatory cells (especially lymphocytes) of fNSIP-, IPF- and control lungs (Fig. 6M, N; Suppl. Fig. E7).

Table 4 – Proteins significantly differentially regulated in fNSIP lungs relative to IPF lung tissue: RF ≥ 1.5 for upregulation, RF ≤ 0.75 for downregulation.

<table>
<thead>
<tr>
<th>Spot no.</th>
<th>Accession number (NCBI)/Protein name</th>
<th>Score Est’d Z (Z-score)</th>
<th>Seq. cov. (%)</th>
<th>Matched peptides</th>
<th>Theor. MW [kDa]</th>
<th>RF_ NSIP/IPF</th>
<th>RF_ IPF/donor</th>
<th>RF_ NSIP/donor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Upregulated Proteins</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3175</td>
<td>gi</td>
<td>3212456: Chain A, crystall structure of human serum albumin (ALB)</td>
<td>2.39</td>
<td>36</td>
<td>24</td>
<td>68.45</td>
<td>1.607</td>
<td>2.1257</td>
</tr>
<tr>
<td>2714*</td>
<td>gi</td>
<td>494296: Chain B, Cathepsin D (CTSD)</td>
<td>2.35</td>
<td>65</td>
<td>16</td>
<td>26.46</td>
<td>1.596</td>
<td>2.3507</td>
</tr>
<tr>
<td>3189</td>
<td>gi</td>
<td>38566176: Phosphoglycerate mutase 1 (PGAM1)</td>
<td>2.41</td>
<td>76</td>
<td>22</td>
<td>28.92</td>
<td>1.5162</td>
<td>0.9823</td>
</tr>
<tr>
<td>2904</td>
<td>gi</td>
<td>5453990: proteasome activator complex subunit 1 (PSME1), proteasome activator 28 subunit alpha (PA28 alpha), 11S regulator complex subunit alpha</td>
<td>1.91</td>
<td>44</td>
<td>12</td>
<td>28.88</td>
<td>1.583</td>
<td>2.2898</td>
</tr>
<tr>
<td>3198†</td>
<td>gi</td>
<td>15277503: ACTB protein, actin beta</td>
<td>2.21</td>
<td>38</td>
<td>16</td>
<td>40.54</td>
<td>1.759</td>
<td>5.1477</td>
</tr>
<tr>
<td>3191</td>
<td>gi</td>
<td>758638: peroxiredoxin 6 (PRDX6)</td>
<td>2.32</td>
<td>70</td>
<td>15</td>
<td>25.13</td>
<td>1.556</td>
<td>1.5126</td>
</tr>
<tr>
<td>2960</td>
<td>gi</td>
<td>9955007: Chain A, Thioredoxin Peroxidase B from red blood cells</td>
<td>2.37</td>
<td>66</td>
<td>20</td>
<td>21.68</td>
<td>1.521</td>
<td>0.6125</td>
</tr>
<tr>
<td>3075</td>
<td>gi</td>
<td>229752: Chain B, Alpha-Ferrous-Carbonmonoxy, Beta-Cobaltous-Deoxy-Hemoglobin (T-State)</td>
<td>2.40</td>
<td>99</td>
<td>15</td>
<td>15.96</td>
<td>1.618</td>
<td>1.5674</td>
</tr>
<tr>
<td>2748</td>
<td>gi</td>
<td>229383: cytochrome b5 fragment (CYB5A)</td>
<td>1.66</td>
<td>87</td>
<td>7</td>
<td>10.01</td>
<td>1.825</td>
<td>3.5195</td>
</tr>
<tr>
<td>Downregulated proteins</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3072</td>
<td>Mixture</td>
<td>0.463</td>
<td>3.0437</td>
<td>6.5791</td>
<td>0.463</td>
<td>3.0437</td>
<td>6.5791</td>
<td></td>
</tr>
<tr>
<td>3198†</td>
<td>1. gi</td>
<td>34043271: keratin, type I cytoskeletal 19, cytokeratin-19 (KRT19)</td>
<td>2.16</td>
<td>57</td>
<td>32</td>
<td>44.07</td>
<td>14.753</td>
<td>14.753</td>
</tr>
<tr>
<td>2. gi</td>
<td>16552261: unnamed protein product, vimentin (VIMT)</td>
<td>2.34</td>
<td>33</td>
<td>14</td>
<td>47.53</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Definition of abbreviations: RF = regulation factor; Est’d Z: Z-score/Profound score: a Z-score of ≥ 1.65 is considered significant at the 5% level (p < 0.05); Seq. cov. (%) = sequence coverage in %; Theor. MW [kDa] = theoretical molecular weight in kDa. * = Cathepsin D heavy chain identified; † = spot migrates with MW ~29 kDa in 2-DE.
alveolar septae in fNSIP lungs (indicated by arrows in Fig. 7A–B and E–F), and in AECII near zones of dense fibrosis in IPF lungs (indicated by arrows in Figs. 7C–D and G–H), as well as in “normal” AECII of control lungs (indicated by arrows in Fig. 7M–P). Additionally, robust expression of PRDX6 was observed in ciliated bronchial cells in IPF lungs (indicated by dashed arrows in Fig. 7I–K), but not in non-ciliated Clara cells (indicated by CC10 staining in Fig. 7J). The same observation was made in bronchioles of fNSIP lungs (not shown) and control lungs (Fig. 7M). Fibroblastic cells of IPF lungs did not reveal pronounced expression of PRDX6, whereas some inflammatory cells in the interstitium of fNSIP-, IPF- and control lungs indicated considerable PRDX6 expression. However, differences in cellular expression of PRDX6 could not be observed in IHC, possibly due to high abundance of this enzyme in the human lung [41]. Immunoblot analysis of BALF samples for PRDX6 revealed reduced protein levels in fNSIP and IPF in comparison to HV, but PRDX6-protein contents were still higher in fNSIP when compared to IPF (Fig. 7Q and R). However, these results were not statistically significant.

