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Research Article

Proteomic analysis of human epithelial lining fluid by microfluidics-based nanoLC-MS/MS: A feasibility study

Microfluidics-based nanoLC-MS/MS (chipLC-MS/MS) was used to identify and quantify proteins in epithelial lining fluid (ELF), collected during bronchoscopy from the main bronchi of chronic obstructive pulmonary disease (COPD) patients and healthy controls using microprobes. ELF is a biofluid that is well suited to study pathophysiological processes in the lung, because it contains high concentrations of biologically active molecules. 1D-PAGE followed by in-gel tryptic digestion and chipLC-MS/MS resulted in identification of approximately 300 proteins. A comparative study of ELF from COPD patients and non-COPD controls using chemical stable isotope labeling (iTRAQ®-8Plex) showed that the levels of lactotransferrin, high-mobility group protein B1 (HMGB 1), alpha 1-antichymotrypsin and cofilin-1 differed significantly in ELF from COPD patients and non-COPD controls (p -values < 0.05). These results were reproduced in another, independent set of ELF samples from COPD patients and non-COPD controls and further validated by immunohistochemistry. This study shows the feasibility of performing chipLC-MS/MS and quantitative proteomics in human ELF.

Keywords:

Chronic obstructive pulmonary disease / Epithelial lining fluid / Mass spectrometry / Microfluidics / Proteomics
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1 Introduction

The prevalence of mortality due to chronic obstructive pulmonary disease (COPD) is increasing and is expected to rank as the 3rd cause of death worldwide in 2020 [1, 2]. Major risk factors for COPD in the western world are smoking or exposure to tobacco smoke in combination with genetic factors [3]. The mechanisms that govern COPD initiation and progression are still poorly understood and no cure is available for this disease, apart from slowing down progression by smoking cessation [4]. Moreover, recent evidence suggests

that cigarette smoke accelerates cellular senescence, which may be implicated in the pathogenesis of COPD [5].

The pathology of COPD encompasses emphysema with destruction of the lung parenchyma as well as fibrosis of the small and large airways leading to irreversible airway obstruction. A subgroup of COPD patients has chronic cough and sputum production, which is a risk factor for disease severity [6]. Other factors correlating with disease severity are infiltration of inflammatory cells, notably lymphocytes, neutrophils and mast cells, alterations of the bronchial epithelium [7] and increased thickness of the airway wall [8, 9]. The severity of COPD is classified in four stages (GOLD stages 1–4) according to international guidelines (www.goldcopd.com) based on measurements of lung function (forced expiratory volume in 1 s percent predicted) but without taking the underlying inflammatory processes into account. Lung function decline in mild to moderate COPD is associated with ongoing inflammation that flares up when inhaled corticosteroids are discontinued [7]. The main approaches to treat COPD patients focus on smoking cessation, symptom relief, physical activity and reduction of complications such as exacerbations

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Abbreviations: BALF, bronchoalveolar lavage fluid; BMP, bronchoscopic microprobe; chipLC, microfluidics-based nanoLC; COPD, chronic obstructive pulmonary disease; ELF, epithelial lining fluid; FA, formic acid; HMGB1, high mobility group protein B1; LPS, lipopolysaccharide; RAGE, receptor for advanced glycation end products; ROS, reactive oxygen species

Colour Online: See the article online to view Figs. 1 and 5 in colour.

Table 1. Characteristics of the COPD patient and non-COPD control for the proteomics study of ELF by SDS-PAGE and chipLC-MS/MS

	COPD subject	Non-COPD subject
Age, years	65	83
Gender	Male	Female
COPD	GOLD stage II	No
FEV ₁ ,% predicted	70	93

FEV₁, forced expiratory volume in 1 s.

[10]. Given the fact that there is no cure for COPD, new biological markers are required to redefine disease severity and disease heterogeneity and to assess treatment efficacy [2].

The use of microfluidics-based nanoLC (chipLC) coupled to mass spectrometry has allowed to identify and quantify proteins in minute sample amounts. Proteomic studies on lung disease have focused on protein analysis in biopsies, biofluids such as serum, bronchoalveolar lavage fluid (BALF), sputum or urine and on cells in culture [11–15]. BALF and sputum are to date the most studied specimens; however, challenges related to the relative inaccessibility of these samples from the lung have hampered progress [16]. Drawbacks of BALF are that it contains not only pulmonary proteins but is prone to contamination with blood resulting in about 50% of plasma-derived proteins [16] while sputum has the drawback of sampling only the upper airways and of being easily contaminated with saliva and bacterial proteins. Epithelial lining fluid (ELF), which forms a thin layer covering the alveoli and the mucosa of small and large airways, constitutes the first barrier between lung tissue and the outer world. ELF, being an extracellular compartment, contains little DNA or RNA but high levels of proteins [17] and is an attractive target for proteomics studies to understand the molecular basis of COPD, due to its direct contact with cigarette smoke and the epithelial layer.

ELF can be obtained by bronchoscopic microprobe sampling (BMP), a technique that uses small adsorptive probes [18, 19]. ELF collected in this way contains higher concentrations of biologically active molecules [20]. Another advantage of microprobe sampling is that the locations from which ELF was obtained are clearly defined [18, 21] and it allows collection of ELF from both central and peripheral airways [22].

In this study, we first provide a qualitative view of the ELF proteome analyzed by SDS-PAGE followed by in-gel tryptic digestion and chipLC-MS/MS. Subsequently, we describe two comparative, quantitative studies (Study I and Study II) using stable isotope labeling (iTRAQ® Reagent-8Plex) followed by 2D chromatography and MS/MS. Statistical analysis showed that proteins related to inflammation, infection, host defense and oxidative stress are differentially expressed in ELF from COPD patients. To strengthen the proteomics data the cellular origin of the observed proteins was assessed by immunohistochemistry in non-COPD and diseased lung tissue.

