Development and application of proteomics as aid for unraveling smoke-induced COPD

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Chapter I

Introduction

Chronic Obstructive Pulmonary Disease: Definition and pathological mechanisms

Chronic Obstructive Pulmonary Disease (COPD) is the fifth cause of death worldwide and is ranked to become the third by 2020 by the World Health Organization (www.who.int/whr) [1]. COPD is a disease of increasing prevalence and mortality worldwide, but despite this it is still not well recognized, hence under-diagnosed and patients with COPD thus suffer from under treatment [2]. COPD often severely limits activities of daily living [3] and impairs health-related quality of life [4].

According to the Chronic Obstructive Lung Disease (GOLD) guidelines [www.goldcopd.org], COPD is defined as "a common preventable and treatable disease that is characterized by persistent airflow limitation that is usually progressive and associated with an enhanced chronic inflammatory response in the airways and the lung to noxious particles or gases. Exacerbations and comorbidities contribute to the overall severity in individual patients".

Airflow limitation in COPD is characterized by a post-bronchodilator Forced Expiratory Volume in one second (FEV_1)/Forced Vital Capacity (FVC) ratio below 0.70. In the past COPD was classified into four stages of disease severity, from mild to very severe (GOLD 1-4) based on FEV_1% predicted [Table 1]. [www.goldcopd.org, report, 2013]. This staging will be used in this thesis, given the timeframe in which the studies were performed.

Table 1. COPD stages based on the GOLD standard definition (GOLD report, February 2013)

GOLD stage	Characteristics (in patients with $FEV_1/FVC < 0.70$)
GOLD 1 : Mild	$FEV_1 \geq 80\%$ predicted
GOLD 2: Moderate	$50\% \leq FEV_1 < 80\%$ predicted
GOLD 3: Severe	$30\% \leq FEV_1 < 50\%$ predicted
GOLD 4: Very Severe	$FEV_1 < 30\%$ predicted

COPD encompasses both chronic bronchitis, small airways disease and emphysema. Chronic bronchitis is defined by the presence of cough and sputum production for at least three months for at least two subsequent years (without including the presence of airway obstruction). Small airways obstruction is one of the hallmarks of COPD, and can be present at an early stage even when FEV_1 is not affected. On the other hand, emphysema is characterized by enlargement of airspaces, destruction of lung parenchyma, and loss of lung elasticity with subsequent closure of small airways [5] [6]. COPD patients frequently suffer from both or all components. In the last decade the clinical heterogeneity of COPD has been extended to other (sub)phenotypes, for example the presence of hyperresponsiveness, being a frequent exacerbator, or suffering from systemic inflammation and other extra pulmonary organ dysfunctions.

The pathogenesis of COPD has been extensively investigated and three pathogenic factors are believed to play a key role in the development and progression of the disease: (1) imbalance in proteases-antiproteases, (2) increased airway inflammation and (3) oxidative stress [7].

As COPD is a very complex and heterogeneous disease, characterized by different clinical and pathological features it is not surprising that the initiating triggering factors are largely unknown. In this thesis we propose that a better understanding of the pathogenesis of COPD is only possible if risk factors responsible for its initiation are being studied in context of the complex clinical and pathological picture of COPD.

Risk factors

Different risk factors contribute to the onset and progression of COPD, first of all active cigarette smoking, but also passive smoking, air pollution, early reduced lung growth and genetic factors. Furthermore, indoor air pollution from biomass fuel is an important cause [8] as well as outdoor air pollution [9] [10], although their role is still poorly understood. Undoubtedly tobacco smoke represents the most important risk factor, however only 15-20% of smokers develop COPD, suggesting that other conditions and the individual susceptibility play an important role. In the Western world, the prevention of cigarette smoke exposure has shown to prevent significantly the onset of COPD [11].

Although cigarette smoking is the primary environmental risk factor, genetic risk factors likely influence the development of COPD.

TNFα is a key player in the inflammatory response, and is elevated in smokers with bronchitis and higher airflow obstruction [12]. With a candidate gene approach, genetic variants in or near the transforming growth factor-β1 (*TGFB1*) gene were linked with the pathogenesis of COPD among cigarette smokers [13] [14] [15] [16] [17]. Linkage analysis was followed by association analysis of single nucleotide polymorphisms in *TGFB1* on chromosome 19q and COPD phenotypes in a family-based sample and a case–control study (cases with severe COPD and control subjects with significant history of smoking but no COPD).

A well-known genetic factor that has been identified to play a role in the pathogenesis of COPD is the gene encoding for *α-1 antitrypsin*. It has been proven that about 1% of COPD patients are *α-1 antitrypsin* deficient, therefore genetically predisposed to develop the disease [18] More recently other genes have been proposed to play a role in this disease, like the gene coding for a disintegrin and metalloprotease *(ADAM)33* [19] [20]. Recent GWAS studies have discovered further candidate genes, that are replicated in several studies like *TNF*, *GST*, *EPHX1*, *SOD3*, *CHRNA* [21] [22] [23] [24].

Mutations in the Glutathione S-Transferases enzymes have been associated with the onset and development of COPD as well. Studies in men with irreversible airflow obstruction *vs* control current smokers (without signs of COPD) showed a polymorphism of *GSTP1* to be associated with COPD [12].

Following α-1 antitrypsin mutations as a proven genetic risk factor for COPD, *α-1 antichymotrypsin*, (another plasma proteinase inhibitor) was studied. Despite two point mutations were identified in patients in different studies, these first results failed to be replicated later on by other groups, making this protein unlikely to be a contributor to the development of the disease [12].

A combined genome-wide analysis data from participants in the COPDGene Study, ECLIPSE, NETT/NAS and GenKOLS studies, confirmed the association to COPD at three different loci: *CHRNA3*, *FAM13A* and *HHIP*. Additionally, 3 other loci were subsequently associated with the disease: *RIN3*, *MMP12* and *TGFB2* [25].

Our current knowledge regarding the inception of this disease is mostly based on data coming from the elderly population [26] since COPD is a disease that mostly manifests later on in life, while little is known about the onset of COPD due to the lack of information regarding the young adult population [27] [28].

Nonetheless, several research groups have shown that the origins of COPD can be already present in early childhood or even in utero [29]. There is increasing evidence that in susceptible subjects, adverse maternal factors may interact with environmental factors altering the growth and development of the airways already before birth or in early childhood [30]. Furthermore, gene-environment interactions may lead to permanent lung injury and consequently the development of COPD. Some deficits in airway function that can be present already after birth can be predictors of airflow limitation in early adult life, a factor that can predispose to developing COPD [31].

Acute effects of cigarette smoking

In the Western world cigarette smoking has been identified as the primary cause for the onset and development of COPD [32] [33]. Cigarette smoke is the source of considerable quantities of free radicals and reactive compounds [34]. Tobacco smoke exposure results in an innate immune response, increasing the susceptibility to infections, in this way causing further adaptive and innate inflammatory responses [35]. Smoking affects the function of immune cells interfering with epithelial cells, macrophages, neutrophils and lymphocytes [36]. Cigarette smoke can impair phagocytic function and chemotaxis, promote the release of reactive oxygen species (ROS) and compromise the natural defenses of the airways against bacteria. Additionally it impairs the production of surfactant proteins (synthetized by alveolar epithelial type II cells) that contribute to host-defense against microbes. Cigarette smoke further interferes with the clearance promoted by ciliary epithelium. As a consequence of all pathways triggered by cigarette smoke, the lung parenchyma is destroyed and immune homeostasis in the lung is distorted leading to further inflammation, continuing a vicious circle that leads to tissue destruction with a lack of repair [37] [38] [39].

In just one cigarette puff, there are 10^4 free radicals directly inhaled into the lung [40] that can imbalance the local antioxidant defenses promoting inflammatory processes. The

imbalance in the oxidant/antioxidant system can enhance the stimulation of cell surface receptors resulting in the amplification of MAPK, NF-kB and AP-1 signaling pathways, further increasing the inflammatory response [41].

The above mentioned findings with respect to damaging effects of cigarette smoke underline how chronic exposure to cigarette smoke constitutes an important etiologic factor for the onset, development and progression of COPD.

The irreversible effects of smoking on the architecture of the lung in case of tissue destruction are the consequence of the accumulation of many years of inhaling harmful particles, however the cumulative result may be due to effects of the very first oxidative stress, protease imbalance and inflammation produced after acute smoking (<24 hours). It is therefore of prime interest to evaluate the very first response of the airways after inhaling cigarette smoke.

One of the earliest manifestations is a breach in the vascular and airway barrier function with recruitment of circulating inflammatory cells to the lungs. The oxidative burden produced in the airways arises very fast, already within one hour of smoking [42] and protein degradation is measurable within 6 hours after smoking [43]. However, the time at which the proteinase/antiproteinase balance is affected still remains unknown.

Over the years different acute models (*in vitro*, *ex vivo*, *in vivo*) have been used to investigate the inflammatory and oxidative processes that occur in COPD due to smoking. In *in vitro* models, the acute response of isolated cells of the lung, such as epithelial cells, fibroblasts, alveolar macrophages or neutrophils to cigarette smoke extract (CSE) has been investigated [44]. Different signaling pathways, involved in epithelial repair processes and upon tissue inflammation occurring in COPD, such as the Wnt signaling pathway, have been investigated to understand their implication in airway remodeling [45]. Furthermore, studies on the changes in mitochondrial morphology in human bronchial epithelial cells under long-term CSE exposure, have been performed in recent years [46] highlighting how these changes, as a consequence of cigarette smoke, could play a role in the pathogenesis of COPD. Despite the usefulness and informative results obtained from *in vitro* studies on cell models, it is not possible to translate these outcomes regarding the inflammatory process, destruction of the lung parenchyma, and the airways changes that occur easily to human subjects affected by such a complex disease as COPD.

Several studies on animal models have been performed to understand the mechanism underlying the pathogenesis of COPD and the effect of acute smoking as well [47]. As summarised by van der Vaart et al [42] investigations were directed towards the effect of acute smoking, namely inflammation and oxidative stress. Guinea pigs, mice and rats are the most commonly used species, from which different specimens were analysed such as Bronchoalveolar Lavage Fluid (BALF), serum, plasma, and lung tissue.

The outcomes from these studies strengthened the effect of acute smoking on factors like *α-1 antitrypsin*, *TNF-α*, and the *Glutathione* system, as already observed in human stud-

ies, though the different models cannot be easily compared due to the different ways the exposure to smoke was performed.

Different knock-out animal models have been investigated in order to explain the alteration of certain proteins under pathological conditions. For instance, a fucose-deficient mice model (Fut8 -/-), linked the lack of fucosylation of TGF-beta1 to the overexpression of matrix metalloproteinsases (MMPs) and with progressive and destructive emphysema. Additionally, this study proposed these results as possible explanation of human emphysema as well [48]. MMPs are a class of proteinases extensively studied for their implication in the development of emphysema and other airway impairments. Further, in a mouse model lacking *mmp*-12, it was demonstrated that *mmp*-12 -/- mice were protected from the development of cigarette smoke-induced emphysema and from the accumulation of lung macrophages [49] [50].

Despite the various *in vitro* and animal studies that have been performed over the last decades, little is known about the acute effect of smoking in humans. Our group has performed the first studies in man to assess the effects of acute cigarette smoking. The purpose of the study performed by van der Vaart et al [51] was to assess the effects of smoking two cigarettes in healthy intermittent smokers. Interestingly the exposure to acute smoking increases partly the inflammatory responses (regarding above all neutrophils and macrophages, phenomena also observed in chronic smokers) but also decreases some aspects of the inflammation, possibly due to the presence of certain molecules (like CO and nicotine) which have anti-inflammatory and immunosuppressive potential.

Additionally, in order to better understand the mechanisms responsible for the changes occurring in the airways after smoke exposure, the impact of smoking cessation on the lungs was studied by our group. Smoking cessation can lead to improvement of respiratory symptoms and airways hyper responsiveness in smokers (irrespective of the presence of COPD or not) and can also slow the decline in lung function [52]. Therefore quitting smoking can improve the accelerated decline of FEV_1 and certain remodeling processes occurring in the lungs. These findings open the route to speculate about the ongoing acute inflammatory processes following smoke exposure and the subsequent remodeling after smoking cessation.

This thesis builds further on these results and expands the findings by studying effects in healthy smokers and those affected by COPD. In order to address the acute effects of smoking, a bi-center cross-sectional study [53] was initiated to gain a better understanding of both the local and systemic inflammatory processes that occur in smoking-induced COPD.

Susceptibility to develop COPD

Among the smokers, only 15-20% are affected by COPD later on in life. Possibly different factors (such as the genetic background) render these subjects more prone, or more "susceptible" to develop COPD.

Parental history of COPD constitutes a strong risk factor for COPD. For example, McCloskey and colleagues demonstrated that siblings of patients with severe COPD have a

significant risk for irreversible airflow obstruction [54]. Likewise, first-degree relatives of early-onset COPD patients show a lower FEV1 and FEV1/FVC when compared with control subjects [55], but only if they had smoked. Additionally, parental history of COPD represents another high risk factor to develop COPD, independently of a positive family history of smoking, personal lifetime smoking or exposure to other sources of smoke [56].

In this thesis we postulate that studying the acute responses to cigarette smoke in young individuals constitutes an interesting model to unravel the pathogenesis of early-onset COPD. In other words, we are not primarily interested in the effects of chronic smoking in older subjects with already existing structural changes due to smoking and ageing. Particularly the comparison of young subjects who will develop COPD later in life (susceptible individuals) versus those who do not (non-susceptible individuals) may give important information. Because there are no tests to discriminate at young age between susceptible and non-susceptible individuals, we can only rely on family history, as described above. Understanding the link between cigarette smoking and the genetic predisposition to develop COPD may lead to a better recognition and discovery of candidate biomarkers for this disease in the future.

Proteomics of epithelial lining fluid: a tool for investigation in COPD

Proteomics is an increasingly important tool in the discovery of biomarkers for human diseases. In COPD as well as in other lung diseases, several proteomics techniques have been applied in different specimens, such as resected lung tissue sections, bronchial biopsies, bronchoalveolar lavage fluid, induced sputum, exhaled breath condensate, nasal lavage fluid, serum, urine, and cultured cells in order to identify potential candidates that are linked to the disease [57].

Up till now, about 40 protein markers have been identified to be associated with COPD through different proteomic techniques [58].

This thesis investigates Epithelial Lining Fluid (ELF) as a new body fluid since this could open new routes towards understanding the processes responsible for the onset and development of COPD. This is, because this biologically active fluid constitutes the very first barrier to inhaled cigarette smoke.

Epithelial lining fluid forms a thin aqueous layer that covers the inner part of the airways and has been suggested as the site of antimicrobial activity against lung infections caused by extracellular pathogens [59]. This first-barrier of defense is therefore an interesting focus of research, particularly because allergens, microbial agents, and toxic agents from cigarette smoke and air pollution are responsible for lung diseases such as atopic asthma, pneumonia, interstitial pulmonary fibrosis or COPD. ELF contains cells and soluble components that play an essential role in this first line of defense against inhaled particles, gases, microbes and allergens. Consequently, it has been proposed that the protein composition of ELF most faithfully reflects the effects of external factors that affect the lung. Changes in ELF

composition may be of primary importance in the early diagnosis and prognosis as well as in the characterization of pathological processes that are related to the progression of lung disorders.

The diagnosis of COPD is currently based on spirometry and the actual treatments are not very effective or even ineffective to slow down or cure the disease. This clinical burden calls for a new methodology to detect new targets for treatment and cure, a methodology that needs to be able to clarify the molecular mechanisms responsible for the pathogenesis of COPD, and, in parallel, for evaluating the influence of the different risk factors, such as cigarette smoking. Proteomics techniques, characterized by high sensitivity and high throughput, could suit this need [60].

Proteomics, defined as the study of the whole proteome, requires only a small amount of specimen to provide qualitative and quantitative information regarding the protein profiles related to a specific disease. In the field of COPD, a proteomic approach could represent an innovative, unbiased way to discover biomarkers for the disease hence providing the clinicians a tool for a better diagnosis and, later on, treatment of COPD [61].