Finally, we focused our research on cytokeratin-19 (KRT19), which occurred on several positions in the 2D-gel, thereby indicating different fold changes/RF-values in the comparisons IPF/fNSIP versus controls (Fig. 1A and B, Tables 2 and 4). First, one KRT19 spot appeared to be equally upregulated in fNSIP- and IPF- versus control lungs according to the DIGE analysis (Fig. 1A, Table 2). Further, KRT19 protein was identified in a protein spot containing a mixture of KRT19 and vimentin (VIME), presumably due to comigration as a result of the similar MW and pI (isoelectric point) of both proteins (indicated in Fig. 1B and Table 4). This spot appeared to be upregulated in IPF- and fNSIP- relative to control lungs, and was nevertheless twice as much downregulated in fNSIP- relative to IPF lungs according to the DIGE analysis (Table 4). We therefore investigated the localization of KRT19 in fNSIP-, IPF- and control lungs by IHC. KRT19 was expressed in an induced fashion in AECII of thickened alveolar septae (Fig. 8A, E) and in AECII overlying areas of uniform dense fibrosis in fNSIP lungs (Fig. 8B, F), as well as in AECII near areas of fibrotic remodelling and bronchiolization in IPF lungs (Supplementary Fig. E8). In contrast, “normal” AECII of control lungs indicated only faint expression of KRT19 (Fig. 8C, G). In IPF/UIP lungs, KRT19 overexpression was (beside AECII) strikingly evident in bronchial structures (bronchiolar basal cells and ciliated and non-ciliated bronchial cells, see Fig. 8D) in areas of bronchiolization and honeycomb cyst formation, which are indicated by KRT5.