2 Materials and methods

2.1 ELF sampling and sample preparation

Epithelial lining fluid was collected from a COPD patient and a non-COPD subject for the SDS-PAGE experiments (see Table 1) and from four COPD patients and four non-COPD controls for Study I and an independent set of four COPD patients and four non-COPD controls for Study II (see Table 2). All subjects gave informed consent for the study, which was approved by the local ethics committee in agreement with the Helsinki declaration of 1964 as revised in 2004.

Following local anesthesia with 30 mL lidocaine (2% w/v), a bronchoscope was introduced via the nose or mouth into the left main bronchus. The outer sheath of

Table 2. Characteristics of the COPD patients and non-COPD controls for the comparative, quantitative proteomics studies of ELF by iTRAQ®-8plex labeling

	Study I		Study II	
	COPD (n = 4)	Non-COPD (n = 4)	COPD (n = 4)	Non-COPD (n = 4)
Age, years	67 (47–74)	61.5 (45–83)	60.5 (53–66)	57.5 (20–87)
Female, number	0	2	2	2
Current smoker, number	1	0	1	2
Ex-smoker, number ^{a)}	3	0	3	1
Inhaled corticosteroids, number ^{b)}	2	0	1	0
Co-morbidity, CCI	1.5 (1–4)	0.5 (0–1)	1 (1–3)	0.5 (0–1)
Pack years, number	37.5 (15–60)	0	36.5 (32–45)	3.5 (2.1–31)
FEV ₁ /FVC,%	61.5 (43–68)	75.0 (73–80)	38 (22–51)	84 (70–99)
FEV ₁ ,% predicted	74 (44–78)	122 (93–134)	36 (19–70)	119 (68–129)

Values are medians (ranges) or numbers.

a) Years stopped smoking in Study I: COPD patient 1: 20 years; COPD patient 2: 2 years; COPD patient 3: 5 years. Years stopped smoking in Study II: COPD patient 4: 10 years; COPD patient 5: 9 years; COPD patient 6: 37 years; non-COPD: 35 years.

b) Number of patients and/or controls under treatment with inhaled corticosteroids.

CCI, Charlson co-morbidity index; FEV₁, forced expiratory volume in 1 s; FVC, forced vital capacity.

the BMP (BC-401C, BC-402C; Olympus, Tokyo, Japan) was guided via the working channel of the bronchoscope into the lumen of the left main bronchus and held at the target position. The inner adsorptive tip was pushed out gently till contacting the airway mucosa and its position was maintained for 10 s allowing ELF adsorption. Participants of the study were asked not to breathe during these 10 s. The tip of the BMP was subsequently withdrawn into the outer sheath and the probe was removed from the bronchoscope. This procedure was repeated to obtain three probes per subject.

The adsorptive tips were cut (about 3 cm in length), inserted into 1.5 mL tubes (Greiner, reaction tubes, 1.5 mL, PP, graduated) containing 1 mL of PBS each and stored on ice. The tubes were fixed on a slowly rotating wheel for 10 min at 4°C to extract proteins. Probes were removed with the help of tweezers and the extracts were centrifuged (Eppendorf Centrifuge 5417R) for 5 min at 3500 rpm at 4°C to remove insoluble material and divided into 200 µL aliquots (Eppendorf tubes) for storage at –80°C.

2.2 Determination of protein concentration

Protein concentration was determined using the Micro BCA Assay (Pierce Protein Research Product, Thermo Scientific) following the manufacturer's protocol in an absorbance plate reader at 550 nm (Molecular Devices, THERMOMax). The total protein concentration in the aliquots was between 150 and 400 µg/mL.

2.3 Sample preparation for SDS-PAGE

One 200 µL aliquot of the ELF extract was thawed and concentrated five- to ten-fold under vacuum (Eppendorf Concentrator 5301). Concentrated extracts were centrifuged (Eppendorf Centrifuge 5417R) for 5 min at 5000 rpm to remove insoluble material. The clear supernatant was transferred to a new tube and 5 µL of five-fold concentrated loading buffer (10% SDS, 10 mM DTT (Sigma), 20% Glycerol (Genfarma bv, Zaan-dam), 0.2M Tris-HCl, pH 6.8 (Duchefa Biochemie, 0.05% Bromophenolblue (Bio-Rad)) were added. The samples were boiled for 3 min prior to SDS-PAGE analysis.

SDS-PAGE was performed in a Mini-Protein III cell (Bio-Rad) using 12.5% gels with 0.1% SDS according to the manufacturer's instructions (all chemicals for polyacryl-amide gels were from Bio-Rad). PageRuler™ Prestained Protein Ladder (Fermentas, # SM0671) was used as molecular weight marker. Proteins were stained with Coomassie Brilliant Blue R concentrate (Sigma), diluted and used as recommended by the manufacturer or with Silver Stain (freshly prepared) as described [23].

2.4 In-gel digestion

In-gel digestion of proteins was performed according to the protocol of Shevchenko et al. [24]. Two lanes (one containing the non-COPD control and one the COPD patient sample)

Figure 1. SDS-PAGE analysis (silver stain) of ELF samples (10 µg of total protein per lane) from a COPD patient (left lane) and a non-COPD control (right lane). Lanes were cut in 43 (1.5 mm wide) slices for in-gel digestion and LC-MS/MS analysis.

from a silver-stained gel (Fig. 1) were cut into 43 equal slices of 1.5 mm width each. Trypsin (Promega, sequencing grade modified trypsin, # V5111) was used (10 µg/mL) for digestion overnight at 37°C with shaking at 450 rpm (Eppendorf Thermomixer). The reaction was quenched by adding 0.75 µL formic acid (FA). The final volume of each sample was about 20 µL.