To our knowledge, proteomics studies on ELF have most frequently used BALF. However, this way of collecting ELF has a number of disadvantages. First, BALF dilutes ELF by a factor of 60-120 [62] and the dilution of ELF may vary from one lavage to the next. Second, the exact location in the bronchial tree from which proteins are derived is not known because BALF is collected from the 4^{th} generation of airways until the alveoli. Analysis of induced sputum might be an alternative to ELF or BALF analysis, since it can be obtained in a non-invasive way. However, the area from which induced sputum is collected is even more variable than that of BALF, and in addition collects specimen from particularly the large airways, while COPD is also present in the small airways and lung parenchyma. Additionally, induced sputum passes the oropharyngeal cavity which contains many bacteria and saliva that may easily contaminate sputum samples.

Bronchoscopic microprobe sampling of ELF is thus a promising technique with several advantages in proteomics research of the lung. This technique uses a small adsorptive pad, which is placed on the mucosal layer of the bronchial wall (2^{nd} generation) under visual control during bronchoscopy. In contrast to the collection of BALF or induced sputum, the area from which ELF is obtained is precisely known. Additionally, there is no dilution of proteins and the risk of contamination with bacteria is minimal, because the probes are protected by an extra sheath within the bronchoscope during entry and exit.

Proteomics of microprobe-sampled ELF may thus open a new area of research in lung disease, particularly in those diseases with a strong inflammatory response to inhaled agents. In that perspective, the response to cigarette smoke and the possible induction of COPD is a particularly promising field for ELF proteomics.

Aims of the studies

In order to better understand the contribution of cigarette smoking to the development of COPD, ELF has been chosen as preferred specimen for proteomics analysis.

We believe that a proteomics approach focusing on ELF represents a new promising tool of investigation that may unravel other aspects of pathogenetic processes leading to COPD than genetic, clinical, cellular and animal studies. The possibility of discovering specific protein markers of COPD in young susceptible individuals and older subjects with established COPD is of great interest for gaining a clearer insight into the initiation phase of COPD. In addition, the comparison of susceptible to non-susceptible individuals may lead to the discovery of "protective" molecules or pathways that are downregulated or upregulated.

In all the recruited individuals studied in this thesis, we first compared COPD patients vs healthy control individuals to provide a first identification of proteins differentially expressed between the two groups. Subsequently we focused on the effect of acute smoking on the protein pattern via qualitative and quantitative proteomic approaches on different groups of young subjects (susceptible or not to develop the disease) and older individuals. We further confirmed the outcomes through other techniques such as immunohistochemistry on lung resection material and immunoassays (ELISA).

Outline of this thesis

In Chapter 2 the method for the collection and processing of Epithelial Lining Fluid (ELF) from COPD patients and healthy controls and its application in proteomics investigations is presented.

Chapter 3 shows the feasibility of performing qualitative and quantitative ELF proteomics leading to the discovery of a number of proteins with differential levels between COPD patients and healthy subjects. Some results were confirmed by immunohistochemistry on lung tissue sections.

In Chapter 4, the patterns of up- and down-regulated proteins in young susceptible and non-susceptible individuals, COPD patients and healthy controls, at baseline and after acute smoking, are assessed. The results from mass spectrometric analyses, ELISA and immunohistochemistry were compared to identify possible biomarkers for susceptibility to COPD.

Chapter 5 represents the summary and general discussion, and underlines the possible future perspectives of this research.

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Chapter II

Proteomics of Epithelial Lining Fluid obtained by Bronchoscopic Microprobe sampling

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Abstract

Epithelial lining fluid (ELF) forms a thin fluid layer that covers the mucosa of the large airways, small airways and alveoli. Since it constitutes the first barrier between the lung and the outer world, it is an interesting target for proteomics studies focusing on lung diseases. Bronchoscopic microprobe (BMP) sampling of ELF is a recently developed technique that uses small probes containing an absorptive tip that are introduced bronchoscopically.

In contrast to other methods used so far for the collection of biofluids from the lung (e.g. bronchoalveolar lavage fluid, induced sputum), this technique has the advantage that ELF is not diluted and contains high concentrations of biomolecules. In addition, the investigated location in the tracheobronchial tree is well defined and there is no contamination with oropharyngeal bacteria or saliva. Despite occasional blood contamination of the probes by scratching the mucosa of the airways, the proteomic analysis of microprobe-sampled ELF opens new possibilities for research in lung diseases. Our work focuses particularly on the induction and progression of cigarette smoke-induced Chronic Obstructive Pulmonary Disease (COPD).

In this chapter we describe the practical aspects of sampling ELF followed by a detailed description of proteomics analysis by LC-MS/MS after protein separation by SDS PAGE and in-gel digestion. As an example, we apply this proteomic platform to the identification and quantification of proteins in ELF from COPD patients and healthy subjects.

Key words: Epithelial Lining Fluid, Bronchoscopic Microsampling Probe, proteomics, 1D-SDS PAGE electrophoresis, mass spectrometry

Abbreviations: ELF (epithelial lining fluid), BALF (bronchoalveolar lavage fluid), COPD (chronic obstructive pulmonary disease), BMP (bronchoscopic microsampling probe), LC (liquid chromatography), MS/MS (tandem mass spectrometry), SDS PAGE (1-dimensional polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate), ARDS (acute respiratory distress syndrome), BCA (Bicinchoninic Acid)

1. Introduction

Proteomics is an increasingly important tool in the discovery of biomarkers for human diseases. Proteomics studies focusing on lung diseases have used resected lung tissue, bronchial biopsies, bronchoalveolar lavage fluid (BALF), induced sputum, exhaled breath condensate, nasal lavage fluid, serum, urine, and cultured cells or their supernatant after stimulation. In the field of COPD proteomic studies have used biopsies, BALF and sputum to detect approximately 40 candidate proteins that may play a role in the pathogenesis of COPD [1],[2],[3],[4],[5],[6]. These proteins have different functions in tissue repair and cell proliferation, the immunological response and inflammation, cytoskeletal function, and protection against oxidants. However, none of these proteins has been validated in the clinical situation of COPD [7].

Epithelial lining fluid (ELF) forms a thin aqueous layer that covers the inner part of the airways and protects them against a hostile outer world. This first-barrier of innate defense is therefore an interesting focus of research, particularly because allergens, microbial agents, and toxic agents from cigarette smoke and air pollution are responsible for lung diseases such as atopic asthma, pneumonia interstitial lung fibrosis or COPD. ELF contains cells and soluble components that probably play an essential role in this first line of defense. Consequently, it has been proposed that the protein composition of ELF most faithfully reflects the effects of external factors that affect the lung and furthermore that detecting changes in this composition is of primary importance in the early diagnosis and prognosis as well as in the characterization of pathological processes that are related to the progression of lung disorders [8].

Proteomics studies on ELF have most frequently used BALF. However, this way of collecting ELF has a number of disadvantages. First, BALF dilutes the ELF by a factor of 60-120 fold [9] which decreases the sensitivity to detect proteins that are not abundantly present in the bronchial tree. Additionally, dilution of ELF may vary from one lavage to the next and there is no accepted method to correct for this variation. Second, the exact location in the bronchial tree from which proteins are derived is not known because BALF is collected from the 4^{th} generation of airways until the alveoli. Third, there is a close relationship between certain proteins in serum and BALF [5,6] which poses questions about the lung specificity of the BALF proteome. Analysis of induced sputum might be an alternative to ELF or BALF analysis, since it can be obtained in a non-invasive way. However, the area from which induced sputum is collected is even more variable than that of BALF. Additionally, induced sputum passes the oropharyngeal cavity which contains many bacteria and saliva that may easily contaminate sputum samples. Finally, sputum requires application of dithiothreitol necessary to liquefy the highly viscous sputum, which may in turn affect proteomics results.

Bronchoscopic microprobe sampling of ELF is thus a very promising technique in proteomics research of the lung. This technique uses a small adsorptive pad, that is placed on the mucosal layer of the bronchial wall (2^{nd} generation) under visual control during

bronchoscopy. It may also be placed in the smaller airways using radiography. In contrast to the collection of BALF or induced sputum, the area from which ELF is obtained is precisely known. Additionally, there is no dilution of proteins and the risk of contamination with bacteria is minimal, because the probes are protected by an extra sheath within the bronchoscope during entry and exit. This collection technique has been used successfully in humans with acute respiratory distress syndrome (ARDS) and small peripheral lung carcinoma [10] as well as in studies measuring drug concentrations in ELF. Microsampling probes have also been used to collect ELF without the help of a bronchoscope in anaesthetized ventilated rabbits followed by proteomic analysis leading to the identification of 43 proteins [11]. More than 50% of these proteins had been reported earlier in relation to lung cancer, lung inflammation and ARDS in human. We believe therefore that proteomics of microprobe-sampled ELF opens a new area of research in lung disease, particularly in those diseases with a strong inflammatory response to inhaled agents. In that perspective, the response to cigarette smoke and the possible induction of COPD is a particularly promising field for ELF proteomics.

In this chapter we describe a methodology of performing proteomics in microprobe-sampled ELF as developed in the context of this thesis. We initially describe how ELF is sampled followed by the extraction of proteins from the probe, sample preparation and protein separation by SDS PAGE with subsequent in-gel digestion of excised protein bands. Identification of proteins by LC-MS/MS of the digests and database search provides an overview of the ELF proteome. To our knowledge, no proteomics studies have been performed in human Epithelial Lining Fluid, so our platform opens new avenues for a better understanding of the onset and progression of COPD and possibly other lung disorders with implications for diagnosis and prognosis as well as for drug discovery and development.

2. Materials and methods

2.1 ELF sampling and sample preparation

Epithelial Lining Fluid is collected from COPD patient and non-COPD subjects for the SDS-PAGE experiments (see Table 1). All subjects gave informed consent for the study,

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

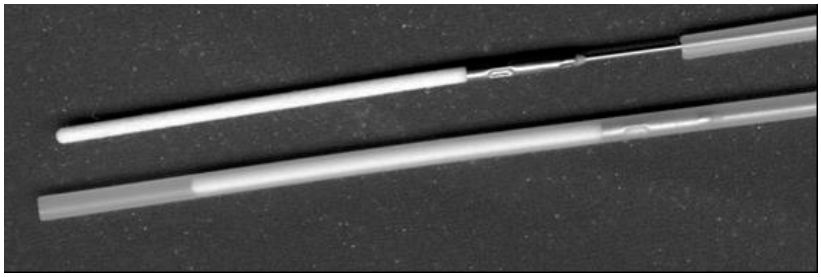

Figure 1: Real representation of a Microprobe Sampling Probe (BC-401)

which was approved by the local ethics committee in agreement with the Helsinki declaration of 1964 as revised in 2004.

Bronchoscopic microsampling probes (BC-401C) were provided by Olympus, Tokyo, Japan (see Figure 1). The BC-401C dimensions are: working length: 1050 mm, fiber rod diameter: 1.1 mm, fiber rod length: 30 mm, maximum insertion portion diameter: 1.8 mm. These probes need a bronchoscopic working channel with an inner diameter of at least 2.0 mm.

Bronchoscopy is performed using established guidelines. Patients are not allowed to drink or eat 5 h prior to bronchoscopy. On arrival, patients receive 40 μg ipratropiumbromide via a spacer. Fifteen minutes later, 30 mL lidocaine (2% w/v) are instilled in the mouth, in the oropharynx and on the vocal cords as well as into the trachea to inhibit coughing. The total lidocain dose is not allowed to exceed 4.5 mg/kg [12]. A flexible bronchoscope with a minimum diameter of the working channel of 2.0 mm is introduced via the nose or mouth into the left main bronchus. The inner adsorptive tip is pushed out gently till contacting the airway mucosa and its position is maintained for 10 seconds allowing ELF adsorption. Participants of the study were asked not to breathe during these 10 seconds. The tip of the BMP is subsequently withdrawn into the outer sheath and the probe is removed from the bronchoscope. This procedure is then repeated to obtain 3 probes per subject. Probes with visual blood contamination were discarded in our studies.

The adsorptive tips were cut (about 3 cm in length), inserted into 1.5 mL tubes (Greiner, reaction tubes, 1.5 mL, 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, Eppendorf AG Hamburg, Germany) 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 is determined using the Micro BCA Assay (Pierce Protein Research Product, Thermo Scientific, The Netherlands) following the manufacturer's protocol in an absorbance plate reader at 550 nm (Molecular Devices, THERMOmax, Minnesota, USA). The total protein concentration in the aliquots is between 150 and 400 μg/mL.

2.3 Sample preparation for SDS-PAGE

One 200 µL aliquot of the ELF extract is thawed and concentrated 5- to 10-fold under vacuum (Eppendorf Concentrator 5301, Eppendorf AG, Hamburg, Germany). Concentrated extracts are centrifuged (Eppendorf Centrifuge 5417R, Eppendorf AG, Hamburg, Germany) for 5 min at 5000 rpm to remove insoluble material. The clear supernatant is transferred to a new tube and 5 µL of 5-fold concentrated loading buffer (10% SDS, 10mM Dithiothreitol (Sigma-Aldirch, The Netherlands), 20% Glycerol (Genfarma bv, Zaandam, The Netherlands), 0.2M Tris-HCl, pH 6.8 (Duchefa Biochemie (Haarlem, The Netherlands), 0.05% Bromophenolblue (BIO-RAD, The Netherlands)) are added. The samples are boiled for 3 min prior to SDS-PAGE analysis.

SDS-PAGE is performed in a Mini-Protein III cell (BIO-RAD, The Netherlands) using 12.5% gels with 0.1% SDS (all chemicals for polyacrylamide gels are from BIO-RAD). PageRuler™ Prestained Protein Ladder (Fermentas, #SM0671, Landsmeer, The Netherlands) is used as molecular weight marker.

SDS-PAGE is performed in 1.0mm thick 12.5% polyacrylamide gels in a mini-Protean III electrophoresis system (BioRad, Veenendal, The Netherlands) at 20mA. The 12.5% polyacrylamide separating gel is prepared by mixing 1.25mL of separating buffer, 2.1mL of acrylamide/bis (30%) stock solution, 1.6mL of water, 50µL of APS (10%) and 2µL of TEMED. The gel is poured between clean glass plates (washed extensively in the following order: ultrapure water, 0.1% SDS added to ultrapure water, ultrapure water, ethanol and dried with clean paper). The separating gel is overlaid with isopropanol until polymerization occurs (around 30-40 min). Isopropanol is then removed and the top of the separating gel is washed extensively with ultrapure water and dried with filter paper. The stacking gel is prepared by initiating polymerization of 1.43mL of water, 0.42mL of acrylamide/bis (30%) stock solution and 0.62mL of stacking buffer through addition of 25µL of APS (10%) and 2.5µL TEMED after inserting the appropriate combs. After approximately 30min, the combs are removed and the wells washed with running buffer (1x). The cassettes are assembled as follows: the inside chamber (cathode chamber) and the outside chamber (anode chamber) are filled with running buffer (1x) covering the top and the bottom of the gel totally. The gel is run (starting voltage of 120V and increasing till 180V) until the tracking dye reaches the bottom of the gel. In order to reveal proteins, the gel is stained in a plastic or glass box with Silver Stain (freshly prepared) as described [13]. Alternatively, the gel is stained with Coomassie Brilliant Blue R concentrate (1g) in 90mL of acetic acid and 250mL of ethanol on a shaker for two hours. Subsequently, the staining solution is replaced with the destaining solution (5% of acetic acid, 50% of methanol or ethanol, 45% of ultrapure water) to clear the gel from the background color and to visualize the bands corresponding to the proteins.

2.4 In-gel digestion

In-gel digestion of proteins is performed according to the protocol of Shevchenko et al. [13] Two lanes (one containing the non-COPD control and one the COPD patient sample, see Table 1 for details about the subjects) from a silver-stained gel (Figure 2) are cut into 43 equal slices of 1.5 mm width each with the help of a clean surgical blade. Trypsin (Promega, sequencing grade modified trypsin, # V5111, Leiden, The Netherlands) is used (10 µg/mL) for digestion overnight at 37°C with shaking at 450 rpm (Eppendorf Thermomixer, Eppendorf AG, Hamburg, Germany). The reaction is quenched by adding 0.75 µL formic acid. The final volume of each sample is about 20 µL.