Fig. 6 – Induction and upregulation of proteasome activator complex subunit 1 (PSME1) in type-II alveolar epithelial cells (AECII) in fibrotic NSIP lungs. Representative immunohistochemistry for proSP-C (A–D, I–K) and PSME1 (E–H, M–O) in serial sections of fNSIP- and IPF lung tissues, and for proSP-C (L) and PSME1 (P) in control lungs. (A–D, E–H) AECII in areas of thickened alveolar septae in fibrotic NSIP lungs (fNSIP; A–D) reveal robust expression of PSME1 (see arrows in E–H). (I–K, M–O) AECII near areas of dense fibrosis (I, J) and active fibrotic remodelling (K) indicate no or only sparse PSME1 expression (see arrows in M, N and O). (L, P) AECII of control lungs indicate no significant expression of PSME1 (indicated by arrows). In general, PSME1 expression is also found in some interstitial inflammatory cells in fNSIP, IPF- and control lungs (G, M–P). Original magnification of photomicrographs A, C, E, G, I, M: ×200 (bar = 100 μm); original magnification of photomicrographs B, D, F, H, J–L, N–P: ×400 (bar = 50 μm).
staining of the basal layer of bronchioles in a serial section (Fig. 8H). Immunoblot analysis of BALF samples for KRT19 revealed significantly enhanced protein contents of a ~25 kDa fragment in lavages of IPF patients, in comparison to fNSIP and HV (p < 0.05 vs. fNSIP; p < 0.01 vs. HV; Fig. 8I and J). Despite reported evidence of CYFRA (cytokeratin 19 fragment, ~37 kDa) in

---

Fig. 7 – Expression analysis of peroxiredoxin 6 (PRDX6) in lungs from patients with fibrotic NSIP and IPF in comparison to Control lungs. Representative immunohistochemistry for proSP-C (A–D), PRDX6 (E–H, I), FoxJ1 (K) and cytokeratin-5 [KRT5] (L) in serial sections of fNSIP- and IPF lung tissues, and for PRDX6 (M, N) and proSP-C (O, P) in control lungs. (A–D, E–H) AECII in areas of thickened alveolar septae in fibrotic NSIP lungs (fNSIP; A, B, E, F) as well as AECII near zones of dense fibrosis in IPF lungs (C, D, G, H) reveal robust expression of PRDX6 (indicated by arrows). (I–L) In IPF lungs, PRDX6 expression is also found in ciliated bronchial cells which are marked by FoxJ1-staining in a parallel section (K) and dashed arrows in Figs. I, J and K. Non-ciliated Clara cells (as indicated by CC10 staining in J) don’t express PRDX6 (I and J). Moderate PRDX6 expression is also observed in some bronchiolar basal cells in IPF lungs (indicated by KRT5-staining in L and arrowheads in I and L). (M–P) In control lungs, PRDX6 is robustly expressed in AECII (indicated by arrows) and ciliated bronchial cells (indicated by dashed arrows). Original magnification of photomicrographs A, C, E, G: ×200 (bar = 100 μm); original magnification of photomicrographs B, D, F, H, I–L and M–P: ×400 (bar = 50 μm). (Q) Representative immunoblot and (R) quantitative immunoblot analysis for PRDX6 of BAL fluid from patients with sporadic fNSIP (n = 7), IPF (n = 6) and healthy volunteers (HV, n = 6). Five μg protein of cell-free BALF samples were concentrated and separated on a 15% SDS-PAGE. Coomassie staining of the blot membrane was used as a loading control. Intensity of bands was densitometrically quantified and presented as column diagram for each category (mean ± SEM).
human BALF of IIP patients [42,43], we could not detect CYFRA as well as full-length KRT19 (~44 kDa) in concentrated lavage samples (10 μg) obtained from our IIP patient cohort.

3.5. Alveolar epithelial endoplasmic reticulum (ER) stress in fibrotic NSIP and IPF

We observed induced expression of stress-induced genes PPIA and LAP3 in AECII of lungs from fNSIP- and IPF patients, as well as encountered induced expression of the antioxidant enzyme PRDX1 and the proteasome activator PMSE1 predominantly in the AECII of fNSIP lungs. Therefore, we finally investigated the localization of expression of typical UPR/ER stress-marker (and -sensor) proteins such as activating transcription factor 6 [ATF6, including activated p50ATF6(N)] and X-box binding protein 1 (XBP1) in fNSIP- in comparison to IPF- and control lungs. AECII near dense zones of uniform fibrosis in fNSIP lungs (Fig. 9A, B) as well as AECII in areas of dense fibrotic remodelling in IPF/UIP lungs (Fig. 9D) indicated robust induction of p50ATF6/ATF6 (Fig. 9E, F, H) and XBP1 (Fig. 9I, J, L) to same extent, in comparison to “normal” AECII of control lungs which revealed no or only sparse expression of ATF6 or XBP1 (Fig. 9M, N, O). Induced and considerable overexpression of p50ATF6/ATF6 and XBP1 was also observed in AECII in areas of thickened alveolar septae of fNSIP lungs (Fig. 9C, G, K). Alveolar macrophages of fNSIP- and IPF lungs revealed also immunostaining for ATF6 (Fig. 9E, F, H), but not for XBP1 (Fig. 9I, J, L).