2.5 ChipLC-MS/MS

Proteins were identified after in-gel digestion by nanoLC-MS/MS using a microfluidics (chip-cube) interface (Agilent, cat. no. G4240A) including a chip (Agilent, cat. no. G4240-62002) with a 40 nL enrichment column (75 µm × 11 mm) and a 75 µm × 150 mm separation column packed with Zorbax 300SB (C-18, 5 µm) chromatographic material. The interface contained a nanoelectrospray tip (2 mm length with conical shape: 100 µm od × 8 µm id) that was coupled online to an ion-trap mass spectrometer (MSD-Trip-SL, Agilent). Injections (3 µL) were performed with an autosampler (Agilent, cat. no. G1389A) equipped with an injection loop of 8 µL and a thermostated cooler maintaining the samples in the autosampler at 4°C during analysis. The chipLC-MS/MS system contained the following additional modules: nanopump (cat. no. G2226A), capillary loading pump (cat. no. G1376A), solvent degasser (cat. no. G1379A). Two eluents (eluent A, 0.1% FA in water and eluent B, 0.1% FA in ACN) were used for the nanopump and one for the capillary pump (0.1% FA, 3% ACN in water). After elution for 5 min with 97% of eluent A, a linear gradient from 3% to 53% of B in 57 min followed by a step gradient from 53 to 90% of eluent B in

5 min was run. Ninety percent of eluent B was maintained for 10 min to regenerate the column.

For acquisition of MS/MS spectra, the following parameters were used: spray voltage: 1.9 kV, drying gas (N_2): 4.0 L/min, dry temperature: 300°C, skimmer: 40.0 V, cap. exit: 200.0 V, Oct. 1: 12.0 V, Oct. 2: 2.50 V, Oct. RF: 200.0 Vpp, Trap drive: 78.0, Lens 1: –5.0 V, Lens 2: –60.0 V; polarity: positive, maximal accumulation time: 15 msec, scan from 50 to 2200 m/z , averages: 4, target mass: 622.0 m/z . The original MS/MS spectra were analyzed with the Data Analysis software (Bruker Daltonics, version 3.4). Compounds were assigned across a retention time window of 18–75 min and deconvoluted with respect to charge states and isotopes.

2.6 Protein identification after in-gel digestion and chipLC-MS/MS

MS/MS spectra were exported as Mascot generic files (Matrix Science, London, UK) and submitted to a web-based version of Mascot (v2.4) to query the SwissProt database (release 2012_07, taxonomy “*Homo sapiens* (human),” 20 232 sequences). The search parameters are listed in Table S4. All listed proteins were identified based on a minimum of two unique peptide sequences with $p < 0.05$ (ions score > 37) and a maximum false discovery rate of 5% (see Supporting Information Table 1).

2.7 Stable isotope labeling

ELF samples containing 50 μ g protein each (about 250–300 μ L) were used for iTRAQ® labeling (see Table 2 for details about the selected subjects). Samples were diluted with 5 mL of 10% ACN in 0.1% aqueous trifluoroacetic acid and concentrated by ultrafiltration (Concentrators, Spin 5K MWCO, 4 mL, Part no. 51855991, Agilent). The retentate was dried under vacuum (Eppendorf Concentrator 5301). After dissolution in 0.5 M triethylammonium bicarbonate (TEAB) and denaturation in 2% SDS, reduction with 50 mM tris-(2-carboxyethyl)phosphine (TCEP) and alkylation with 200 mM (MMTS), each sample was digested overnight at 37°C with trypsin (Promega, sequencing grade modified trypsin, # V5111) at an enzyme to protein ratio of 1:6. Subsequently each sample was labeled (ABSciex, iTRAQ® Reagent-8Plex) for 2 h at room temperature according to the manufacturer's protocol [25]. Four COPD (labeled with tags 113, 114, 115 and 116, respectively) and four non-COPD control samples (labeled with tags 117, 118, 119 and 121, respectively) were labeled. The individually labeled digests were combined into a single sample mixture of equal original protein amount of 50 μ g. In order to remove excess iTRAQ® reagent and pre-fractionate to reduce complexity, the peptide mixture was subjected to strong-cation exchange chromatography (PolyLC [410 992–5400], 2.1 $\times$ 200 mm column, Columbia, Maryland, USA) at 0.2 mL/min (AKTA Purifier 10 with frac-900 fraction collector, GE Healthcare BioSciences AB, Uppsala, Sweden)

using the following buffers: A: 5 mM KH_2PO_4/H_3PO_4 pH 3, 25% ACN and B: 5mM KH_2PO_4/H_3PO_4 pH 3, 25% ACN, 1.0 M KCl. The gradient was divided into three segments: 0–15% B [12 column volumes; one column volume corresponds to 0.69 mL], 15–50% B (3 column volumes), 50–100% B (5 column volumes) at a gradient slope of 10 mM KCl/min. The resulting peptide-containing fractions (45 out of a total of 60 fractions, 0.2 mL per fraction) were pooled, according to their UV absorbance at 280 nm to obtain 24 fractions of approximately equal peptide amount and dried under vacuum in a centrifuge (Eppendorf Concentrator 5301).