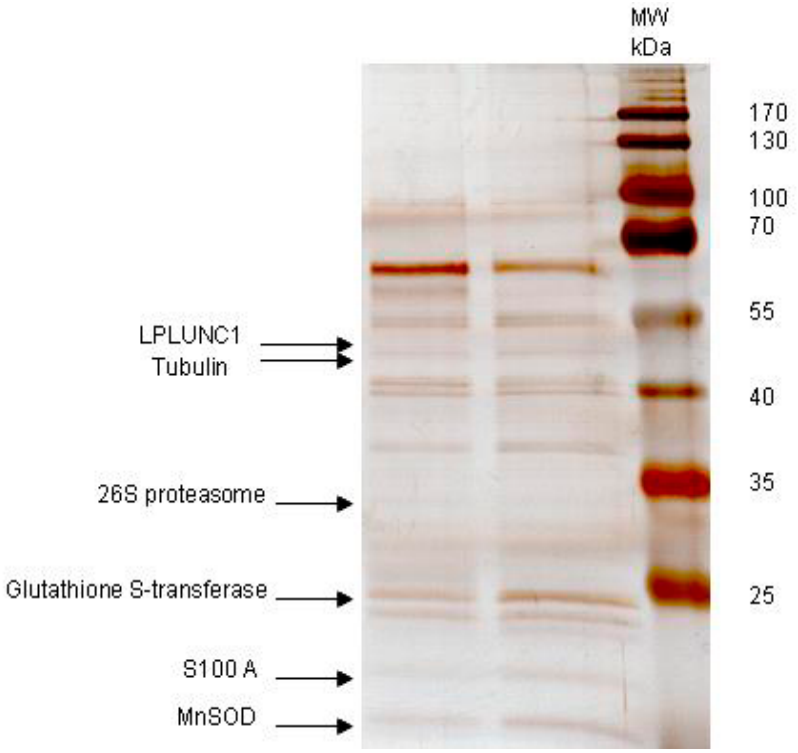

Figure 2. SDS-PAGE analysis (silver stain) of ELF samples (10 µg of total protein per lane) from a COPD patient (left lane) and a non-COPD control (right lane). Lanes were cut in 43 (1.5 mm wide) slices for in-gel digestion and LC-MS/MS analysis

2.5 ChipLC-MS/MS

Proteins are identified after in-gel digestion by nanoLC-MS/MS using a microfluidics (chip-cube) interface (Agilent, cat. n° G4240A) including a chip (Agilent, cat. n° 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 contains a nanoelectrospray tip (2 mm length with conical shape: 100 µm OD × 8 µm ID) that is coupled on-line to an ion-trap mass spectrometer (MSD-Trap-SL, Agilent, Amstelveen, The Netherlands). Injections (3µL) are performed with an autosampler (Agilent, cat. n° G1389A) equipped with an injection loop of 8µL and a thermostated cooler

maintaining the samples in the autosampler at 4°C during analysis. The chipLC-MS/MS system contains the following additional modules: nanopump (cat. n° G2226A), capillary loading pump (cat. n° G1376A), and solvent degasser (cat. n ° G1379A). Two eluents (eluent A, 0.1% formic acid in water and eluent B, 0.1% formic acid in acetonitrile) are used for the nanopump and one for the capillary pump (0.1% formic acid, 3% acetonitrile 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 is run. 90% of eluent B is maintained for 10 min to regenerate the column.

For acquisition of MS/MS spectra, the following parameters are 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 are analyzed with the Data Analysis software (Bruker Daltonics, version 3.4,Germany). Compounds are 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 are exported as Mascot generic files (Matrix Science, London, U.K.) and submitted to a web-based version of Mascot (v2.4) to query the SwissProt data base (release 2012_07, taxonomy "*Homo sapiens* (human)", 20,232 sequences). The search parameters are listed in Table 2. All listed proteins were identified based on a minimum of 2 unique

Table 2. Search parameters for protein identification based on data from the proteomics study of ELF by SDS-PAGE and chipLC-MS/MS using Mascot (v2.4)

Taxonomy	Homo sapiens
Database	SwissProt (release 2012_07, 20232 sequences)
Peptide Charge	1+,2+,3+
Variable Modifications	Carbamidomethyl (C) Oxidation (M)
Quantitation	None
Enzyme	Trypsin
Allow up to	3 missed cleavages
Peptide tolerance	+/- 1.6 Da
MS/MS tolerance	+/- 0.8 Da
Data format	Mascot generic
Instrument	ESI-TRAP
Decoy	enabled

peptide sequences with $p < 0.05$ (ions score > 37) and a maximum false discovery rate of 5% (see *Supplementary Table S1 in the Online Repository* https://www.dropbox.com/s/ky0c4mpstmle11n/Chapter%202_Supplementary%20Table%20S1_Thesis%20LF%202015.xls?dl=0).

3. Results

To map the ELF proteome, individual samples from a COPD patient and a non-COPD control are subjected to protein separation by SDS-PAGE (Figure 2). Forty-three bands from each lane are cut, subjected to trypsin digestion and subsequently analyzed by chipLC-MS/MS resulting in a total of 269 identified proteins (1349 peptides) of which 193 proteins are identified in both ELF samples. Analysis of the ELF sample from the COPD patient result in 239 identified proteins while 223 proteins are identified in the non-COPD control sample. Forty-six of the 269 proteins are only identified in the COPD sample, while 30 are only identified in the non-COPD control sample (see *Supplementary Table S1 in the Online Repository*).

The identified proteins are classified in three groups:

1- proteins identified in the COPD but not in the non-COPD control sample
2- proteins identified in the non-COPD control but not in the COPD sample
3- proteins identified both in COPD and healthy control samples

Annotation of the proteins that are only identified in ELF from the COPD patient revealed a number of potentially interesting functions. For example, a number of members of the palate, lung and nasal epithelium clone protein (PLUNC) protein superfamily (LPLC1, accession number *Q8TDL5* and BPIL1, accession number *Q8N4F0*), that has been reported to be implicated in host defense [14] are identified next to nicotin acid-based structural proteins (nicotinate phosphoribosyltransferase, accession number *Q6XQN6* and nicotinamide phosphoribosyltransferase, accession number *P43490*) and neutrophil cytosol factor 1 (NCF-1, accession number *P14598*), that are related to the host response to bacterial infections. Proteins S100A9 and S100A8 (accession numbers *P06702* and *P05109* respectively) that are members of the calgranulin superfamily [15] identified in ELF from the COPD patient, underline the importance of inflammation in the onset/progression of the illness. Superoxide dismutase (MnSOD, accession number *P04179*) an anti-oxidant protein [16] and the enzyme leukotriene A-4 hydrolase (LTA-4 hydrolase, accession number *P09960*) [17] are also only identified in the bands of the COPD sample, while glutathione S-transferase [16], a detoxifying enzyme that protects against oxidative stress, as induced by cigarette smoking, is only identified in ELF from the healthy control.

ELF from the COPD patient contained also a number of Ras-related proteins (RAB1B, RAB30, RAC1, RAP1A, RAC2). This may indicate involvement of EPAC proteins in

obstructive airways diseases as described earlier [18] and their anti-inflammatory effect in alveolar macrophages [19,20]. Many proteins involved in proteasome activation and regulation (PSME3, PSMB10, PSMB4; accession numbers: *P61289*, *P40306*, *P28070* respectively) are identified in ELF from the COPD patient, a result that may reflect the involvement of this system during conditions of inflammation and oxidative stress [21].

Although most of the tubulin protein family members are identified in ELF from both COPD and healthy individuals (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) are absent in ELF from the COPD patient. Tubulins are a class of GTP-binding proteins that are the major constituents of microtubules. Their interaction with actin filaments is fundamental for the structure of the cytoskeleton and the induction of cytoskeletal changes in cells. It is interesting to note that cofilin-1, another actin-binding protein, is found to be increased in ELF from COPD patients in our quantitative, comparative study later on.

4. Discussion

This is the first detailed proteomics study of human Epithelial Lining Fluid (ELF) with a focus to decipher changes that are related to Chronic Obstructive Pulmonary Disease (COPD). Proteomic analysis of ELF by SDS-PAGE followed by in-gel digestion and microfluidics-nanoLC-MS/MS resulted in identification of more than 300 proteins in samples from COPD patients and healthy controls.

ELF is thus a promising sampling technique for proteomic analysis in relation to pulmonary diseases first of all due to the higher protein content (150-400 µg/mL) when compared to other biofluids from the lung, such as BALF, exhaled breath condensate or induced sputum. Second, it is possible to sample ELF from exactly defined locations in the lung.

The identified proteins (*Supplementary Table S1*) belong to families that are involved in various aspects of COPD pathogenesis, such as inflammation, oxidative stress, bacterial infection and the protease-antiprotease balance. One of the identified proteins in ELF from a COPD patient belongs to the PLUNC (palate, lung and nasal epithelium carcinoma associated protein) family. PLUNC proteins show specific expression in lung tissue [22] and may be involved in the airway inflammatory response upon exposure to irritants. They are also associated with tumor progression and play a role in the innate immune response of the upper airways [23]. Furthermore they are involved in host defense against bacteria by direct antibacterial or by indirect LPS-sensing/neutralizing activity [22,24,25] Whereas LPLC1 (accession number *Q8TDL5*) is mostly expressed in trachea and nasal septal epithelium, BPI (accession number *P17213*) is a product of inflammatory cells, such as polymorpho-

nuclear leukocytes [26]. LPLC1 is primarily expressed in bronchial epithelium as compared to peripheral lung tissue and has been identified in nasal mucus, BALF and sputum [1,14]

Neutrophil cytosol factor 1 (NCF-1, accession number *P14598*) is only identified in ELF from the COPD patient pointing to the well-known involvement of the neutrophils in this illness. NCF-1 is a cytosolic subunit of neutrophil NADPH oxidase (nicotinamide adenine dinucleotide phosphate-oxidase, NOX), a complex responsible of the production of superoxide anions that is activated during the respiratory burst [27], and oxidative stress is a hallmark of COPD [28]. Even though involvement of NCF-1 in COPD is not clear, its presence in the diseased sample only can underline the bacterial colonization that occurs during the illness. Its role is plausible, since it was shown previously in several studies that activated neutrophils are increased in bronchial biopsies and sputum of COPD patients, and that there is a significant correlation between the number of circulating neutrophils and COPD severity as well as the rate of lung function decline [29].

Nicotin acid-based structural proteins (Nicotinate phosphoribosyltransferase, and Nicotinamide phosphoribosyltransferase), that are only identified in ELF from the COPD patient, are also related to the response to bacterial infection because of their role as cofactors in NAD synthesis, which is essential for the energy metabolism and many other essential biochemical pathways related to bacterial development and survival [30].

Manganese superoxide dismutase (MnSOD) is only identified in ELF from the COPD patient, confirming the importance of oxidative stress in the development of COPD. Superoxide dismutase converts superoxide anions into molecular oxygen and hydrogenperoxide (notably one of the major source of oxidants is cigarette smoke). This enzyme is widely expressed in alveolar type II epithelial cells and alveolar macrophages and has been found to be increased in alveolar pneumocyte II cells of smokers and patients with COPD [31]. This finding is not surprising considering its main functions, namely scavenge and eliminate reactive oxigen species, that in our particular case are generated by exposure to oxidants in cigarette smoke [28]. Another potent antioxidant system in the airways (together with superoxide dismutase as a candidate defense system) is based on the balance of intracellular glutathione (GSH). It has been shown that alteration of the balance between GSSG (the oxidized form of glutathione) and the reduced form (GSH) is related to oxidative stress in lung epithelial cells and that this occurs after cigarette smoke exposure, which decreases the levels of intracellular GSH [32]. Glutathione-S transferase (GSTs) are a superfamily of metabolic enzymes that are involved in the detoxification of reactive compounds, for example in tobacco smoke. It is interesting to note that GST A1-A2 has been identified in ELF of the healthy control only [33]. So far the relevance of their increase in ELF of smokers is unclear, but our finding corroborates published data showing an increased GST gene expression in the bronchial epithelium of smokers without COPD [16] and how cigarette smoking affects this important regulatory enzyme family [34]. Moreover there exists an association of single nucleotide polymorphisms in GST genes with COPD.

The enzyme leukotriene A-4 hydrolase (LTA-4 hydrolase) hydrolyzes LTA-4 to form LTB-4 (a potent chemoattractant of neutrophils), which is found to be increased in exhaled breath condensate and sputum of COPD patients, notably during exacerbations [35]. It has also been demonstrated that this enzyme is increased in circulating neutrophils of asthmatic patients [36] (remarkably a key feature that characterizes COPD is the inflammatory process with a persistent infiltration of neutrophils). Indeed different LTB-4 antagonists are being developed for the treatment of inflammatory diseases, especially asthma and COPD [37].

Two proteins of the S100 family, S100A9 and S100A8, were identified in ELF from the COPD patient but not in ELF from the healthy control. S100 proteins are known to be expressed in epithelial cells and macrophages in chronic inflammations [23] and to be released by phagocytes after interaction with microtubules exhibiting pro-inflammatory activity [38]. Their presence in ELF of the COPD patient may thus be related to the presence/progression of COPD. The identified proteins belong to a subgroup of the S100 protein family the calgranulins. Calgranulins contribute to experimental and clinical lung inflammation by their elevated concentrations in the cytoplasm of neutrophils. Release of calgranulins from neutrophils upon activation contribute to it's the antibacterial activity [39]. Furthermore they are overexpressed at local sites of inflammation [40] and act as "amplifiers" of inflammation, influencing cytokine induction [41]. Calgranulins are thus important targets to modulate inflammation [42]. This increased expression in ELF of COPD patients is further confirmed in our quantitative study later on, where both S100 A8 and A9 were differentially expressed in the set of COPD samples in comparison to the healthy controls.

Our initial study revealed proteasome–related proteins PSMD14, PSMD1, PSMC6, PSME3, PSMB10, PSMB4 in ELF from the COPD patient. Next to its intracellular function to degrade ubiquitinilated proteins within the cell in an ATP-dependent reaction, the 26S proteasome has also been shown to exist outside the cell (after lung injury) and to contribute to proteolysis of proteins in the alveolar space [43]. It has been demonstrated that the ubiquitin-proteasome system plays a crucial role in the homeostasis of muscle tissue as response to hypoxemia [44]. Further studies concerning the pathophysiological role of these proteins are thus warranted notably in relation to cachexia, which is often associated with COPD.

The ELF of the COPD patient contained several small GTP-ase related proteins (RAB1B, RAB33B, RAB30, RAC1, RAP1A, RAB6A, RAC2), which have previously been implicated in the development and progression of lung carcinoma [45]. Both in COPD and in the development of lung cancer this class of proteins points towards the involvement of the EPAC protein family, specific guanine nucleotide exchange factors for the Ras GTPase homologues (RAP1 and RAP2) [19]. Recent evidence shows the relevance of Epac in lung fibroblast and airway smooth muscle cell proliferation [46]. Since EPAC proteins are regulated by cAMP and induce the activation of Ras GTPases, a better understanding of their pro-inflammatory properties can lead to a better comprehension of airways disease pathogenesis, in particular of COPD and asthma.

5. Conclusions

In summary we present here a detailed methodology for the collection and treatment of Epithelial Lining Fluid (ELF) and the suitability of this biofluid for proteomic investigation in pulmonary disease, specifically COPD.

This feasibility study of human epithelial lining fluid (ELF) with a focus on COPD and cigarette smoking represents a first approach to perform qualitative studies on pulmonary diseases and opens new routes of the usefulness of ELF for clinical investigations.

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Chapter III

Quantitative proteomic analysis of human Epithelial Lining Fluid by microfluidics-based nanoLC-MS/MS: a feasibility study

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Abstract

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 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. 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.

Abbreviations: ELF (epithelial lining fluid), BALF (bronchoalveolar lavage fluid), COPD (chronic obstructive pulmonary disease), BMP (bronchoscopic microprobe), HMBG1 (high-mobility group box 1), FA (formic acid), NADPH oxidase (nicotinamide adenine dinucleotide phosphate-oxidase), MnSOD (manganese superoxide dismutase), ROS (reactive oxygen species), FEV_1 (Forced Expiratory Volume in 1 second), CCI (Charlson co-morbidity index), BSA (bovine serum albumin), TLR (toll-like receptor), LPS (lipopolysaccharide).