4. Discussion

We performed a comparative proteome analysis of lung tissue from patients with fNSIP and IPF and normal control lung tissue in order to identify differentially regulated proteins in fNSIP and IPF relative to controls as well as between IPF and fNSIP itself. The latter aim was challenging, because it had been reported that gene expression profiles of IPF- and NSIP lungs were quite similar and that only few differences between the two types of

Fig. 8 – Expression analysis of cytokeratin 19 (KRT19) in lungs from patients with fibrotic NSIP and IPF in comparison to Control lungs. Representative immunohistochemistry for cytokeratin 19 [KRT19] (A, B) and proSP-C (E, F) in fibrotic NSIP lungs (fNSIP), and for KRT19 (C) and proSP-C (G) in control lung tissues, and for KRT19 (D) and cytokeratin 5 [KRT5] (H) in IPF lungs. (A, B, E, F) In fNSIP, robust KRT19 overexpression was observed in hyperplastic AECl in areas of thickened alveolar septae (A, E) and in AECl in areas of dense uniform fibrosis (B, F). (C, G) AECl in control lungs indicate a weak basal expression of KRT19 (indicated by arrows), whereas bronchial epithelium reveal a notable constitutive KRT19 expression in normal control lungs (indicated by hashmark in C). (D, H) In IPF lungs, strong expression of KRT19 is not only observed in hyperplastic AECl (not shown), but also very dominantly in bronchiolar basal cells (indicated by KRT5 staining in H), as well as ciliated—and non-ciliated bronchial cells of abnormal bronchioles in bronchiolized areas in IPF lungs. Original magnification of photomicrographs A and E: ×100 (bar = 200 μm); original magnification of photomicrographs B and F: ×200 (bar = 100 μm); original magnification of photomicrographs C and G: ×400 (bar = 50 μm); original magnification of photomicrographs D and H: ×50 (bar = 500 μm). (I) Representative immunoblot and (J) quantitative immunoblot analysis for KRT19 of BAL fluid from patients with sporadic fNSIP (n = 7), IPF (n = 6) and healthy volunteers (HV, n = 6). 10 μg protein of cell-free BALF samples were concentrated and separated on a 10% SDS-PAGE. Coomassie staining of the blot membrane was used as a loading control. Intensity of bands was densitometrically quantified and presented as column diagram for each category (mean ± SEM). * (p < 0.05) for fNSIP versus HV, ** (p < 0.01) for IPF versus HV.
IIP were found [10,11,31]. The challenge to analyze three different proteomes was overcome by the 2D-DIGE method, by separating and comparing the differently labelled IPF-, fNSIP- and healthy control lung proteome (IPF: Cy3, NSIP: Cy5, control: Cy2) on one and the same 2D-gel. Spot matching and quantification were improved in comparison with traditional 2D-gel-based techniques.

Our study is limited by the relatively low number of lung tissue samples for each patient category, especially for fNSIP and donor lung materials. It has, however, to be kept in mind, that both, IPF and fNSIP, are orphan diseases, with (idiopathic) fNSIP being even much less frequent as compared to IPF. To somewhat circumvent this problem, we used pooled samples of organ donor lungs as well as of explanted end-stage lungs from each patient category, in order to reduce the (everlasting) biological variation from patient to patient, and to identify robust and “real” differences. In addition, most of the relevant results/proteomic signatures were further confirmed by IHC in individual samples, and by additional analysis of BALF obtained from additional IPF- and fNSIP patients. We therefore believe that inclusion of a larger number of samples would not greatly change our findings and conclusions.