2.8 Comparative protein analysis by LC-MS/MS and MALDI TOF/TOF

Dried fractions were dissolved in 100 μ L 2% ACN/0.1% TFA. Four microliters were trapped on a pre-column (300 μ m $\times$ 5 mm, C18 PepMap300) and separated on a C18 capillary column (C18 PepMap 300, 75 μ m $\times$ 150 mm, 3 μ m particle size) mounted on an Ultimate 3000 nanoflow LC system (Dionex, Amsterdam, The Netherlands). Solutions of 0.05% TFA in water (i) and 80% ACN, 0.05% TFA in water (ii) were used for elution using a two-step gradient from 4 to 40% B in 50 min and from 40 to 60% B in 10 min at a flow rate of 300 nL/min. The column effluent was mixed 1:4 v/v with a solution of 2.3 mg/mL α -cyano-4-hydroxycinnamic acid (LaserBio Labs, Sophia-Antipolis, France) in 60% ACN/0.07% TFA. Fractions of 12 s were spotted on blank MALDI targets with a Probot system (Dionex, Amsterdam, The Netherlands). Mass spectrometric analysis was carried out on a 4800 Proteomics Analyzer MALDI-TOF/TOF instrument (Applied Biosystems, Foster City, CA, USA; 4000 Series Explorer v3.5 software) using fixed laser intensity with a uniformly random spot search pattern. MS data were acquired automatically over a mass range of 900–5000 Da in the positive-ion reflector mode. In each MS spectrum the 15 most abundant ions with a signal-to-noise level above 100 and 20 in the m/z range 900–2000 and 2000–5000, respectively, were selected as precursors for MS/MS fragmentation (2 kV acceleration voltage; air as collision gas at 4×10^{-7} Torr; precursor mass window set at 200 resolution (full width at half maximum); metastable suppression enabled). MS/MS acquisition was set to a minimum of 1000 and a maximum of 2000 shots per spectrum with 50 shots per sub-spectrum. The stop-condition criteria were set to a minimum S/N of 100 on at least seven peaks per spectrum after the minimum of 1000 shots. Peak lists of the acquired MS/MS spectra were generated using default settings and a S/N threshold of 10. Delay time was determined automatically based on the mass focus (m/z).

2.9 Protein identification and quantification

Proteins were identified based on the acquired peptide MS/MS spectra using Protein Pilot® software v2.0 (Applied

Biosystems). The search was performed against the IPI Human database (IPI_v3_72, May 2010, 86 417 entries). The ProteinPilot® Cutoff score was 1.3, which corresponds to a confidence limit of 95% at the peptide level. User defined options were: (i) cysteine alkylation (methyl methanethiosulfonate), (ii) trypsin digestion, and (iii) thorough identification search. The results were exported to Microsoft Excel.

Protein identifications were subsequently confirmed using Mascot (version 2.1, Matrix Science, London, UK) searching against the human sequence database (IPI Human database, IPI v3_72, May 2010, 86 417 entries) and subsequently validated in Scaffold® version 3.0 (version_3_00_07, www.proteomesoftware.com). The search parameters in Mascot are listed in Supporting Information Table S5. Protein identifications were based on at least two unique peptides identified independently, with a probability higher than 95% and a false discovery rate of less than 5% at the protein level.

2.10 Statistical analysis

In order to assess whether there is a statistically significant difference between protein levels in ELF from COPD patients versus non-COPD controls, the following approach was used:

- (i) Calculation of peak areas and determination of the ratios of the peak areas for all tags.
- (ii) Normalization of peak area ratios to iTRAQ-113.
- (iii) Transformation of calculated ratios to $\log_{10}$ to approximate a normal distribution.
- (iv) Calculation of *p*-values of the log-transformed ratios between COPD and non-COPD control samples for each peptide using a two-tailed Students *t*-test.
- (v) Exclusion of all peptides with non-significant ratio differences (*p*-value > 0.05).
- (vi) Visual inspection of reporter ion spectra of significantly differing proteins and representation of peak areas in the form of box-and-whisker plots.

2.11 Immunohistochemistry

Immunohistochemistry was performed to compare lung transplant tissue from five COPD patients (current smokers), five COPD patients (ex-smokers), five non-COPD controls (current smokers) and five non-COPD controls (never smokers) (see Table 3 for details about patients and controls). Three-micrometer thick lung sections were cut from selected formalin-fixed paraffin-embedded tissue blocks and mounted on to APES (aminopropylethoxysilane)-coated glass slides (Sigma-Aldrich, Deisenhofen, Germany). Sections were deparaffinized (2 × 10 min with xylol, 2 × 100%, 2 × 96%, 1 × 70% ethanol and 1 × rinsed in demineralized water) and subsequently washed in PBS. Antigen retrieval was performed by microwave treatment for lactotransferrin, high mobility