Key words: Chronic Obstructive Pulmonary Disease, epithelial lining fluid, mass spectrometry, microfluidics, stable isotope labeling

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 severe disease [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 (FEV_1 % 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 [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 [11,12]. BALF and sputum are to date the most studied specimen. However, challenges related to the relative inaccessibility of these samples from the lung have hampered progress [13]. 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 [13] 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 [14] and is an attractive target for proteomic 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, a technique that uses small adsorptive probes [15,16]. ELF collected in this way contains higher concentrations of biologically active molecules [17]. Another advantage of microprobe sampling is that the locations from which ELF was obtained are clearly defined [16,18] and it allows collection of ELF from both central and peripheral airways [19].

In this study we describe two comparative, quantitative studies (Study I and II) using stable isotope labeling (iTRAQ® Reagent-8Plex) followed by two-dimensional 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 4 COPD patients and 4 non-COPD controls for Study I and an independent set of 4 COPD patients and 4 non-COPD controls for Study II (see Table 1). 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 the bronchoscope (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 of the microprobe was pushed out gently till contacting the airway mucosa and its position was maintained for 10 sec allowing ELF adsorption. Participants of the study were asked not to breathe during these 10 sec. The tip of the microprobe was subsequently withdrawn into the outer sheath and the probe was removed from the bronchoscope. This procedure was repeated to obtain 3 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.

Table 1. 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[1]	3	0	3	1
Inhaled corticosteroids, number*	2	0	1	0
Co-morbidity, CCI	1.5 (1-4)	0.5 (0-1)	1.0 (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)	38.0 (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. * Number of patients and/or controls under treatment with inhaled corticosteroids. [1] Years stopped smoking in study I: COPD patient 1:20 years; COPD patient 2 years: 2; COPD patient 3 years: 5. Years stopped smoking in study II: COPD patient 4: 10 years; COPD patient 5: 9 years; COPD patient 6 years: 37; non-COPD: 35 years.

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 Stable isotope labeling

ELF samples containing 50 µg protein each (about 250-300 µL) were used for iTRAQ® labeling. Samples were diluted with 5 mL of 10 % acetonitrile in 0.1 % aqueous trifluoracetic acid and concentrated by ultrafiltration (Concentrators, Spin 5K MWCO, 4 mL, Part n° 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 200mM methyl methanethiosulfonate (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 [20]. 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 individu-

ally labeled digests were combined into a single sample mixture of equal original protein amount of 50 µg. 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 x 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% acetonitrile and B: 5mM KH_2PO_4/H_3PO_4 pH 3, 25% acetonitrile, 1.0 M KCl. The gradient was divided into three segments: 0-15% B [12 column volumes (CVs); one column volume corresponds to 0.69 mL], 15-50% B (3 CVs), 50-100% B (5 CVs) 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).

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

Dried fractions were dissolved in 100 µL 2% ACN/0.1% TFA. 4 µL were trapped on a pre-column (300 µm x 5 mm, C18 PepMap300) and separated on a C18 capillary column (C18 PepMap 300, 75 µm x 150 mm, 3 µm particle size) mounted on an Ultimate 3000 nanoflow liquid chromatography system (Dionex, Amsterdam, The Netherlands). Solutions of 0.05% TFA in water (A) and 80% ACN, 0.05% TFA in water (B) were used for elution using a two-step gradient from 4-40% B in 50 min and from 40-60% 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 sec 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 was 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, FWHM); 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).

2.5 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, 86417 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 (MMTS), (ii) trypsin digestion, (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, 86417 entries) and subsequently validated in Scaffold® version 3.0 (version_3_00_07, www.proteomesoftware.com). The search parameters in Mascot are listed in Table 2. Protein identifications were based on at least 2 unique peptides identified independently, with a probability > 95% and a false discovery rate < 5% at the protein level.

Table 2. Search parameters for the comparative, quantitative proteomics studies of ELF by iTRAQ®-8plex labeling using Mascot (v 2.1)

Database	Human IPI (IPI_v3_72, downloaded May 2010, 86417 entries)
Taxonomy	Homo Sapiens
Enzyme	Trypsin
Maximum missed cleavages	2
Fixed modifications	Carbamidomethyl (C) iTRAQ (K) iTRAQ (N-term)
Variable modifications	Deamidation (NQ) Oxidation (M)
MS/MS fragment tolerance	0.4 Da
Precursor tolerance	100 ppm
Peptide charge	1+
Monoisotopic mass	Enabled
Instrument	MALDI TOF/TOF

2.6 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 Student t-test
V) Exclusion of all peptides with non-significant ratio differences (p-values > 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

2.7 Immunohistochemistry

Immunohistochemistry was performed to compare lung transplant tissue from 10 COPD patients (5 current and 5 ex-smokers) and 10 non-COPD controls (5 current and 5 never smokers) (see Table 3 for demographic/characteristics). Three-µm 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 x 10 min with xylol, 2 x 100%, 2 x 96%, 1 x 70% ethanol and 1x rinsed in demineralized water) and subsequently washed in PBS. Antigen retrieval was performed by microwave treatment for lactotransferrin, 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 3-times with PBS. Antigen retrieval for alpha 1-antichymotrypsin (Serpin 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 3 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 Corporation, Taipei, Taiwan). Serpin A3: monoclonal antibody (M04, clone 3F5, purified mouse immunoglobulin, H00000012-M04, Abnova Corporation, Taipei, Taiwan). HMGB1: monoclonal antibody (M03, clone

Table 3. Characteristics of subjects for immunohistochemistry in lung tissue

	COPD current smokers (n=5)	COPD ex-smokers (n=5)	non-COPD current smokers (n=5)	non-COPD never smokers (n=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.

1B11, purified mouse immunoglobulin, H00003146-M03, Abnova Corporation, Taipei, Taiwan). 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 3 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 3 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 3 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% H_2O_2 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.

Table 4. List of proteins that were differentially expressed (p-value <0.05) between COPD patients and non-COPD controls in Study I.

PROT ID	Peptide Sequence	% Conf	p-value
Lactotransferrin	QVLLHQQAK	99	0.050001
HMGB1 High mobility group protein B1	FKDPNAPK	95	0.002797
HMGB1 High mobility group protein B1	TYIPPKGETK	98	0.007503
HMGB1 High mobility group protein B1	GKFEDMAK	99	0.017289
HMGB1 High mobility group protein B1	FKDPNAPK	99	0.035467
S100A9 Protein S100-A9	DLQNFLK	99	0.047091
S100A8 Protein S100-A8	GNFHAVYR	99	0.024526
S100A8 Protein S100-A8	GNFHAVYR	99	0.047001
PRR4 Proline-rich protein 4	HPPPPPFQNQQRPPR	98	0.043183
S100A11 Protein S100-A11	IAVFQK	99	0.038573
CFL1 Cofilin-1	VFNDMK	98	0.016863
CTSD Cathepsin D	VGFAEAAR	98	0.000939
CTSD Cathepsin D	VGFAEAAR	99	0.005474
CTSD Cathepsin D	QVFGEATK	99	0.022463
CTSD Cathepsin D	QVFGEATK	99	0.050849
SERPINA3 Isoform 1 of Alpha-1-antichymotrypsin	ADLSGITGAR	99	0.036503
SERPINB1 Leukocyte elastase inhibitor	EATTNAPFR	99	0.050304
FABP5 Fatty acid-binding protein, epidermal	FEETTADGR	99	0.039586
immunoglobulin heavy constant alpha 1	VAAEDWK	99	0.042706

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.

Table 5. List of proteins that were differentially expressed (p-value <0.05) between COPD patients and non-COPD controls in Study II.

PROT ID	Peptide Sequence	%Conf	p-value
Serum Albumin	QEPERNECFLQHK	95	0.000271
Serum Albumin	QEPERNECFLQHK	99	0.001393
Serum Albumin	QEPERNECFLQHK	97	0.006680
Serum Albumin	QEPERNECFLQHK	99	0.010684
Serum Albumin	QEPERNECFLQHK	95	0.011268
Serum Albumin	QEPERNECFLQHK	99	0.014682
Serum Albumin	ADDKETCFAEEGKK	99	0.016724
Serum Albumin	FKDLGEENFK	99	0.032723
Serum Albumin	AAFTECCQAADK	99	0.040482
Serum Albumin	KQTALVELVK	99	0.046894
Serum Albumin	SLHTLFGDK	99	0.047762
Lactotransferrin	CVPNSNER	99	0.025997
Lactotransferrin	CVPNSNER	99	0.046806
Lactotransferrin	CVPNSNER	98	0.049095
Lysozyme C	GISLANWM	99	0.020879
Lysozyme C	GISLANWM	99	0.022927
Lysozyme C	GISLANWM	99	0.028264
Lysozyme C	GISLANWM	99	0.029096
Lysozyme C	GISLANWM	99	0.032404
Lysozyme C	TPGAVNACHLSC	99	0.033321
Lysozyme C	WESGYNTR	99	0.049890
TPPP3 Tubulin polymerization-promoting protein family member 3	VINYEEFKK	99	0.004726
TPPP3 Tubulin polymerization-promoting protein family member 3	VINYEEFKK	99	0.006257
TPPP3 Tubulin polymerization-promoting protein family member 3	KFAIHGDPK	99	0.010858
TPPP3 Tubulin polymerization-promoting protein family member 3	VINYEEFKK	98	0.034053

Table 5. (continued)

PROT ID	Peptide Sequence	%Conf	p-value
TPPP3 Tubulin polymerization-promoting protein family member 3	FAIHGDPK	99	0.044395
SERPINB3 Isoform 1 of Serpin B3	VLHFDQVTENTTGK	99	0.012023
SERPINB3 Isoform 1 of Serpin B3	LLTEFNK	99	0.013593
SERPINB3 Isoform 1 of Serpin B3	VDLHLPR	99	0.015634
SERPINB3 Isoform 1 of Serpin B3	DNTAQQIK	99	0.017242
SERPINB3 Isoform 1 of Serpin B3	STDAYELK	99	0.018278
SERPINB3 Isoform 1 of Serpin B3	DNTAQQIK	99	0.019286
SERPINB3 Isoform 1 of Serpin B3	VEESYDLK	98	0.020738
SERPINB3 Isoform 1 of Serpin B3	VLHFDQVTENTTGK	99	0.023973
SERPINB3 Isoform 1 of Serpin B3	LLTEFNK	99	0.024826
SERPINB3 Isoform 1 of Serpin B3	TNSILFYGR	99	0.028996
SERPINB3 Isoform 1 of Serpin B3	GLVLSGVLHK	99	0.033519
SERPINB3 Isoform 1 of Serpin B3	STDAYELK	99	0.048145
PRDX1 Peroxiredoxin-1	ATAVMPDGQFK	98	0.003473
PRDX1 Peroxiredoxin-1	IGHPAPNFK	99	0.021833
PRDX1 Peroxiredoxin-1	TIAQDYGVLK	99	0.027750
PRDX1 Peroxiredoxin-1	IGHPAPNFK	99	0.031206
PRDX1 Peroxiredoxin-1	IGHPAPNFK	99	0.038753
PRDX1 Peroxiredoxin-1	GLFIIDDK	99	0.047778
PRDX1 Peroxiredoxin-1	ATAVMPDGQFK	99	0.048031
PRDX1 Peroxiredoxin-1	GLFIIDDK	99	0.048352
Lipocalin2	VVSTNYNQH	99	0.007674
Lipocalin2	CDYWIR	98	0.009438
Lipocalin2	VVSTNYNQH	99	0.009718
Lipocalin2	ITLYGR	97	0.010305
Lipocalin2	ITLYGR	97	0.010801
Lipocalin2	MYATIYELK	99	0.017940
Lipocalin2	SYPGLTSY	97	0.018663
Lipocalin2	CDYWIR	98	0.022056
Lipocalin2	ITLYGR	98	0.023392
Lipocalin2	AMVFFK	96	0.027884
Lipocalin2	MYATIYELK	99	0.030108
Lipocalin2	VVSTNYNQH	99	0.037927
Lipocalin2	ELTSELK	99	0.048133

Table 5. (continued)

PROT ID	Peptide Sequence	%Conf	p-value
Lipocalin2	MYATIYELK	99	0.049614
Heat shock 70 kDa protein 1	ATAGDTHLGGEDFDNR	99	0.018112
Heat shock 70 kDa protein 1	ATAGDTHLGGEDFDNR	99	0.018294
Trypsin2	NKPGVYTK	99	0.011682
Trypsin2	TLNNDIMLIK	99	0.019314
Trypsin2	HNIEVLEGNEQFINAAK	99	0.020050
Trypsin2	TLNNDIMLIK	99	0.029460
Trypsin2	TLNNDIMLIK	99	0.034270
Trypsin2	NKPGVYTK	99	0.036753
Trypsin2	NKPGVYTK	97	0.038890
Trypsin2	TLNNDIMLIK	99	0.044381
Alpha-enolase	IEEELGSK	99	0.003068
Alpha-enolase	YDLDFK	96	0.008774
Alpha-enolase	IEEELGSK	99	0.019121
Alpha-enolase	YDLDFK	96	0.024167
Alpha-enolase	AVEHINK	99	0.034646
Alpha-enolase	KLNVTEQEK	99	0.034814
Alpha-enolase	AVEHINK	99	0.048284
HMGB1 High mobility group protein B1	GKFEDMAK	99	0.031723
Serotransferrin	DGAGDVAFVK	99	0.041425
Annexin A1	VLDLELK	97	0.008810
Annexin A1	SEDFGVNEDLADSDAR	99	0.013807
Annexin A1	NALLSLAK	99	0.017334
Annexin A1	SEDFGVNEDLADSDAR	99	0.031210
Aldolase A	AAQEEYVK	99	0.032018
Aldolase A	ADDGRPFPQVIK	99	0.038973
Aldolase A	AAQEEYVK	99	0.041378
Antileukoproteinase	CLDPVDTPNPTR	99	0.017052
Antileukoproteinase	SAQCLR	98	0.021319
Antileukoproteinase	CLDPVDTPNPTR	99	0.028522
Antileukoproteinase	CLDPVDTPNPTR	99	0.033911
Antileukoproteinase	AGVCPPK	99	0.043484
Antileukoproteinase	SCVSPVK	99	0.044010
Antileukoproteinase	SCVSPVK	98	0.046398
Antileukoproteinase	AGVCPPK	98	0.047679

Table 5. (continued)

PROT ID	Peptide Sequence	%Conf	p-value
Cystatin-B	AKHDELTYF	97	0.036163
Calcyphosin	FLDNFDSSEK	99	0.004338
Calcyphosin	EAVIAAAFAK	99	0.009422
Calcyphosin	EAVIAAAFAK	99	0.019265
Calcyphosin	SLDADEFR	99	0.022335
Calcyphosin	FLDNFDSSEK	99	0.025640
Uteroglobin	KLVDTLPQKPR	98	0.040892
Uteroglobin	KLVDTLPQKPR	99	0.044009
Peptidyl-prolyl cis-trans isomerase A	FEDENFILK	99	0.026676
Peptidyl-prolyl cis-trans isomerase A	HVVFGK	97	0.043256
PRB4 Basic salivary proline-rich protein 4	FLISGKPEGR	99	0.005636
PRB4 Basic salivary proline-rich protein 4	FLISGKPEGR	99	0.007615
PRB4 Basic salivary proline-rich protein 4	FLISGKPEGR	99	0.009605
Glutathione S-transferase P	ASCLYGQLPK	99	0.011144
ubiquitin C	IQDKEGIPPDQQR	99	0.004345
ubiquitin C	IQDKEGIPPDQQR	99	0.032706
Cofilin-1	CTLAEK	97	0.045054
Peroxiredoxin-6	LAPEFAK	97	0.038869
Prothymosin alpha	EVVEEAENGR	99	0.010827
Prothymosin alpha	EVVEEAENGR	99	0.012952
Prothymosin alpha	EVVEEAENGR	99	0.017906
Prothymosin alpha	EVVEEAENGR	99	0.020773
Prothymosin alpha	EVVEEAENGR	99	0.024886
Isoform 6 of Calpastatin	QAEPELDLR	97	0.002275
Beta-2-microglobulin	IQVYSR	97	0.031638
Calmodulin 2	MKDTDSEEEIR	99	0.018726
Calmodulin 2	MKDTDSEEEIR	99	0.029427
SERPINA3 Isoform 1 of Alpha-1-antichymotrypsin	ADLSGITGAR	99	0.023235
SERPINA3 Isoform 1 of Alpha-1-antichymotrypsin	NLAVSQVVHK	99	0.026457
ALDH1A1 Retinal dehydrogenase 1	SLDDVIK	98	0.003881
ALDH1A1 Retinal dehydrogenase 1	VAFTGSTEVGK	99	0.011807
ALDH1A1 Retinal dehydrogenase 1	VAFTGSTEVGK	99	0.013266
ALDH1A1 Retinal dehydrogenase 1	SLDDVIK	98	0.018902
ALDH1A1 Retinal dehydrogenase 1	LADLIER	99	0.037141