In line with previously performed transcriptional profiling of IPF and NSIP lungs [10,11,31], our results certainly indicate that both diseases are “proteomically” similar, suggesting similar pathogenetic principles and disease processes. In accordance with our previous published proteome analysis of IPF- and control lungs [33], the present data set certainly suggests that ER stress as well as a general stress-response play a crucial role in the pathogenesis of both IPF and fNSIP. Among the upregulated proteins in IPF and fNSIP were stress-induced proteins, such as leucine aminopeptidase (LAP3) and peptidylprolyl isomerase A (PPIA), the latter being also described to be involved in the UPR [44]. With its chaperone and PPIase activities, PPIA ascertains the correct folding and conformation of nascent or denatured proteins, and also provides protection against environmental insults. LAP3 has been reported to catalyze the initial processing of antigenic proteins in the cytoplasm, which is a necessary step for antigen presentation and recognition by cytotoxic T lymphocytes (CTLs) [34]. As expected, both stress-induced genes

![Fig. 9](#)

**Fig. 9** – Induction of markers for the Unfolded Protein Response (UPR) in type-II alveolar epithelial cells (AECII) in fibrotic NSIP and IPF lungs. Representative immunohistochemistry for proSP-C (A–D), activating transcription factor 6 [ATF6] (E–H) and X-box binding protein 1 [XBP1] (I–L) in serial sections of fNSIP- and IPF lung tissues, and for proSP-C (M), ATF6 (N) and XBP1 (O) in control lungs. (A–D), (E–H), (I–L) AECII in areas of dense uniform fibrosis in fibrotic NSIP lungs (fNSIP; A, B, E, F, I, J) as well as AECII in areas of active fibrotic remodelling in IPF lungs (D, H, L) reveal very strong expression of ATF6 and XBP1 to same extent (AECII are indicated by arrows). Alveolar macrophages of fNSIP and IPF lungs (indicated by hashmarks) reveal also expression of ATF6 (F and H), but not of XBP1 (J and L). Expression of ATF6 and XBP1 is also observed in AECII in areas of thickened alveolar septae in NSIP lungs (see arrows in C, G, K). (M, N, O) In control lungs, no or only sparse expression of ATF6 and XBP1 is observed in the AECII (indicated by arrows). Original magnification of photomicrographs A, E, I: ×200 (bar = 100 μm); original magnification of photomicrographs B–D, F–H, J–L: ×400 (bar = 50 μm).
were observed to be predominantly overexpressed in the AECII in the fibrotic lung: AECII of thickened alveolar septae and near areas of dense uniform fibrosis in fNSIP lungs as well as AECII near dense fibrotic regions in UIP/IPF lungs showed robust PPIA- or LAP3 expression, whereas “normal” AECII of control lungs indicated no or only a basal, lower expression of both enzymes (Figs. 2 and 3). Importantly, PPIA expression is not only induced by the UPR, but also induced in response to a wide variety of stressors including cancer [45]. PPIA is upregulated in many cancers [45,46], and probably functions in maintaining the conformation of oncogenic proteins. It has also been suggested that PPIA exerts in tumor cells an anti-apoptotic function by sequestering cytochrome c [47]. Upregulation of PPIA was also observed in “abnormal” bronchiolar basal cells of