group protein B1 (HMGB1) and cofilin-1 as follows: after preheating the solution (1 mM EDTA, pH 8.0) at 100°C, the slides were inserted into a plastic container and heated in a microwave oven for 15 min at 300 W, cooled in 1 mM EDTA, pH 8.0 at room temperature and washed three times with PBS. Antigen retrieval for alpha 1-antichymotrypsin (Serp A3) was performed in 0.1 M Tris-HCl, pH 9.0 at 80°C overnight. Samples were cooled to room temperature for 30 min and washed with PBS. Endogenous peroxidase activity was blocked by incubating the slides for 30 min at room temperature with 0.3% H₂O₂ (Merck, Germany) in PBS (500 µL 30% H₂O₂ in 50 mL PBS). After washing three times with PBS, sections were incubated with the following primary antibodies. Cofilin-1: CFL1 monoclonal antibody (M04, clone 1A1, purified mouse immunoglobulin, H00001072-M04, Abnova, Taipei, Taiwan). Serpin A3: monoclonal antibody (M04, clone 3F5, purified mouse immunoglobulin, H00000012-M04, Abnova). HMGB1: monoclonal antibody (M03, clone 1B11, purified mouse immunoglobulin, H00003146-M03, Abnova). Lactotransferrin: monoclonal antibody (clone 1C6, NB120–10109, Novus Biologicals, LLC, Littleton, CO, USA) diluted 1/100 in PBS containing 1% BSA for 1 h at room temperature. Sections were subsequently washed three times with PBS and incubated with a peroxidase-labeled secondary anti-mouse antibody (antiserum raised in rabbits, DAKO, Glostrup, Denmark, 1/100 diluted in PBS/1% BSA + 1% antiserum) for 30 min at room temperature.

After washing three times with PBS, sections were incubated with peroxidase-labeled goat anti-rabbit antiserum (DAKO, Glostrup, Denmark, 1/100 diluted in PBS/1% BSA + 1% antiserum) for 30 min at room temperature. After washing the sections three times with PBS, peroxidase activity was visualized by incubation with 50 mL of 3,3-diaminobenzidine as substrate in the presence of 50 µL 30% H₂O₂ for 10 min at room temperature followed by rinsing with demineralized water. Sections were counterstained with haematoxylin for approximately 2 min, rinsed in tap water, dehydrated in 70, 96 and 100% ethanol and then dried. Finally sections were mounted with mounting medium and covered with a cover slip.

A semi-quantitative analysis for cofilin-1 was performed in a blinded fashion by two persons (ML and LF). The 20 stained tissue sections were scored based on staining intensity on a scale from 0 to +3 (0 = negative; +1 = weak; +2 = positive; +3 = strongly positive). In case of uncertainty on the level of staining a score of 0.5 was assigned.

3 Results

3.1 ELF analysis by SDS-PAGE and chipLC-MS/MS

To map the ELF proteome, samples from a COPD patient and a non-COPD control were subjected to protein separation by SDS-PAGE (Fig. 1). Forty-three bands from each lane were cut, subjected to trypsin digestion and subsequently analyzed by chipLC-MS/MS resulting in a total of 269 identified

Table 3. Characteristics of subjects for immunohistochemistry in lung tissue

	COPD current smokers (<i>n</i> = 5)	COPD ex-smokers (<i>n</i> = 5)	Non-COPD current smokers (<i>n</i> = 5)	Non-COPD never smokers (<i>n</i> = 5)
Female, number	0	0	4	4
Age, years	69 (44–72)	72.5 (65–76)	65 (58–68)	65 (36–70)
Pack years, number	36 (27–58)	30 (8–50)	25 (13–38)	0
Stop smoking, years	0	17	0	0
FEV ₁ , % predicted	75.5 (50–78)	67 (50–74)	91 (76–118)	114 (101–127)

Values are medians (ranges), or numbers.

FEV₁, forced expiratory volume in 1 s.

proteins (1349 peptides) of which 193 were identified in both ELF samples. Analysis of the ELF sample from the COPD patient resulted in 239 identified proteins while 223 proteins were identified in the non-COPD control sample. Forty-six of the 269 proteins were only identified in the COPD sample, while 30 were only identified in the non-COPD control sample (see Supporting Information Table 1). Annotation of proteins that were only identified in ELF from the COPD patient revealed a set of potentially interesting functions. A member of the nicotinic acid-related structural protein family (nicotinate phosphoribosyltransferase, accession number *Q6XQN6* and nicotinamide-phosphoribosyltransferase, accession number *P43490*) that is related to the host response to bacterial infections was identified. The presence of protein *S100A8* (accession number *P05109*), a member of the calgranulin superfamily [26], may reflect the chronic inflammation associated with COPD development and progression. Presence of the enzyme leukotriene A-4 hydrolase (LTA-4hydrolase, accession number *P09960*) [27] indicates a key role of oxidative stress in COPD, while glutathione S-transferases A2 (accession number *P09210*), A3 (accession number *Q16772*) and theta-1 (accession number *P30711*) [28], detoxifying enzymes that protect against oxidative stress, were only identified in ELF from the non-COPD control.

ELF from the COPD patient contained a number of Ras-related proteins (RAB1B, RAB10, RAB35; accession numbers *P62820* or *Q9H0U4*, *P61026* and *Q15286*, respectively). This may indicate involvement of EPAC proteins in obstructive airways diseases [29] and their anti-inflammatory effect in alveolar macrophages [30, 31]. Many proteins involved in proteasome activation and regulation (Proteasome activator complex subunit 3 and proteasome subunit beta type-4; accession numbers *P61289* and *P28070*, respectively) were only identified in ELF from the COPD patient, a result that points towards the involvement of the proteasome during conditions of inflammation and oxidative stress.

Although most of the tubulin protein family members were identified in ELF from the COPD patient and the non-COPD control (TBA1B, TBA1A, TBB5, TBA1C, TBB2A, TBB3, TBA8; accession numbers: *P68363*, *Q71U36*, *P07437*, *Q9BQE3*, *Q13885*, *Q13509*, *Q9NY65*, respectively), some types such as TBB2B and TBA4A (accession numbers: *Q9BVA1* and *P68366*, respectively) were not detected in ELF from the COPD patient.