Table 5. (continued)

PROT ID	Peptide Sequence	%Conf	p-value
Gelsolin	VVEHPEFLK	99	0.036583
Profilin-1	EGVHGGLINK	99	0.026815
Profilin-1	EGVHGGLINK	95	0.032537
Serine protease 3	NRPGVYTK	99	0.006073
Serine protease 3	NRPGVYTK	99	0.008088
Serine protease 3	NRPGVYTK	99	0.011810
Serine protease 3	NRPGVYTK	98	0.043466
Ig G	LPVVLANGQIR	99	0.040428
Transaldolase	LVPVLSAK	99	0.025288
WAP four-disulfide core domain 2	VSCVTPNF	98	0.011594
WAP four-disulfide core domain 2	VSCVTPNF	98	0.040977
immunoglobulin heavy constant alpha 2	QVQLVQSGAEVK	99	0.011782
DYNLL1 Dynein light chain 1, cytoplasmic	DIAAHIK	99	0.004109
DYNLL1 Dynein light chain 1, cytoplasmic	DIAAHIK	99	0.010728
DYNLL1 Dynein light chain 1, cytoplasmic	DIAAHIK	99	0.015364
DYNLL1 Dynein light chain 1, cytoplasmic	DIAAHIK	99	0.016026
nucleobindin 1	APAAHPEGQLK	99	0.017418
immunoglobulin heavy constant gamma 2	GLPAPIEK	98	0.010103
immunoglobulin heavy constant gamma 2	GLPAPIEK	98	0.026745
Lupus La protein	EGIILFK	98	0.033084
Peroxiredoxin-1, 2	SKEYFSK	98	0.009565
Peroxiredoxin-1, 2	SKEYFSK	99	0.011544
Peroxiredoxin-1, 2	QITVNDLPVGR	99	0.020629
Peroxiredoxin-1, 2	QITVNDLPVGR	99	0.036862
Glutathione S-transferase A2, A1	YNLYGK	98	0.024061
Glutathione S-transferase A2, A1	SHGQDYLVGNK	99	0.032452
Glutathione S-transferase A2, A1	YNLYGK	98	0.048564
Heat shock 70 kDa protein 1, 71 kDa	TTPSYVAFTDTER	99	0.014592
Heat shock 70 kDa protein 1, 71 kDa	TTPSYVAFTDTER	99	0.034436
Trypsin 2, 3	EQFINAAK	98	0.017703
Trypsin 2, 3	EQFINAAK	99	0.017712
Trypsin 2, 3	EQFINAAK	98	0.018843
Trypsin 2, 3	EQFINAAK	98	0.028696
immunoglobulin heavy constant mu	GPSVFPLAPCSR	99	0.013499
ALDH3A1 Aldehyde dehydrogenase	VTLELGGK	99	0.018278
ALDH3A1 Aldehyde dehydrogenase	VTLELGGK	99	0.022224

3. Results

Shotgun proteomics with stable isotope labeling using the iTRAQ® 8-plex reagents and MALDI-TOF/TOF mass spectrometry 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.

Each study resulted in a number of proteins with statistically significantly different concentration levels in ELF between COPD patients and non-COPD controls (see Table 4 and 5). The levels of four proteins, lactotransferrin (accession number *P02788*), alpha 1-antichymotrypsin (accession number *P01011*), cofilin-1 (accession number *P23528*) and high mobility group protein B1 (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, 3 for HMGB1, 4 for Cofilin-1 and 2 for Alpha 1-antichymotrypsin in Study I.

In Study II, 21 peptides for Lactotransferrin, 4 for HMGB1, 4 for Cofilin-1 and 3 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 >95% confidence of identification were selected for the final quantification.

Lactotransferrin was increased in ELF of COPD patients (Figure 1, panels A and B) based on the analysis of two unique peptides that were found in Study I and II (QVLLHQQAK 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 of lactotransferrin in ELF may be related to its antimicrobial properties [21]. In fact it has been demonstrated that secretion of antimicrobial proteins by epithelial cells is increased upon airway inflammation, which supports our findings [22]. 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 (a protein part 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 3 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 (Figure 3) 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 II) showed that HMGB1 is reduced in ELF of COPD patients relative to non-COPD controls (Figures 1, panels C and D), which contrasts with an earlier study in BALF of COPD patients [23]. Analysis with Scaffold® showed that the selected peptides were specific for HMGB1, excluding a possible affiliation with similar proteins

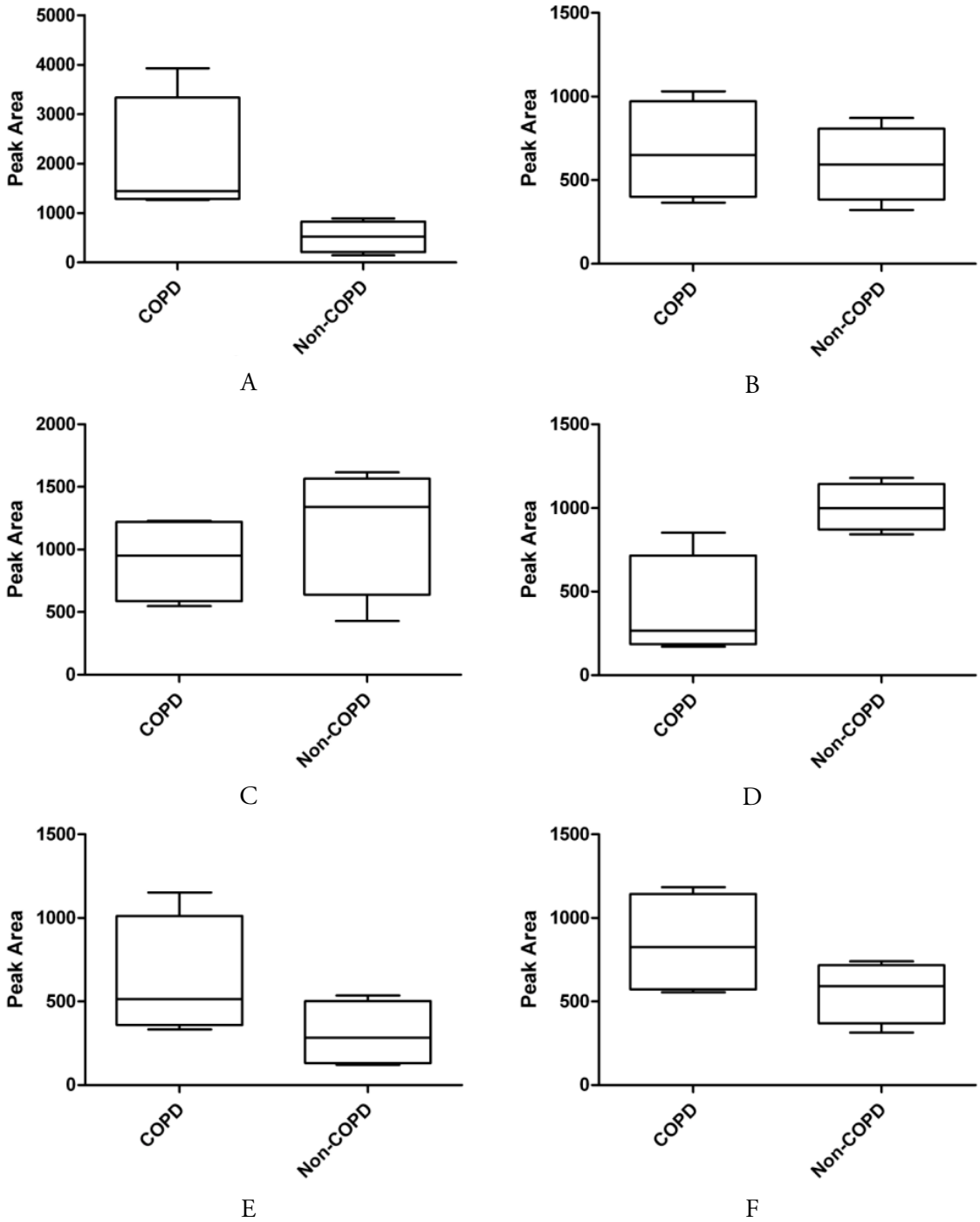


Figure 1. Box and whisker plots of the levels of three proteins that differed significantly in ELF from COPD patients versus non-COPD controls in two independent studies (Study I and Study II). Lactotransferrin (panel A, Study I, peptide QVLLHQQAK and B, Study II, peptide CVPNSNER); HMGB1 (panel C, Study I, peptide GKFEDMAK and panel D, Study II, peptide GKFEDMAK); Serpin A3 (panel E, Study I, peptide ADLSGITGAR and panel F, Study II, peptide ADLSGITGAR). Each error bar represents the variation inside the group

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

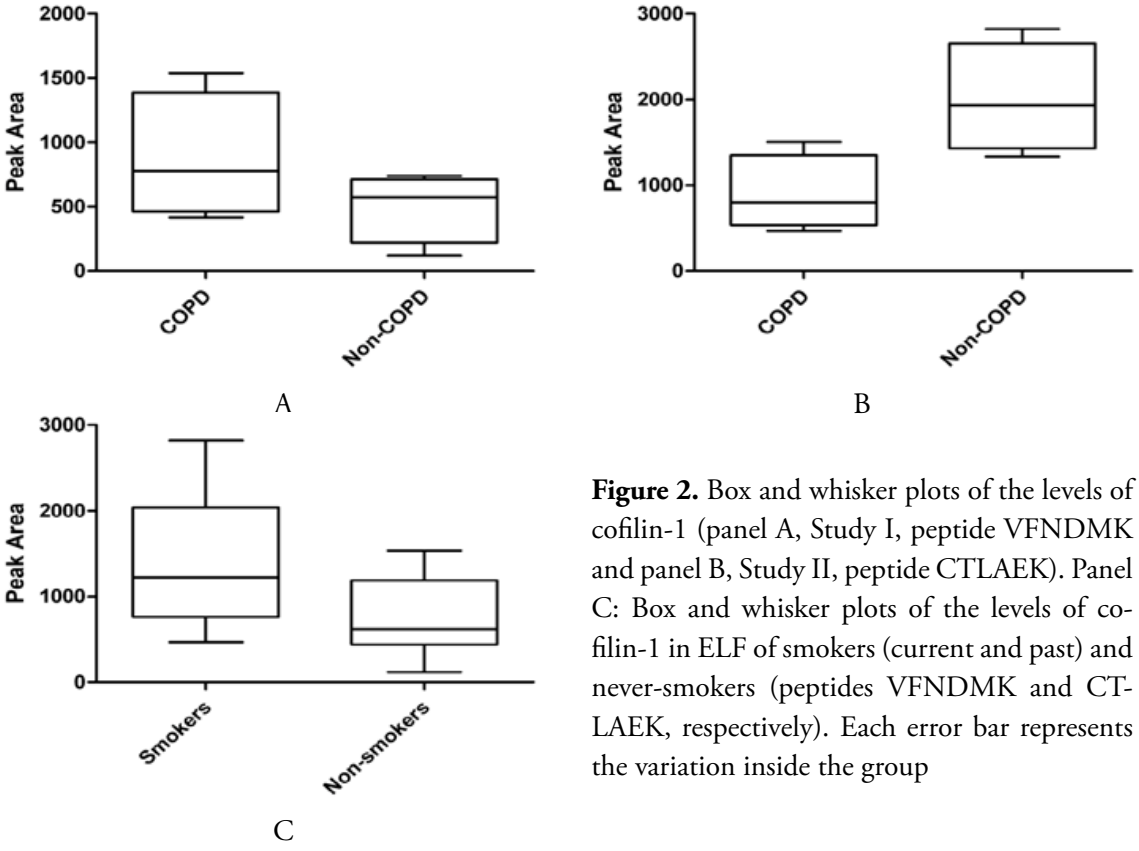
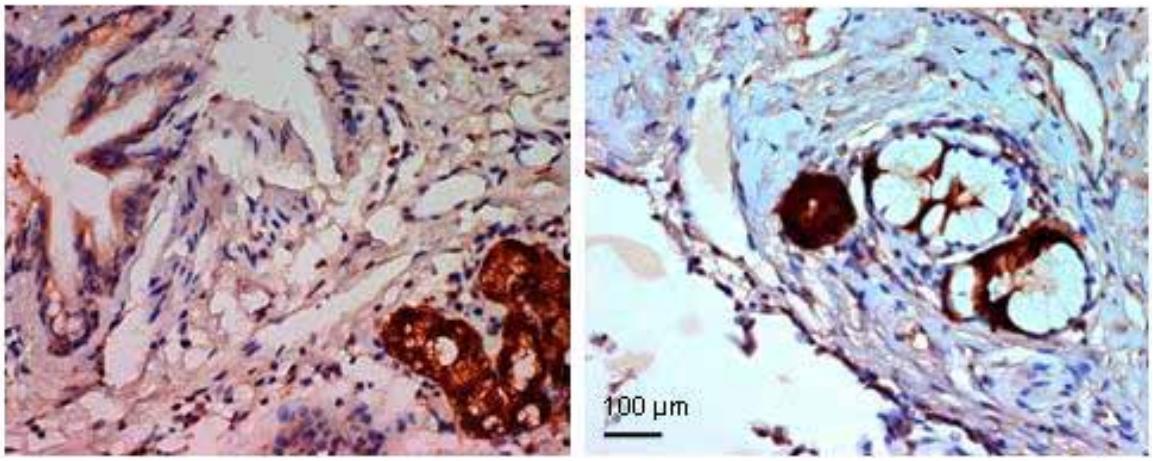


Figure 2. Box and whisker plots of the levels of cofilin-1 (panel A, Study I, peptide VFNDMK and panel B, Study II, peptide CTLAEK). Panel C: Box and whisker plots of the levels of cofilin-1 in ELF of smokers (current and past) and never-smokers (peptides VFNDMK and CTLAEK, respectively). Each error bar represents the variation inside the group

(Figure 4). HMGB1 is thus likely released into ELF due to leakage from epithelial cells associated with necrosis and/or cellular turnover.