IPF lungs, but not in the basal layer of normal bronchioles in control lungs (Fig. 2I–N). We suggest that induced overexpression of PPIA in basal cells may be one cause for the (observed) exaggerated, proliferative character of this cell type in IPF and thus govern the process of bronchiolization in this disease. As expected, antioxidant enzymes such as peroxiredoxin 1 (PRDX1) were observed to be upregulated in fNSIP and IPF, presumably as a response to (reported) increased oxidative stress in IPF’s [48-50]. Moreover, it has recently been shown that homozygous Prdx1(−/−) knockout mice are more susceptible to bleomycin-induced lung fibrosis in comparison to wild-type mice, with marked increases in pulmonary ROS levels in diseased knockout mice, thus underscoring the crucial role of Prdx1 in protection against pulmonary fibrosis because of its antioxidant actions [51]. For validation of upregulated PRDX1 expression, we analyzed its cellular distribution in fNSIP, IPF- and normal control lungs by IHC. Interestingly, we found PRDX1 to be mainly expressed in ciliated bronchial cells and in alveolar macrophages in fNSIP, IPF- and control lungs (with pronounced upregulation in fNSIP- and IPF lungs vs. controls), whereas “hyperplastic” AECII in IPF- as well as “normal” AECII in control lungs did not reveal a notable expression of PRDX1 (Fig. 4A–K). Much to our surprise, we observed a prominent induction of PRDX1 in hyperplastic AECII in areas of thickened alveolar septae in some fNSIP lungs (Fig. 4L–Q, Suppl. Fig. E6I–P). Of note, “hyperplastic” AECII near areas of dense uniform fibrosis in fNSIP lungs did—similar to the AECII of UIP/IPF lungs—never reveal a significant PRDX1 expression. Taken together, the impressive finding of an AECII-localized upregulation of PRDX1 exclusively in NSIP typical areas such as thickened alveolar septae in fNSIP lungs may represent an attempt by these AECII to adjust to the microenvironment in a manner that is advantageous to survival. According to this notion, induction of ER stress sensed by the ATF6 and IRE1/XBP1-pathways was also encountered in AECII in areas of thickened alveolar septae in fNSIP lungs (Fig. 9). Oxidative stress is known to disrupt protein folding through formation of protein carbonyls; and oxidative stress and ER stress-response are tightly interconnected through the PERK/Nrf2 pathway which induces cytoprotective and antioxidant acting genes such as peroxiredoxins in response to increased ROS levels and oxidant stress [52,53]. Furthermore, it has been already shown that Nrf2 is induced and upregulated in AECII of both NSIP- and IPF lungs, whereas “normal” AECII in controls did not indicate notable protein levels of this transcription factor [49,54]. Additionally, antioxidants have been reported to reduce ER stress and improve protein secretion in an in vitro model of protein misfolding [55]. Although purely speculative at present, the observed upregulation of PRDX1 exclusively in AECII in areas of thickened alveolar septae in fNSIP lungs could represent a protection- and survival mechanism of these AECII against oxidant-mediated cell injury and apoptosis, thereby resulting in preservation of septated alveolar structure in this histologic subtype.