3.2 Comparative quantitative proteomics using iTRAQ® labeling

Shotgun proteomics with stable isotope labeling using the iTRAQ® 8-plex reagents and MALDI-TOF/TOF MS lead to the identification of 138 proteins based on 1745 iTRAQ®-labeled peptides with a confidence level of >95% for Study I, and 161 proteins (1205 iTRAQ®-labeled peptides with a confidence level of >95%) for Study II (see Supporting Information Tables 2 and 3).

Each study resulted in a number of proteins with statistically significantly different concentration levels in ELF between COPD patients and non-COPD controls (see Supporting Information Tables 6 and 7). The levels of four proteins, lactotransferrin (accession number *P02788*), alpha 1-antichymotrypsin (accession number *P01011*), cofilin-1 (accession number *P23528*) and HMGB1 (accession number *P09429*), were significantly different ($p < 0.05$) in both studies.

The protein sequence selected for quantification was based on unique peptides identified in both studies. In particular, 13 unique peptides were identified for lactotransferrin, three for HMGB1, four for cofilin-1 and two for alpha 1-antichymotrypsin in Study I.

In Study II, 21 peptides for lactotransferrin, four for HMGB1, four for Cofilin-1 and three for alpha 1-antichymotrypsin were identified, respectively.

Some of the peptides were redundantly identified, but only unique peptides specific for that protein with $p < 0.05$ and confidence of identification >95% were selected for the final quantification.

Lactotransferrin was increased in ELF of COPD patients (Fig. 2A and B) based on the analysis of two unique peptides that were found in Study I and Study II (QVLL-HQQAK and CVPNSNER). Comparative sequence analysis using Scaffold® established that there is no ambiguity with respect to other proteins belonging to the same superfamily, such as serotransferrin. The increase in lactotransferrin may be related to its antimicrobial properties [32]. In fact it has been demonstrated that secretion of antimicrobial proteins by epithelial cells is increased upon airway inflammation, which supports our findings [33]. A search against a sequence database covering eubacteria excluded the possibility that this protein was of bacterial origin (data not shown). Although not statistically significant in Study I, we also found lysozyme C

Figure 2. Box and whisker plots of the levels of three proteins that differed significantly in ELF from COPD patients versus non-COPD controls in two independent studies (Study I and Study II). Lactotransferrin ((A) Study I, peptide QVLLHQQAK and (B) Study II, peptide CVPNSNER); HMGB1 ((C) Study I, peptide GKFEDMAK and (D) Study II, peptide GKFEDMAK); Serpin A3 ((E) Study I, peptide ADLSGITGAR and (F) Study II, peptide ADLSGITGAR). Each error bar represents the variation inside the group (see Supporting Information Table 7).

(a protein of the innate immune system that also exhibits antibacterial properties) to be increased in ELF from COPD patients ($p < 0.05$ in Study II) based on three unique peptides (GISLANWM, TPGAVNACHLSC and WESGYNTR). Immunohistochemistry (IHC) of lactotransferrin revealed a strong positive staining in glands in lung tissue from COPD patients and non-COPD controls (see Supporting Information Fig. 1) indicating that higher levels of lactotransferrin in ELF are likely due to increased secretion by epithelial cells in COPD patients.

Our analyses (Study I and Study II) showed that HMGB1 is reduced in ELF of COPD patients relative to non-COPD controls (Fig. 2C and D), which contrasts with an earlier study in BALF of COPD patients [34]. Analysis with Scaffold®

showed that the selected peptides were specific for HMGB1, excluding a possible affiliation with similar proteins such as HMGB2. Immunohistochemical analysis of lung tissue from COPD patients and non-COPD controls (current, ex and never smokers) showed that HMGB1 is mainly present in the nuclei of epithelial cells and macrophages without visible differences in abundance (see Supporting Information Fig. 2). HMGB1 is thus likely released into ELF due to leakage from epithelial cells associated with necrosis and cellular turnover.

The alpha 1-antichymotrypsin (Serpin A3) level was increased in ELF from COPD patients (Fig. 2E and F) in both studies. Since immunohistochemistry of lung tissue showed protein expression in alveolar macrophages (high levels) as

Figure 3. Box and whisker plots of the levels of cofilin-1. (A) Study I, peptide VFNDMK and (B) Study II, peptide CTIAEK. (C) Box and whisker plots of the levels of cofilin-1 in ELF of smokers (current and past) and never-smokers (peptides VFNDMK and CTIAEK, respectively). Each error bar represents the variation inside the group (see Supporting Information Table 7).

Figure 4. Immunohistochemistry of cofilin-1 in lung tissue from (A) a COPD patient (current smoker), (B) a non-COPD control (never-smoker) and (C) a non-COPD control (current smoker), (D) and (E) are negative staining controls.

well as in epithelium and endothelium (moderate levels) without visible differences in abundance in lung tissue from COPD patients and non-COPD controls (current, ex and never smokers) (see Supporting Information Fig. 3), we conclude that secretion of alpha-1-antichymotrypsin is increased in COPD patients.

Remarkably, elevated levels of cofilin-1 were present in ELF from COPD patients in Study I (Fig. 3A), while they appeared to be decreased in Study II (Fig. 3B). Comparative sequence analysis using Scaffold® established that there is no ambiguity with respect to other proteins. These, at first sight, contradictory results, were reconciled when taking the

smoking history of the subjects into account. The ELF control samples used in Study I were all from never-smokers (see Table 2), while two of the controls in Study II were current smokers and one was an ex-smoker, although without COPD. When reclassifying our samples into smokers (current and past) and never-smokers, we found that cofilin-1 is significantly increased in ELF from smokers, irrespective of them having COPD (Fig. 3C). These results were confirmed by immunohistochemistry (Fig. 4). In fact, a semi-quantitative, blinded immunohistochemical analysis of lung tissue from current smokers with or without COPD showed increased staining compared with non-COPD controls who

Figure 5. Semi-quantitative scoring of staining intensity of the immunohistochemical analysis of cofilin-1 in lung tissue. COPD curr = COPD patients (current smokers); COPD ex = COPD patients (former smokers); Ctrl curr = non-COPD controls (current smokers); Ctrl non = non-COPD controls (never smokers). Each dot represents a single patient or control. All analyses were performed by two independent investigators in a blinded fashion (ML and LF).

never smoked (Fig. 5). This corroborates previously published data showing that cofilin-1 is increased in lung tissue of non-COPD smokers [26].