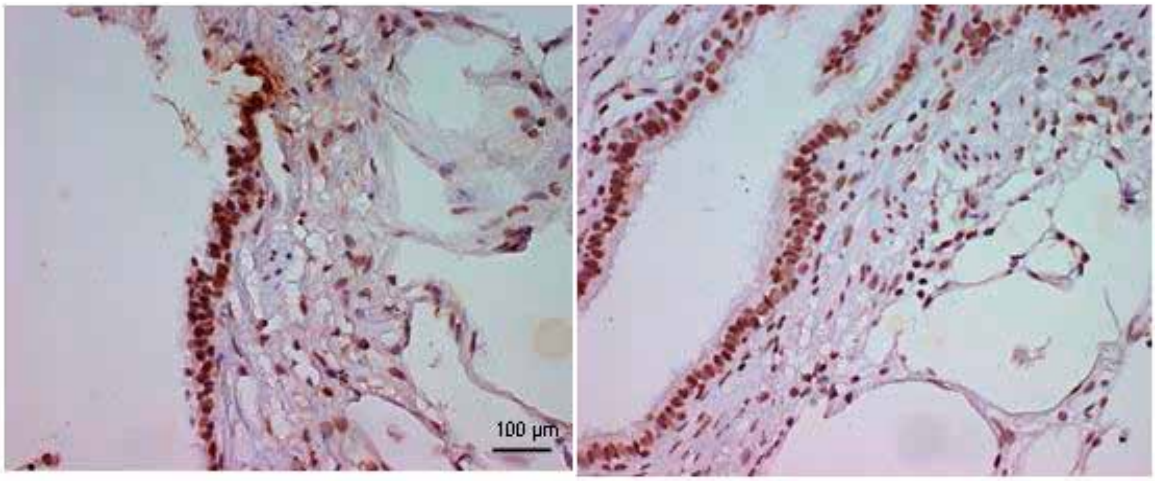
The alpha 1-antichymotrypsin (Serpin A3) level was increased in ELF from COPD patients (Figure 1, panels E and F) in both studies. Since immunohistochemistry of lung tissue showed protein expression in alveolar macrophages (high levels) as well as in epithelium and endothelium (moderate levels) without visible differences in abundance in lung tissue from COPD patients and non-COPD controls (current, ex and never smokers) (Figure 5), 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 (Figure 2, panel A), while they appeared to be decreased in Study II (Figure 2, panel B). Comparative sequence analysis using Scaffold® established that there is no ambiguity of our identification 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 where all from never-smokers (see Table 1), 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 (Figure 2, panel C). These results were confirmed by immunohistochemistry



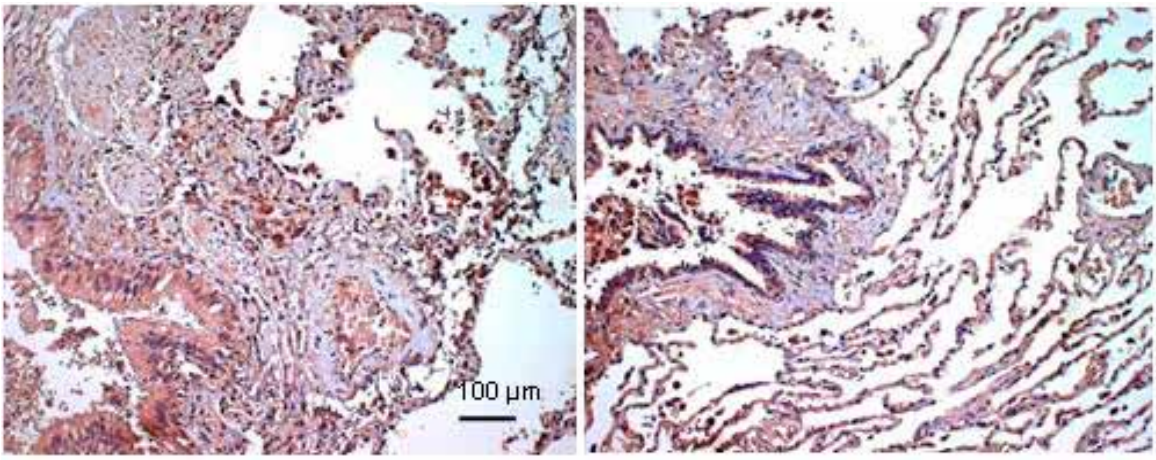
A B

Figure 3. Immunohistochemistry of lactotransferrin in lung tissue from (A) a COPD patient (current smoker), (B) a non-COPD control (never-smoker).



A B

Figure 4. Immunohistochemistry of HMGB1 in lung tissue from (A) a COPD patient (current smoker), (B) a non-COPD control (never-smoker).



A B

Figure 5. Immunohistochemistry of alpha 1-antichymotrypsin in lung tissue from (A) a COPD patient (current smoker), (B) a non-COPD control (never-smoker).

(Figure 6). 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 never smoked (Figure 7). This corroborates previously published data showing that cofilin-1 is increased in lung tissue of smokers without COPD [24].

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 Table 6 A and B).

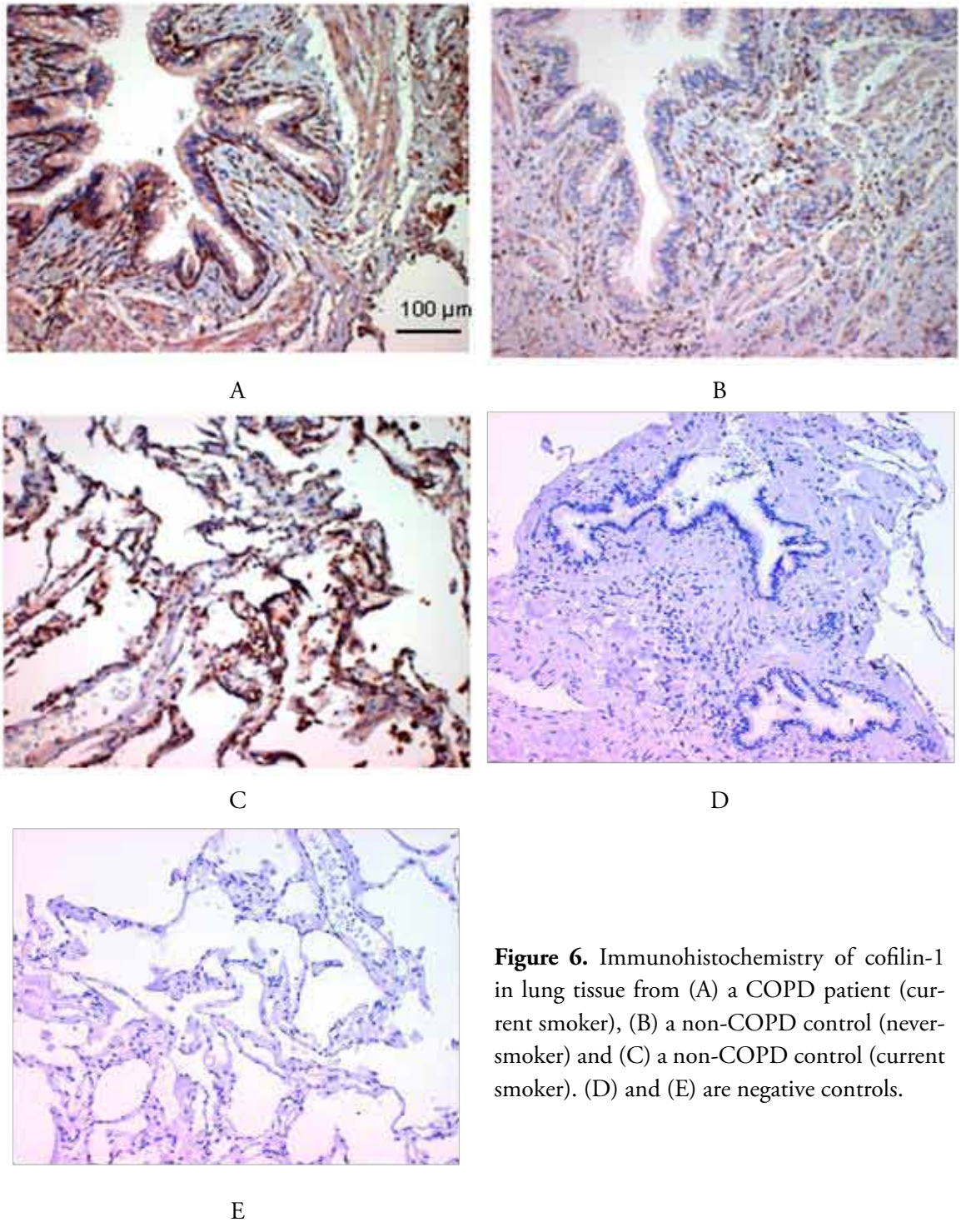


Figure 6. Immunohistochemistry of cofilin-1 in lung tissue from (A) a COPD patient (current smoker), (B) a non-COPD control (never-smoker) and (C) a non-COPD control (current smoker). (D) and (E) are negative controls.

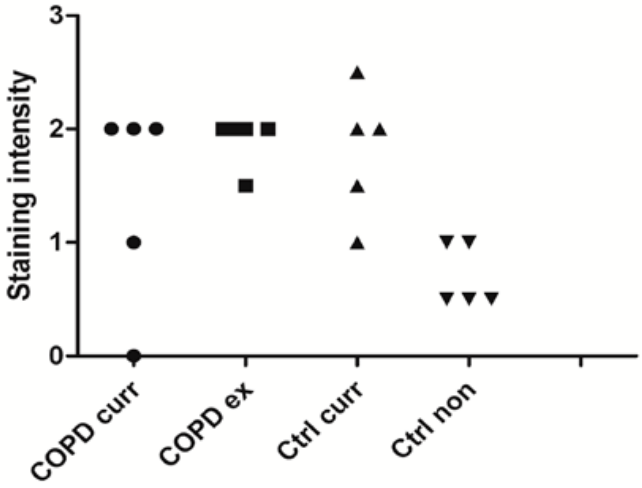


Figure 7. Semi-quantitative scoring of staining intensity of the immunohistochemical analysis of cofilin-1 in lung tissue. COPD curr = COPD patients (current smokers); COPD ex = COPD patients (former smokers); Ctrl curr = non-COPD controls (current smokers); Ctrl non = non-COPD controls (never smokers). Each dot represents a single patient or control. All analyses were performed by 2 independent investigators in a blinded fashion (ML and LF).

4. Discussion

Chemical stable isotope labeling (iTRAQ® 8-plex) in combination with 2-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 (4 COPD patients and 4 non-COPD controls) and Study II (a different set of 4 COPD patients and 4 non-COPD controls) showed that lactotransferrin, cofilin-1, high mobility group protein B1 (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 (Figure 3). Lactotransferrin is present in different secretory fluids (e.g. milk, saliva, nasal secretions) and is, together with lysozyme C, the most abundant antimicrobial protein in human airways [21]. 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 [21].

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 [25]. 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 [25,26]. Recently, it has been proven that free iron ions may be involved in the pathogenesis of airways disease, notably COPD and asthma [27]. Interestingly, lactotransferrin

Table 6A. Coefficient of variation of the significantly different peptides of Lactotransferrin, HMGB1, Cofilin-1 and Alpha 1-antichymotrypsin in Study I.

Protein	Peptide sequence	STDEV	Average	CV
Lactotransferrin	QVLLHQQAK	0.213058	0.472786	0.450645
Lactotransferrin	QVLLHQQAK	0.24353	0.541586	0.449661
Lactotransferrin	QVLLHQQAK	0.141302	0.249071	0.567315
Lactotransferrin	QVLLHQQAK	0.105459	0.235643	0.447539
Lactotransferrin	QVLLHQQAK	0.150204	0.337943	0.444467
Lactotransferrin	QVLLHQQAK	0.148902	0.422586	0.35236
Lactotransferrin	QVLLHQQAK	0.144559	0.247857	0.583235
Lactotransferrin	QVLLHQQAK	0.147934	0.31	0.477206
Lactotransferrin	QVLLHQQAK	0.15309	0.192643	0.794681
Lactotransferrin	QVLLHQQAK	0.087661	0.444457	0.197232
HMGB1	GKPDAAK	0.542795	1.752557	0.309716
Cofilin-1	VFNDMK	0.264903	0.843243	0.314148
Cofilin-1	VFNDMK	0.159527	0.621329	0.256751
Alpha 1-antichymotrypsin	ADLSGITGAR	0.429097	1.049871	0.408714
Alpha 1-antichymotrypsin	ADLSGITGAR	0.328629	0.808871	0.406281

Table 6B. Coefficient of variation of the significantly different peptides of Lactotransferrin, HMGB1, Cofilin-1 and Alpha 1-antichymotrypsin in Study II.

Protein	Peptide sequence	STDEV	Average	CV
Lactotransferrin	CVPNSNER	0.435043	1.074771	0.404777
Lactotransferrin	CVPNSNER	0.296897	0.919329	0.32295
Lactotransferrin	CVPNSNER	0.576454	1.233886	0.467186
Lactotransferrin	CVPNSNER	0.303257	0.897843	0.337761
HMGB1	GEHPGLSIGDVAK	0.382222	0.7069	0.540701
HMGB1	GKFEDMAK	0.581184	1.408971	0.412488
Cofilin-1	CTLAEK	0.245076	0,985443	0,248696
Cofilin-1	CTLAEK	0.225495	0.918657	0.245461
Alpha 1-antichymotrypsin	ADLSGITGAR	0.808442	0.981429	0.82374

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 [28]. 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 [29-31].

Alpha 1-antichymotrypsin (Serpin A3), a member of the acute-phase protein family, was shown to be increased in ELF from COPD patients (Figures 1, panels E 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 (Figure 5). 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 [32]. Even though alpha 1-antichymotrypsin is primarily produced in the liver, there is evidence of its secretion from alveolar epithelial cells and alveolar macrophages [33,34].

High mobility group protein B1 (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 [35] demonstrated its pro-inflammatory activity by binding to cytokines like IL-1β and TNF-α. It was surprising to find that the level of HMGB1 was increased in the non-COPD controls of our studies (Figures 1, panels C and D) in contrast to published data on BALF from COPD patients [23]. Our quantitative results are based on the analysis of 3 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 7 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 [36]. 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 (Figure 4). 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 [23]. Taking the pluripotency of HMGB1 into account, its interaction with

different types of receptors (not only RAGE, but also TLR) 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 [37,38].

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 [39]. 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 [40]. 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 ROS and a decline in ATP levels resulting in the formation of cofilin-actin rods [41]. 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 [41]. Klemke *et al.*, showed the link between the formation of oxidized cofilin and oxidative stress, by direct incubation of the protein with H_2O_2 [42]. 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 Ser^3) 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 epithelial lining fluid (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 anyway the feasibility of proteomics investigation in human ELF and represents a first approach to perform quantitative studies on pulmonary diseases using this biofluid. Despite in an explorative way, 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.

Acknowledgements

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Chapter IV

Susceptibility to COPD: differential proteomic profiling after acute smoking

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Abstract

Cigarette smoking is the main risk factor for COPD, yet only a subset of smokers develops COPD. Family members of patients with severe early-onset COPD have an increased risk to develop COPD and are therefore defined as "susceptible individuals".

Here we perform unbiased analyses of proteomic profiles to assess how "susceptible individuals" differ from age-matched "non-susceptible individuals" in response to cigarette smoking.

Epithelial lining fluid (ELF) was collected at baseline and 24 hours after smoking 3 cigarettes in young individuals susceptible or non-susceptible to develop COPD and older subjects with established COPD. Controls at baseline were older healthy smoking and non-smoking individuals. Five samples per group were pooled and analysed by stable isotope labelling (iTRAQ®) in duplicate. Six proteins were selected and validated by ELISA or immunohistochemistry.

After smoking, 23 proteins increased or decreased in young susceptible individuals, 7 in young non-susceptible individuals, and 13 in COPD in the first experiment; 23 proteins increased or decreased in young susceptible individuals, 32 in young non-susceptible individuals, and 11 in COPD in the second experiment. SerpinB3 and Uteroglobin decreased after acute smoke exposure in young non-susceptible individuals exclusively, whereas Peroxiredoxin I, S100A9, S100A8, ALDH3A1 decreased both in young susceptible and non-susceptible individuals, changes being significantly different between groups for Uteroglobin with iTRAQ and for Serpin B3 with iTRAQ and ELISA measures. Peroxiredoxin I, SerpinB3 and ALDH3A1 increased in COPD patients after smoking.

We conclude that smoking induces a differential protein response in ELF of susceptible and non-susceptible young individuals, which differs from patients with established COPD. This is the first study applying unbiased proteomic profiling to unravel the underlying mechanisms that induce COPD. Our data suggest that SerpinB3 and Uteroglobin could be interesting proteins in understanding the processes leading to COPD.

Abbreviations: ELF= epithelial lining fluid; COPD= chronic obstructive pulmonary disease; ALDH3A1= Aldehyde dehydrogenase 3A1; CC16= Clara cell 16; BALF= bronchoalveolar lavage fluid

Keywords: COPD, acute smoking, epithelial lining fluid, proteomics

1. Introduction

Chronic obstructive pulmonary disease (COPD) is a major leading chronic disease and the only one with increasing prevalence and mortality worldwide. It is characterized by chronic, progressive airflow limitation [1]. The pathology of COPD includes a complex network of inflammation, oxidative stress, tissue damage, remodelling and repair [2]. It comprises many detrimental processes that contribute to disease progression, a progression that is relentless and without a cure. Further research in this area is thus important, since a better understanding of COPD pathogenesis will enable the development of new and more effective treatments for the prevention and progression of COPD. Proteomics is an emerging scientific research field with important advances in proteomic instrumentation and methodology leading to the possibility to identify in small quantities of biological material an entire set of proteins important for the pathophysiology of a complex disease like COPD [3]. In COPD a relative low number of proteomic studies has been performed [3], using different methods [4,5], in biological materials like bronchoalveolar fluid [6–9], induced sputum [10–13] and exhaled breath condensate [14]. Although promising disease-specific and severity-related biomarkers came out [4], not one study focused on the very first phase of the induction of COPD.