Among the down-regulated proteins in fNSIP and IPF (Table 3), we identified the anti-coagulant protein annexin A5 (ANXA5), consistent with the decreased fibrinolytic activity in ILD [56]. ANXA5 is also involved in autophagosome maturation and thus directly in autophagy which is a pathway responsible for the degradation of unwanted intracellular materials. Therefore, ANXA5 is crucial for cell survival [38,57]. In line with reported oxidant-antioxidant imbalance in IPF’s [50], we observed a downregulation of some antioxidant acting enzymes, namely glutathione transferase and haptoglobin. Haptoglobin is a hemoglobin-binding acute-phase protein which possesses anti-inflammatory and antioxidative properties. Haptoglobin decreases hemoglobin-driven oxidative stress: It forms a complex with hemoglobin in order to protect the organs from damage by hemoglobin, while making the hemoglobin accessible to degradative enzymes [58,59].

Another very interesting downregulated protein in fNSIP and IPF was serum amyloid P component (SAP), a member of the pentaxin family, and which has been shown to inhibit fibrocyte differentiation in vitro [39,40]. In line with this observation, SAP has been recently described to reduce bleomycin-induced lung fibrosis in the mouse through attenuating bone marrow-derived mesenchymal cell accumulation and collagen synthesis [60]. Additionally, SAP has been also shown to inhibit pro-fibrotic alternative (M2) macrophage activation and accumulation in models of pulmonary and renal fibrosis [61,62]. Another function of SAP is to scavenge nuclear material (i.e. DNA) from damaged circulating cells and to clear apoptotic and necrotic cell debris [63,64]. Interestingly, our analysis of BALF-SAP protein contents revealed significantly reduced protein levels for IPF only, as compared to healthy volunteers (HV), whereas BALF of fNSIP patients contained SAP levels similar to HV (Fig. 5)—the latter finding being in contrast to the Dige results employing lung tissue (Table 3). On the other hand, this result is in line with reduced or no appearance of fibroblast foci in fNSIP and suggests, that BALF of fNSIP patients contained SAP levels similar to HV (Fig. 5)—the latter finding being in contrast to the Dige results employing lung tissue (Table 3). On the other hand, this result is in line with reduced or no appearance of fibroblast foci in fNSIP and suggests, that BALF of fNSIP patients contained SAP levels similar to HV (Fig. 5)—the latter finding being in contrast to the Dige results employing lung tissue (Table 3). On the other hand, this result is in line with reduced or no appearance of fibroblast foci in fNSIP and suggests, that BALF of fNSIP patients contained SAP levels similar to HV (Fig. 5)—the latter finding being in contrast to the Dige results employing lung tissue (Table 3). On the other hand, this result is in line with reduced or no appearance of fibroblast foci in fNSIP and suggests, that BALF of fNSIP patients contained SAP levels similar to HV (Fig. 5)—the latter finding being in contrast to the Dige results employing lung tissue (Table 3). On the other hand, this result is in line with reduced or no appearance of fibroblast foci in fNSIP and suggests, that BALF of fNSIP patients contained SAP levels similar to HV (Fig. 5)—the latter finding being in contrast to the Dige results employing lung tissue (Table 3). On the other hand, this result is in line with reduced or no appearance of fibroblast foci in fNSIP and suggests, that BALF of fNSIP patients contained SAP levels similar to HV (Fig. 5)—the latter finding being in contrast to the Dige results employing lung tissue (Table 3). On the other hand, this result is in line with reduced or no appearance of fibroblast foci in fNSIP and suggests, that BALF of fNSIP patients contained SAP levels similar to HV (Fig. 5)—the latter finding being in contrast to the Dige results employing lung tissue (Table 3).
which has been originally described as a regulatory subunit of the proteasome implicated in antigen processing [34]. Recently, PSME1 has also been suggested to improve clearance of misfolded, oxidized proteins. Whereas overexpression of PSME1 in vitro was found to significantly attenuate H₂O₂-induced accumulation of protein carbonyls and apoptosis in cultured cardiomyocytes [66], the reverse approach, knockdown of PSME1 in vitro, forwarded enhanced susceptibility to oxidant cell injury [67]. Similar to conventional antioxidant enzymes such as peroxiredoxins or glutathione transferase, PSME1 is also a target of the cytoprotective antioxidant transcription factor Nrf2, and induced in response to oxidative stress [68]. Only recently, induction of proteasome activator complex subunit 1 was also shown to be involved in the removal of modified proteins during the process of differentiation [69]. Thus, the observed induction of PSME1 in AECII in areas of thickened alveolar septae and AECII to comigration as a result of similar MW and pI of both larly high level in lung.

In fNSIP versus IPF, but was upregulated in both fNSIP and IPF proteins was the onliest significantly downregulated spot in (Table 4). Using IHC, a very strong expression of PRDX6 was with no changes in PRDX6 expression between IPF and controls upregulation of PRDX6 in fNSIP compared to IPF and controls, particularly dominating in basal and luminal bronchiolar cells in areas of bronchiolization, including epithelial abnormalities such as squamous metaplasia lining the honeycomb-regions (Fig. 8, Suppl. Fig. E8). Thus, KRT19 upregulation in IPF versus fNSIP highlighted the phenomenon of aberrant epithelial repair in IPF such as the bronchiolization-process which is a hallmark of the UIP pattern [1,3,15,17]. In line with this, increased BALF and serum levels of cytokeratin 19 fragment (CYFRA) have been reported to reflect ongoing epithelial injury and repair in IPF [42,43]. However, we could not detect CYFRA or full-length KRT19 in concentrated lavage samples by immuno-blotting. Instead, we observed significantly elevated protein levels of a ~25 kDa fragment of KRT19 in BALF of IPF patients in comparison to fNSIP and HV [Fig. 8I, J], possibly reflecting only the process of epithelial instability and apoptosis in IPF, but not extensive regeneration of epithelial cells. KRT19 has been described as a caspase-3 substrate [74,75] and is cleaved by it in response to induction of apoptosis by anisomycin [76].