The reproducibility of the quantification for the chosen peptides was confirmed for all the selected proteins (coefficient of variation of the peptide area ratios < 1; see Supporting Information Table 9).

4 Discussion

4.1 ELF proteomics by SDS-PAGE and chipLC-MS/MS

This is the first detailed proteomics study of human ELF based on chipLC-MS/MS with a focus on deciphering changes at the proteome level that are related to COPD. Proteomic analysis of ELF by SDS-PAGE followed by in-gel digestion and chipLC-MS/MS resulted in identification of 269 proteins in samples from COPD patients and non-COPD controls. The identified proteins (see Supporting Information Table 1) belong to families that are involved in various aspects of COPD pathogenesis, such as inflammation, oxidative stress, bacterial infection, the acute-phase response and the protease-antiprotease balance [35].

A potent antioxidant system in the airways is based on glutathione (GSH). It has been shown that alterations in the balance between GSSG, the oxidized form of glutathione, and its reduced form (GSH) are related to oxidative stress in lung epithelial cells and that exposure to cigarette smoke decreases the levels of intracellular GSH [36]. It is interesting to note that GST A1, A2, A3 and theta-1 were only identified in ELF of the

non-COPD control [37]. Our finding corroborates published data showing increased GST gene expression in the bronchial epithelium of smokers without COPD [28], polymorphisms that associate with COPD development [38] and the effect of cigarette smoke on this important regulatory enzyme family [39].

One protein of the S100 family, S100A8, was only identified in ELF from the COPD patient. S100 proteins are known to be expressed in epithelial cells and macrophages during chronic inflammation and to be released by phagocytes, after interaction with microtubules, exhibiting pro-inflammatory activity [40]. The identified S100 proteins belong to the calgranulin subgroup known to contribute to experimental and clinical lung inflammation with an elevated concentration in the cytoplasm of neutrophils. Release of calgranulins from neutrophils upon activation is related to their antibacterial activity [41]. Their over expression at sites of inflammation [42] and their action as “amplifiers” of inflammation, affecting cytokine induction, underline a possible role in the development of COPD. Calgranulins may thus constitute important therapeutic targets to modulate inflammation [43, 44].

The qualitative proteomics study revealed the presence of proteasome-related proteins in ELF from the COPD patient. The 26S proteasome has been shown to exist outside the cell (after lung injury) and to contribute to proteolysis of proteins in the alveolar space. Furthermore, it has been demonstrated that the ubiquitin-proteasome system plays a crucial role in maintaining homeostasis of muscle tissue in response to hypoxemia as well as during inflammation and oxidative stress [45, 46].

Some members of the tubulin family (TBB2B and TBA4A), a class of GTP-binding proteins that are the major constituents of microtubules, were only detected in ELF from the non-COPD control. The interaction between tubulins and actin filaments is fundamental for the structure of the cytoskeleton and the induction of cytoskeletal changes. It is of importance to note that cofilin-1, another actin-binding protein, was found to be increased in ELF of current or past smokers (see below), indicating that exposure to cigarette smoke can influence the rearrangement of the cytoskeleton.

4.2 Quantitative ELF proteomics using iTRAQ labeling

Chemical stable isotope labeling (iTRAQ® 8-plex) in combination with two-dimensional liquid chromatography and MALDI-TOF/TOF mass spectrometry allowed assessing differential protein levels when comparing ELF from COPD patients versus non-COPD controls. Comparison of the ELF proteome in Study I (four COPD patients and four non-COPD controls) and Study II (a different set of four COPD patients and four non-COPD controls) showed that lactotransferrin, cofilin-1, HMGB1 and alpha 1-antichymotrypsin (serpin A3) were significantly different.

Lactotransferrin, an iron-binding protein that is significantly increased in ELF from COPD patients, has bactericidal

and bacteriostatic properties, giving it a major role in the first-line defense against microbial infections and in the prevention of systemic inflammation. Immunohistochemistry of lung tissue from COPD patients showed that lactotransferrin is primarily expressed in submucosal glands as well as in serous and acinar cells (see Supporting Information Fig. 1). Lactotransferrin is present in different secretory fluids (e.g. milk, saliva, and nasal secretions) and is, together with lysozyme C, the most abundant antimicrobial protein in human airways [32]. Previous studies have demonstrated higher concentrations of these two proteins in the airways of patients suffering from cystic fibrosis as well as in patients with COPD, asthma, chronic bronchitis, and pneumonia [32].