In the past, investigating the acute response to cigarette smoking has been put forward as an attractive approach to understand the pathogenesis of COPD [15,16]. This so called acute smoking model is attractive because inflammatory responses of the lung to cigarette smoke can be investigated in a standardised and dynamic way. Although highly standardised, the acute smoking results in human studies demonstrate remarkably high inter-individual differences to cigarette smoking [16,17]. This variation may be due to methodological issues of assessing inflammatory responses, however, it could also reflect a really different response between individuals. In this perspective, it is important to acknowledge that only 20-30% of the smokers develop COPD, suggesting that a specific genetic background plays a role in the pathogenesis of COPD [18]. Indeed previous studies have suggested that family members of patients with severe early-onset COPD have an increased risk to develop COPD with smoking [19], and can therefore be labelled as "susceptible individuals". Thus far the mechanisms that lead to development of COPD in susceptible smokers remain largely unknown.

In this study we hypothesize that the acute smoking model is an attractive tool to better understand the essential differences between susceptible and non-susceptible individuals. This will especially be important in young individuals with a low number of pack-years smoking since they still have clean and uncompromised lungs. In other words, we hypothesize that ageing and lifelong smoking leads to altered airways not reflecting the very first aberrant response to cigarette smoking at young age. For this reason, we set out to investigate the onset of COPD in an acute smoking experiment in young healthy individuals, being

susceptible" or "non-susceptible" to develop COPD. To address this point, we profiled proteins in epithelial lining fluid (ELF), prior to and 24h after a controlled smoking episode in susceptible and non-susceptible young individuals. In addition, we compare these results with those in older subjects with established COPD. We chose to investigate ELF because this biologically active fluid constitutes the very first barrier to cigarette smoke, and because proteomic analysis of undiluted ELF recovered by a bronchoscopic microsampling probe contains many proteins associated with lung disease [20].

2. Materials and methods

2.1 Subjects

This study was part of a larger multi-centre study (www.clinicaltrials.gov, NCT00807469) [21]. Subjects were recruited at the outdoor clinic of the University Medical Centre Groningen (UMCG). Young (18-45 years) subjects were divided in those who were susceptible or not susceptible to develop COPD. Susceptibility was based on family history: not susceptible refers to subjects with smoking family members who are at least 45 years old yet without having COPD. Susceptible individuals needed to have a high prevalence of COPD in smoking family members older than 45 years: 2 out of 2, 2 out of 3, 3 out of 3, 3 out of 4, or 4 out of 4. All subjects were "party smokers" with <10 pack-years smoking, who were able to stop smoking for at least two days and start smoking on request. Old (>45 years) subjects with established COPD (GOLD II) and > 10 pack-years smoking were included for comparison. In addition, two control groups of old individuals were included: subjects with normal lung function despite > 10 pack/years smoking (healthy smokers), and subjects with normal lung function and no smoking history (healthy non-smokers).

The study was approved by the Medisch Ethische Commissie Universitair Medisch Centrum Groningen (METc 2008-136), and all subjects gave their written informed consent.

2.2 Smoking, ELF collection and sample preparation

Young susceptible and non-susceptible individuals and old COPD patients participated in the acute smoking experiments. The healthy smoking and non-smoking individuals did not perform smoking experiments and served as controls for COPD patients at baseline. All subjects were not allowed to smoke for at least two days prior to the experiments. Immediately before smoking exhaled CO was measured to ascertain that individuals had not smoked recently, and immediately after smoking to confirm that all individuals inhaled cigarette smoke sufficiently. If subjects had an exhaled CO >5ppm, indicating recent cigarette smoking, they were not allowed to participate in the acute smoking experiment. In the acute smoking experiment, all subjects smoked 3 Marlboro® cigarettes within one hour under supervision; always at the same time of the day between 9 and 11 A.M. Data from subjects

who did not inhale sufficiently (exhaled CO <2ppm) was not included. Bronchoscopy was performed both 24 hours after smoking and 6 weeks later in a stable phase to obtain baseline data. All bronchoscopies were carried out according to international guidelines [22]. ELF was collected at the mucosa of the left main bronchus using 3 microsampling probes (BC-401C; Olympus, Tokyo, Japan) [23].

2.3 Stable Isotope Labelling

ELF samples containing 50 µg total protein were used for iTRAQ labelling. The procedure was performed as previously described [24,25]. Briefly, each tryptic digested sample was labelled (iTRAQ® Reagent 4-plex, ABSciex, Foster City, CA, USA) according to the manufacturer's protocol. The individually labelled digests were then combined into a single sample mixture and subjected to strong-cation exchange chromatography (AKTA Purifier, GE Healthcare Biosciences AB, Uppsala, Sweden). The resulting peptide-containing fractions were separated by reversed-phase chromatography (Ultimate 3000 nanoflow liquid chromatography system, Dionex, Amsterdam, The Netherlands). Fractions of 12 sec were spotted on MALDI targets (Probot, Dionex, Amsterdam, The Netherlands) and mass spectrometric analysis was carried out on a 4800 Proteomics Analyzer MALDI TOF/TOF instrument (Applied Biosystems, Foster City, CA, USA) controlled by the 4000 Series Explorer v3.5 software.

Proteins were identified using Protein Pilot® software v4.0 (Applied Biosystems). The identification was performed using the IPI Human database (IPI v3.83). The Protein Pilot® cut-off score was 1.3, corresponding to a confidence limit of 95% at the peptide level. Protein identifications were based on at least 2 unique peptides identified independently. A probability higher than 95% and a false discovery rate lower than 5%, were accepted. The experiments were repeated with the same set of samples. ProQuant software was used to calculate the intensity of 3 reporter ions (m/z: 115, 116 and 117, Figure 1) and to divide them by the intensity of the 4^{th} reporter ion (m/z: 114) for each measured compound. All ratios were transformed into natural logarithms and plotted against the number of peptides subjected to MS/MS analysis. Gaussian curves were fitted on the smoothed histograms (histogram between -1 and +1 with 200 steps, smoothed using a Savitzky-Golay algorithm) and standard deviations (SD) were determined. Proteins with natural log-transformed ion ratios differing by at least 2.5×SD (98.8% confidence) were considered significantly different from the random variation. Visual explanation of the applied method is presented in Figure 1 in the Supplementary Materials. All data pre-processing work was done on a personal computer equipped with a +3600 MHz AMD processor and 4 GB of RAM, using MATLAB 7.11.0.584 (R2010b).

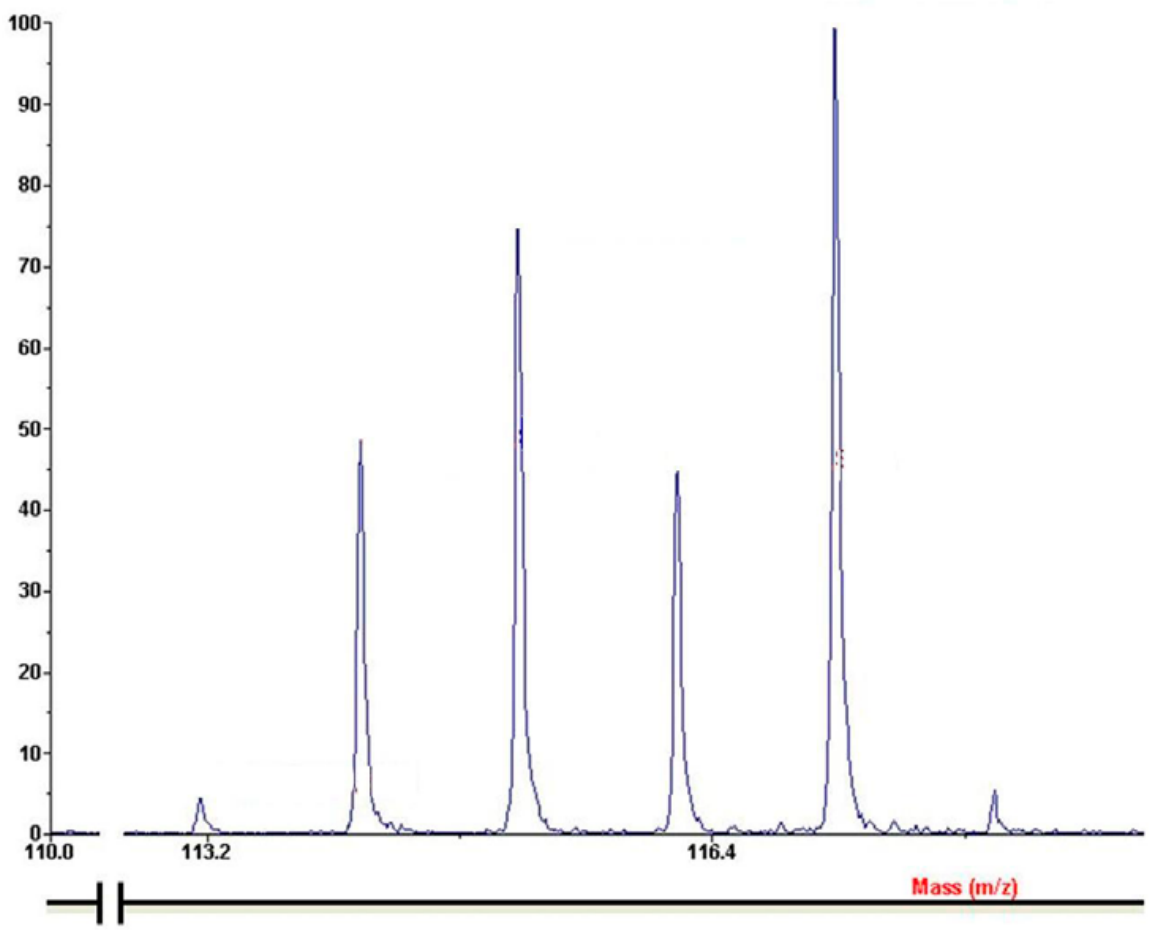

Figure 1. Reporter ion pattern of Peroxiredoxin I (peptide LVQAFQFTDK). Peaks at 114, 115, 116 and 117 represent the group of young non-susceptible after acute smoking; young non-susceptible at baseline, young susceptible after acute smoking and young susceptible at baseline, respectively.

2.4 ELISA

Due to methodological problems with the commercially available ELISA kits, we were unable to obtain results for S100A8 and ALDH3A1. The other four selected proteins were all above the detection limit of the ELISA. Commercially available ELISA kits from Uscn Life Science Inc. (China) were used following the manufacturer's protocols. Briefly, 100 uL of undiluted ELF were incubated for 2 hours at 37 °C in microtitre plates precoated with the specific monoclonal antibody. Subsequently a biotin-conjugated polyclonal antibody was added, followed by a TMB substrate solution and finally the reaction was stopped adding 50 uL of a sulphuric acid solution. The absorbance of each sample and calibration curve was read at 450 nm. The protein concentration in the samples was determined comparing the absorbance values of the samples to the standard curve.

Statistical analyses were performed using SPSS (version 16.0; SPSS, Chicago IL). Baseline differences between young non-susceptible versus young susceptible individuals and between old healthy smokers versus non-smokers and COPD were tested using Mann-Whitney U

tests. Changes associated with smoke exposure (before and after acute exposure to cigarette smoke) within the group of young non-susceptible individuals, young susceptible individuals and COPD patients were tested using Wilcoxon tests. P-values < 0.05 were considered statistically significant.

2.5 Immunohistochemistry

Immunohistochemistry of Aldehyde dehydrogenase 3A1 was performed to compare lung tissue from COPD patients who underwent lung transplantation (5 current smokers and 5 ex-smokers) and non-COPD controls who underwent surgery for lung cancer (5 current smokers and 5 never/ex-smokers). Three-µm thick lung sections were cut from selected formalin-fixed paraffin-embedded tissue blocks; immunostaining and quantification was performed as previously described [26]. Anti-ALDH3A1, SAB1405446 (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands) was used as primary antibody. Sections were scored semi-quantitatively.

3. Results

3.1 Subjects

A total of 25 subjects were selected for the iTRAQ experiments, 5 participants per group (Table 1 upper section). There was no significant difference in the clinical characteristics between the young susceptible and non-susceptible subjects, although there was a trend for higher age in the former group (p=0.16). COPD patients had a higher age than the old healthy smokers and non-smokers (p=0.009 and p=0.006, respectively). The COPD patients demonstrated airway obstruction compatible with GOLD stage II and 6 (out of 8) subjects demonstrated signs of emphysema (CO diffusion < 80% predicted). To verify the proteins detected by iTRAQ, eighteen additional subjects divided over the above groups were additionally included to enhance the numbers in the ELISA experiments, resulting in a total of 43 participating subjects (Table 1 lower section).

3.2 Proteomics

General results

Pooled ELF samples (n=5 per group) labelled with stable isotopes (iTRAQ®4-plex) were analysed by mass spectrometry in duplicate (Table 1-2 in the Supplementary Materials). In the group of young subjects 64 overlapping proteins were identified; in the older group 70 proteins (Figure 2 in the Supplementary Materials). At baseline, 6 overlapping proteins were differentially expressed between young susceptible and young non-susceptible individuals; and 7 between old healthy smokers and never-smokers (Table 3 and figure 3 in the

Table 1. Characteristics of the participating subjects

	Acute smoking experiment			Baseline controls	
	Young healthy susceptible	Young healthy non-susceptible	Old COPD	Old healthy smokers	Old healthy never-smokers
A. Subjects participating in the iTRAQ study					
Male/Female, n	3 / 2	0 / 5	0 / 5	3 / 2	1 / 4
Age, years	29 (18-42)	20 (19-39)	66 (55-74)	50 (47-53)	49 (45-53)
Pack years, n	0 (0-8)	2 (0-9)	23 (21-46)	38 (11-52)	0
FEV_1, % pred	103 (97-108)	109 (98-117)	74 (49-80)	111 (105-32)	111 (109-122)
FEV_1/FVC, %	80 (76-94)	81 (77-91)	54 (32-60)	80 (74-85)	76 (75-82)
TLC, % pred	25 (23-28)	22 (16-25)	39 (38-55)	36 (32-37)	33 (31-36)
CO diffusion, mmol/min/kPa	84 (80-97)	87 (62-98)	71 (40-86)	84 (74-96)	106 (84-117)
B. Subjects participating in the ELISA study					
Male/Female,n	3/4	0/6	0/8	6/3	8/5
Age, years	29 (18-42)	21 (19-39)	66 (55-74)	54 (47-70)	54.5 (45-70)
Pack years, n	0 (0-8)	2 (0-9)	28 (20-49)	39 (11-52)	0
FEV_1, % pred	108 (100-116)	109 (98-117)	68 (49-80)	110 (101-121)	111 (93-122)
FEV_1/FVC, %	78 (76-94)	82 (77-91)	52 (32-60)	78 (70-85)	78 (74-82)
TLC, % pred	25 (23-28)	22 (16-25)	38.5 (33-55)	36 (32-41)	36 (31-43)
CO diffusion, mmol/min/kPa	85 (84-102)	88 (62-98)	64 (40-91)	88 (83-117)	106 (84-119)

Values are medians (ranges) or numbers.

Table 2. Summary of iTRAQ® comparisons from pooled ELF samples

	Acute smoking comparisons			Group comparisons at baseline	
	Young healthy susceptible	Young healthy Non-susceptible	Old COPD	Young healthy susceptible vs non-susceptible	Old healthy smokers vs never-smokers
Peroxiredoxin I	0.29	0.50	6.9	σ <2.5	σ <2.5
Uteroglobin	σ <2.5	0.50	0.1	σ <2.5	σ <2.5
SerpinB3	σ <2.5	0.40	11.6*	2.42	σ <2.5
S100A9	0.39	0.50	σ <2.5	3.51	σ <2.5
S100A8	0.35	0.46	σ < 2.5	2.93	σ <2.5
ALDH3A1	0.29	0.29	16.7	σ <2.5	7.80

Data are expressed as median of ratios (of peptides for one protein that are discriminatory between samples): after smoking / before smoking (left section) or group comparisons (right section). σ <2.5: peptides of that protein did not reach a statistically significant difference. *: based on one peptide.