Taken together, our DIGE data including validation experiments suggest that there are consistent, but only few differences between IPF and fNSIP at the proteomic level. In comparison to IPF, the proteomic signature of fNSIP was enriched for genes which are functionally associated with defense mechanisms against oxidative— and ER stress and thus antiapoptotic strategies. Importantly, this finding is in line with the reported transcriptomic signature in NSIP lungs, revealing downregulation of the proapoptotic p53 kinase HIPK2 in this entity versus IPF lungs—and thus reduced apoptosis in NSIP. With regard to normal control lungs, HIPK2 was upregulated in both NSIP and IPF [10]. Additionally, the obtained fNSIP signature of increased antioxidant capacity in alveolar epithelium is in line with previous IHC-studies reporting decreased expression of apoptotic proteins (p53, p21) in (alveolar) epithelial cells of NSIP- in comparison to IPF lungs [23,24]. We therefore conclude that the impressive finding of an AECII-localized upregulation of antioxidant acting enzymes as well as of factors involved in the removal of oxidized proteins in fNSIP lungs may represent an attempt of these AECII to survive under conditions of persistent ER- and oxidative stress (Fig. 10), thereby resulting in the maintenance of septated alveolar structure in this distinct histologic subtype.

Taken together, our data indicate that central molecular events in the pathogenesis of IPF and fNSIP are localized to the alveolar epithelium, and put forward antioxidant therapeutic approaches to inhibit detrimental oxidant-mediated reactions (which may also originate from chronic ER stress) in these fatal diseases.

4.1. Correlation of proteomic changes with functional data

With regard to SAP, we performed a linear regression analysis between SAP-BALF levels and DLCO/SB values from fNSIP and IPF patients, and observed no significant correlation of SAP-BALF levels with DLCO/SB [Suppl. Fig. E9B]. Importantly, we observed a significant correlation of BALF-SAP with DLCO/SB (r² = 0.50, p = 0.0224) when IPF patients and HV were included in the analysis [Suppl. Fig. E9C]. Further linear regression
In conclusion, we demonstrated for the first time a comparative proteome analysis of subpleural lung tissue from patients with sporadic IPF and fNSIP, with explanted donor lungs serving as controls. We observed that the histologically different presentations of UIP/IPF and fNSIP were proteomically similar and that only few protein expression changes exist between IPF and fNSIP. Our data suggest that ER stress and a general stress-response as well as the decline of antioxidant capacity in alveolar epithelium are key in the pathogenesis of IPF and fNSIP. Finally, we conclude that signatures of enhanced protection mechanisms against oxidative—and ER stress distinguish fNSIP from IPF, and may explain the better outcome and survival in patients with fNSIP in comparison to IPF patients (Fig. 10). These changes seem to be correlated with disease severity.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jprot.2013.04.033.

Competing interests

The authors declare that they have no competing interests.

Acknowledgment

We thank Dr. Franka Garcia Prado (TOPLAB GmbH) for her valuable help and discussion, and we thank the technicians Silke Härdel, Stefanie Hezel and Leonie Steinbach for scanning of numerous immunostained lung tissue sections.

This work has been funded by grants from the German Centre for Lung Research (DZL) and from the Bundesministerium für Bildung und Forschung ("German Network for Diffuse Parenchymal Lung Diseases", GOLDnet), as well as from the European Community’s Seventh Framework Programme (FP7/2007–2013) under grant agreement no. HEALTH-F2-2007-202224 eurIPFnet.

5. Concluding remarks

studies indicate that BALF levels for PRDX1 and -6 positively correlate with DLCO/SB in patients with fNSIP and IPF [Suppl. Figs. E10/E11A–C], and BALF contents for 25 kDa-KRT19 fragment displayed a significant inverse correlation with DLCO/SB in both categories [Suppl. Figs. E12A + C]. Additionally, BALF levels of PRDX6 and KRT19 fragments also correlated significantly with FVC in IPF patients [Suppl. Figs. E13A + B]. Despite the low number of patient subjects used for analyses, these preliminary correlation tests might suggest a biological relationship between SAP, PRDX1 and -6, and cleaved KRT19 fragments with the severity of lung disease in IPF and/or fNSIP.

Hence, these correlation studies offer “antioxidant” (PRDX1 and -6) or “antifibrotic” (SAP) signatures as novel biomarkers to monitor disease progression and severity in different IIPs. Future research should focus on confirming these findings and suggestions in BALF and serum in a bigger cohort of IPF- and fNSIP patients, and if this panel of proteins may be used to differentiate IPF from NSIP (and also from other IIPs), and to indicate prognosis.

REFERENCEs