Lactotransferrin kills bacteria by binding iron with very high affinity, which is essential for survival of bacteria and for stabilization of the bacterial membrane, explaining the increased levels in COPD patients, where a continuous inflammatory process is ongoing rendering patients susceptible to bacterial infections. The capability of lactotransferrin to scavenge free iron ions is also important for development of the adaptive immune response as well as for the reduction of oxidative stress, since metal ions catalyze the generation of free radicals [28, 47]. Recently, it has been proven that free iron ions may be involved in the pathogenesis of airways disease, notably COPD and asthma [35]. Interestingly, lactotransferrin binds to lipopolysaccharide (LPS) with high affinity, thus preventing its association with other LPS-binding proteins and blocking the transfer of LPS to the CD14 receptor on the surface of macrophages [48]. Lactotransferrin exhibits anti-inflammatory activity through this mechanism by down-regulating the release of cytokines from monocytes and reducing recruitment and activation of immune cells at inflammatory sites [49–51].

Alpha 1-antichymotrypsin (Serpine A3), a member of the acute-phase protein family, was shown to be increased in ELF from COPD patients (Fig. 2E and F). Immunohistochemistry of lung tissue showed that this protein was primarily and extensively present in alveolar macrophages as well as in epithelium and endothelium (see Supporting Information Fig. 3). The plasma concentration of alpha 1-antichymotrypsin can increase up to 100-fold during inflammation or infection induced by cytokines. This serine proteinase inhibitor, that principally targets cathepsin G, is also involved in the inflammatory response associated with tumor growth and invasiveness and there is evidence that it can be produced by lung adenocarcinomas [52]. Even though alpha 1-antichymotrypsin is primarily produced in the liver, there is evidence of its secretion from alveolar epithelial cells and alveolar macrophages [53, 54].

HMGB1, a member of the class of “alarmins,” is not only a DNA-binding protein but has also been shown to be involved in the development and progression of acute lung injury. Different studies [55] demonstrated its pro-inflammatory activity by binding to cytokines such as IL-1 β and TNF- α . It was surprising to find that in our studies the level of HMGB1 was increased in non-COPD controls (Fig. 2, panels C and D) in contrast to published data on BALF from COPD patients [34].

Our quantitative results are based on the analysis of three peptides, one of which (GKFEDMAK) overlapped between the two studies. Analysis with Scaffold® showed that the selected peptides were specific for HMGB1, excluding a possible affiliation with similar proteins such as HMGB2. Since our quantitative results were reported as ratios with respect to the 113 label, we verified the influence of normalization on the final result by repeating the data analysis with normalization to each of the other seven labels (data not shown). This confirmed that normalization did not affect the overall result showing an increased level in ELF from non-COPD controls as compared to COPD patients. Further studies are required to address this apparent discrepancy.

Extracellular HMGB1 is one of the main diffusible signals of necrosis. HMGB1 binds to the receptor for advanced glycation end products (RAGE) with high affinity, while it remains tightly bound to DNA during apoptosis [56]. Immunohistochemistry of lung tissue showed that HMGB1 is localized in the nuclei of epithelial cells indicating that its presence in ELF is due to release from these cells (see Supporting Information Fig. 2). It is noteworthy that the function of HMGB1 may be altered by post-translational modifications (acetylation, phosphorylation, oxidation and methylation) that can, for example, affect the binding to RAGE. The differential occurrence of modifications may provide one possible explanation for the conflicting findings obtained by quantitative proteomics in ELF (this study) and those reported by others using ELISA assays in BALF [34]. Taking the pluripotency of HMGB1 into account, its interaction with different types of receptors (not only RAGE, but also toll-like receptor) and its involvement in a range of signaling pathways, it is important to keep in mind that this protein alone has only minimal pro-inflammatory activity [57, 58].

Cofilin-1, a protein that we found to be increased in ELF and lung tissue from smokers (current and past), irrespective of COPD, is an actin depolymerizing factor, mainly involved in cytoskeletal changes and is the major form of cofilin in non-muscle tissue. After binding to cofilin, actin is converted into its active monomer and transported to the nucleus, where it can activate RNA polymerase II and promote gene transcription [59]. Cofilin-1 is found in association with actin rods during cellular stress, in the nucleus or in the cytoplasm, and protects actin until the stress is over [60]. Previous data show that the levels of active cofilin and ADP-actin increase dramatically in response to oxidative stress due to an elevated level of reactive oxygen species (ROS) and a decline in ATP levels resulting in the formation of cofilin-actin rods [61]. It is thus important to maintain the level of ATP above a certain threshold in order to allow a cell to recover. In this balance the contribution of cofilin-1 is essential, because a large amount of active cofilin can be sequestered by the rods thus reducing depletion of ATP, since ATP hydrolysis is associated with actin turnover, which is in turn promoted by cofilin [61]. Klemke et al. showed the link between the formation of oxidized cofilin and oxidative stress, by direct incubation of the protein with H₂O₂ [62]. Oxidized cofilin, despite its binding to F-actin, was no longer capable to promote

depolymerization, creating a link between oxidative stress caused by ROS and cellular hyporesponsiveness. Recently, a phosphorylated (on Ser³) inactive form of cofilin-1 was found in response to ROS, further emphasizing the influence of stress factors, such as exposure to tobacco smoke, on the regulation of cellular functions by cofilin.

Our study of human ELF shows the power of combining different methods (microfluidics-based nanoLC-MS/MS, iTRAQ® labeling and immunohistochemistry) to compare expression levels of different proteins in samples of limited availability and to elucidate their localization in lung tissue. Due to the limited number of patients and controls and the lack of well-matched COPD and non-COPD samples, this should be considered a pilot study that requires verification in larger sample sets. Our study demonstrates the feasibility of proteomics investigation in human ELF and represents a first approach to perform quantitative studies on pulmonary diseases using this biofluid. Despite their exploratory character, our results show the possibility to identify proteins related to biological or clinical features of COPD, underlying the power of combining the novelty of using ELF and different analytical methods of analysis.

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