Supplementary Materials). After acute smoking of 3 cigarettes, the number of differentially expressed proteins showing overlap between the first and second experiment was 9 proteins in the group of the young susceptible individuals, 3 in the young non-susceptible individuals and 3 in the COPD patients (Table 4 and figure 4 in the Supplementary Materials).

The complete list of all proteins and relative peptides identified and quantified with high confidence (>95%) is reported in Table 8 in the *Online repository*.

Proteomics: selection of differential proteins

The following proteins were selected for further analysis with ELISA or immunohistochemistry based on the following criteria: 1) significant up- or down-regulation in both iTRAQ experiments, 2) quantification with 2 or more statistically significantly different peptides (p value < 0.02), 3) biological function that might be implicated in the onset and progression of COPD.

- Peroxiredoxin I (accession number Q06830),
- Uteroglobin (CC16, accession number P11684),
- SerpinB3 (SCCA1, accession number P29508),
- S100A8 (MRP8, Calgranulin A, accession number P05109),
- S100A9 (MRP14, Calgranulin B, accession number P06702),
- Aldehyde dehydrogenase 3A1 (ALDH3A1, accession number P30838).

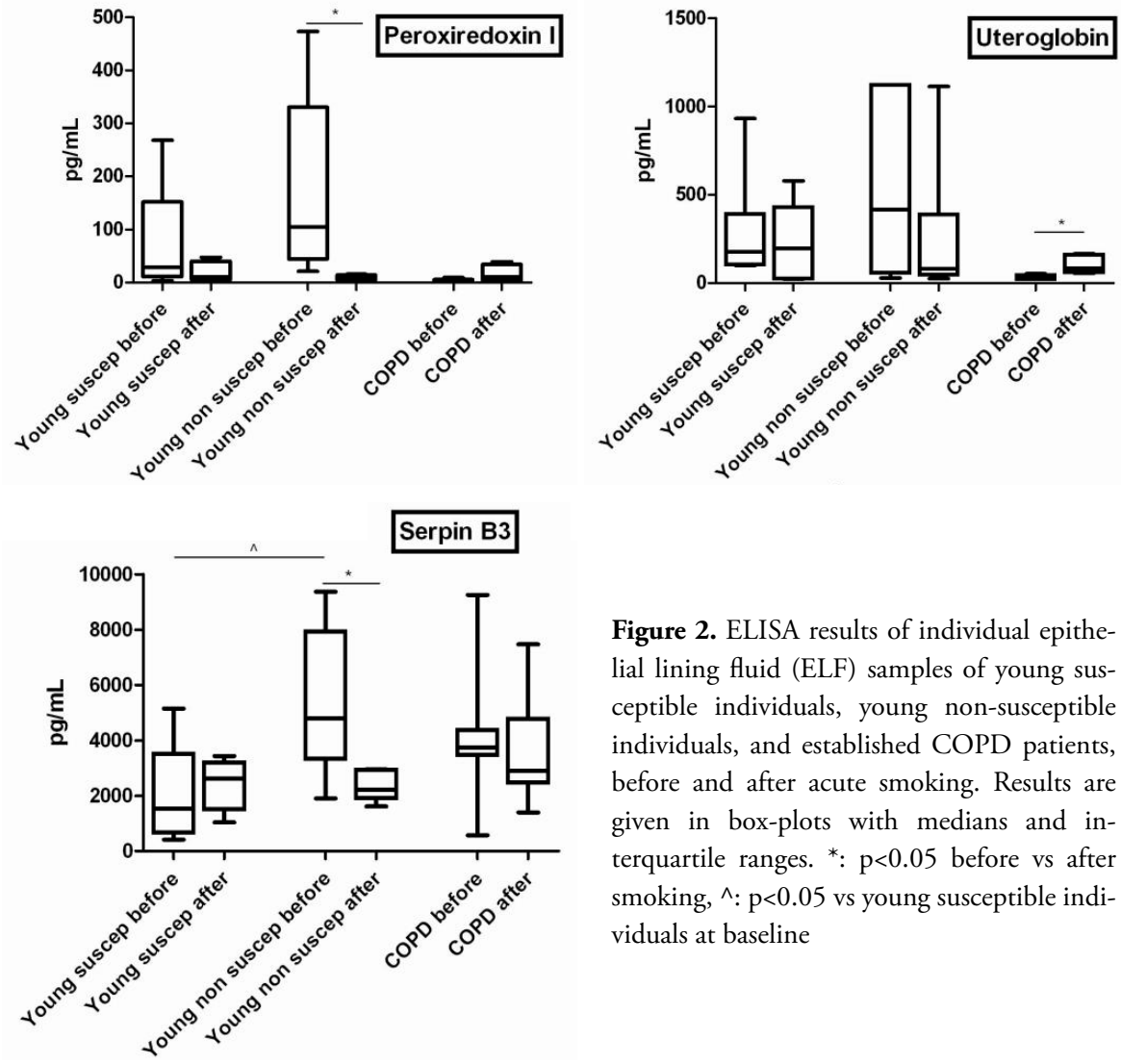

Figure 2. ELISA results of individual epithelial lining fluid (ELF) samples of young susceptible individuals, young non-susceptible individuals, and established COPD patients, before and after acute smoking. Results are given in box-plots with medians and interquartile ranges. *: $p<0.05$ before vs after smoking, ^: $p<0.05$ vs young susceptible individuals at baseline

Proteomics: comparison between groups at baseline

There were no significant differences in Peroxiredoxin I, Uteroglobin and ALDH3A1, between young susceptible and young non-susceptible individuals, while SerpinB3, S100A9, and S100A8 levels were higher in the young susceptible group (Table 2). Old healthy smokers showed higher levels of ALDH3A1 and Peroxiredoxin I than old healthy non-smokers.

Proteomics: comparison before and after acute smoking

In the young susceptible individuals levels of Peroxiredoxin I, S100A9, S100A8 and ALDH3A1 decreased after acute smoke exposure (Table 2) while all selected proteins were down-regulated in the young non-susceptible group. On the contrary, Peroxiredoxin I, SerpinB3, and ALDH3A1 were up-regulated in the old COPD patients, whereas Uteroglobin was down-regulated after acute smoke exposure (Table 2).

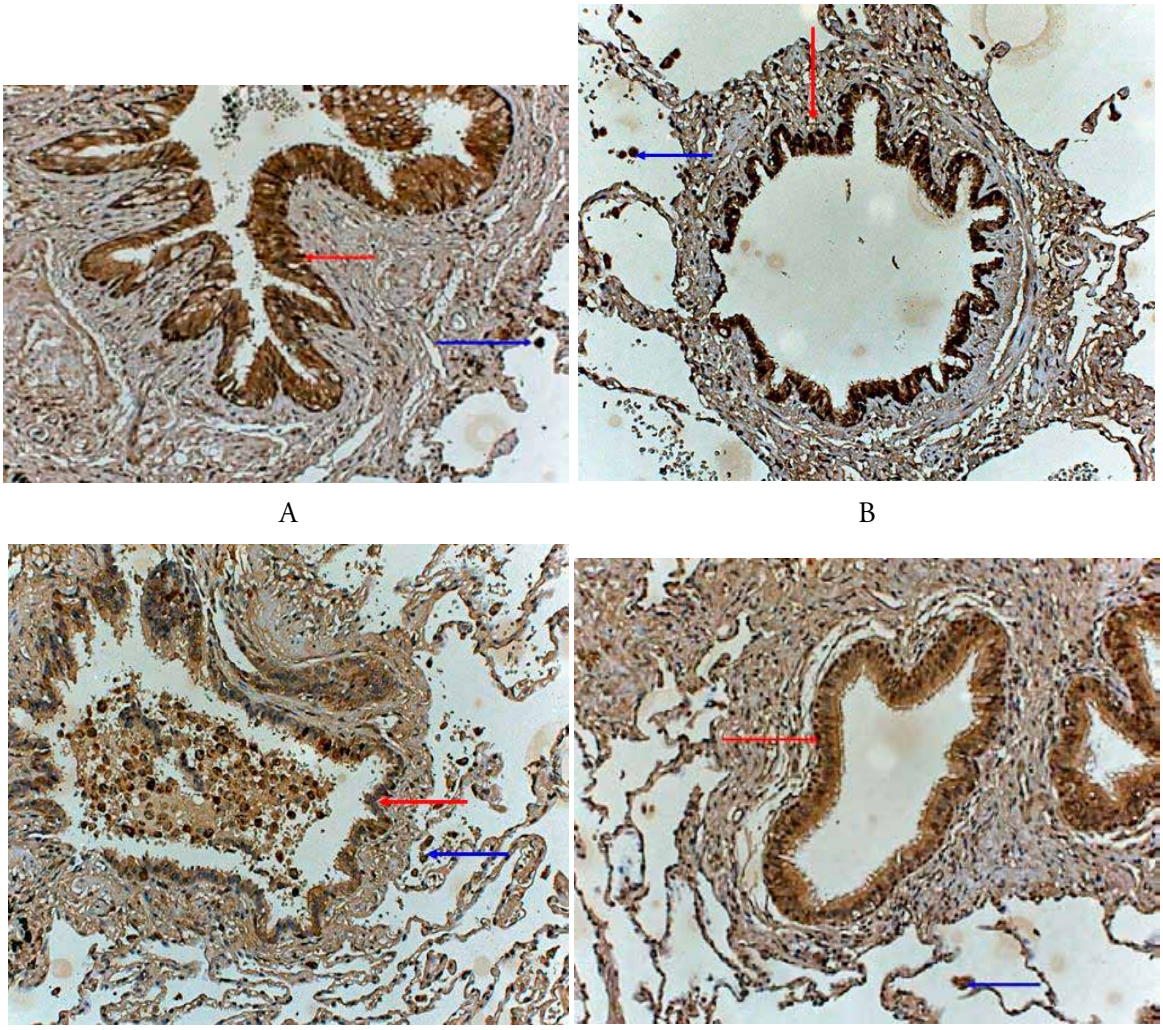

Figure 3. Immunohistochemistry of aldehyde dehydrogenase 3A1. Panel A: immunostaining of a COPD patient current smoker. Panel B: immunostaining of a healthy control current smoker. Panel C: immunostaining of a COPD patient ex-smoker. Panel D: immunostaining of a healthy control non-smoker. All COPD patients are GOLD STAGE II. The red arrows indicate epithelial cells and blue arrows indicate macrophages, more or less positive for ALDH3A1.

ELISA: comparison between groups at baseline

Young susceptible individuals showed a trend for lower SerpinB3 concentrations in ELF than young non-susceptible individuals (p=0.056).There were no significant differences between old healthy smokers versus non-smokers, nor between COPD patients and the two old healthy groups.

ELISA: comparison before and after acute smoking

In young susceptible individuals, expression of the selected proteins did not differ significantly before and after acute smoking (Table 3). In the young non-susceptible individuals Peroxiredoxin I and S100A9 concentrations were lower after smoking (p=0.043, and p=0.028,

Table 3. ELISA results of non-pooled ELF

		Acute smoking experiment			Baseline controls	
		Young susceptible	Young Non-susceptible	Old COPD	Old healthy smokers	Old healthy never-smokers
PRDX I, pg/ml						
	Before	28.5 (3.3-268)	105 (21-473)	3.8 (0.4-8.8)	36.5 (1.8-227)	13 (1.1-164)
	After	10.5 (0.07-48)	10.5 (1.9-15.5)*	10.8 (2.4-39)^		
Uteroglobin, pg/ml						
	Before	176 (100-933)	415 (29-1123)	24 (21-52)	409 (17-1484)	134 (29-764)
	After	195 (21-580)	81 (25-1115)	84 (54-166)		
SerpinB3, pg/ml						
	Before	1536 (417-5152)#	4803 (1900-9371)	3745 (567-9254)	3935 (798-4454)	2476 (821-4904)
	After	2609 (1040-3439)	2210 (1610-2955)	2907 (1398-7474)		
S100A9, µg/mL						
	Before	0.24 (0.01-0.96)	0.43 (0.17-2.80)	0.63 (0.37-0.87)	0.9 (0.2-5.2)	1 (0.3-2.2)
	After	0.72 (0.22-0.75)	0.18 (0.05-0.39)*	0.54 (0.10-1.90)		

Values are medians (ranges).*$p<0.05$ vs before. ^$p=0.063$ vs before. Bold: significant difference in acute smoke response between two groups. #$p=0.056$ vs young non-susceptible subjects.

respectively), whereas SerpinB3 showed a similar trend (p=0.08) (Figure 2).The comparison between young non-susceptible and susceptible individuals regarding their acute smoking response showed a significant difference in the change of SerpinB3 with smoking (Table 3, Mann Whitney U test, p=0.016). In the COPD patients Peroxiredoxin I tended to increase after acute smoking (p=0.063).

Due to experimental issues no quantifiable results were obtained for S100A8 (Table 6 in the Supplementary Materials); regarding ALDH3A1 no statistically significant differences between the groups were observed (Table 7 in the Supplementary Materials).

Immunohistochemistry confirmation: ALDH3A1
A semi-quantitative analysis was performed in a blinded fashion (by authors LF and ML) on ALDH3A1 expression in lung resection material of 5 COPD patients (current smokers), 5 COPD patients (ex-smokers), 5 healthy controls (current smokers), and 5 healthy never/ex-smokers (Table 5 in the Supplementary Materials). ALDH3A1 protein expression was clearly associated with smoking status (Figure 5 in the Supplementary Materials). Highest expression of ALDH3A1 was observed in macrophages and epithelial cells of COPD patients (current smokers), followed by healthy subjects (current smokers), and COPD patients (former smokers). The lowest expression was seen in healthy individuals and never smokers (Figure 3).

4. Discussion

This is the first study to apply an unbiased proteomic approach to better understand the mechanisms underlying the development of COPD. iTRAQ analysis of ELF after acute smoke exposure demonstrated (in duplo) 9 proteins to be increased or decreased in young susceptible individuals, 4 proteins in the young non-susceptible individuals, and 3 in COPD patients. Six proteins were selected based on significant up- or down-regulation in two iTRAQ experiments, identification and quantification with two or more statistically significant peptides, and a biological function that might be implicated in the onset and progression of COPD. Of interest, two proteins (SerpinB3, Uteroglobin) decreased after smoking of 3 cigarettes in young non-susceptible individuals while remaining stable in young susceptible individuals. Four proteins (Peroxiredoxin I, S100A9, S100A8, ALDH3A1) decreased both in young susceptible and non-susceptible individuals. Peroxiredoxin, SerpinB3 and ALDH3A1 increased in COPD patients after a comparable smoke exposure. These differentially expressed proteins may play a role in protection against oxidative stress, anti-inflammatory responses and metabolizing toxic compounds, thus constituting plausible candidates involved in COPD development.

What might be the function of the above described differential proteins in relation to COPD more specifically? SerpinB3 inhibits several types of proteases and plays a role in modulating inflammation, programmed cell death and fibrosis [27]. S100A8 and S100A9 proteins, so called calgranulins, are known for their antimicrobial activity and their role as pro-inflammatory mediators in acute and chronic inflammation [28–30]. Uteroglobin may play a role in reducing airway inflammation and protecting against oxidative stress, in addition to its immunosuppressive and anti-tumor qualities [31]. Peroxiredoxins are known to control the response to oxidants and to play an anti-inflammatory role [32]. They are highly expressed in the healthy lung [28], and constitute a powerful defence against oxidative stress by decomposing peroxides, one of the major components of the tar phase of cigarette smoke. Finally, ALDH3A1 is one of the aldehyde dehydrogenases involved in the detoxification of carcinogenic aldehydes associated with cigarette smoke [33].

We found four proteins to decrease upon acute smoking irrespective of COPD susceptibility and hypothesize that these proteins play a role in orchestrating the normal inflammatory response to smoke exposure. In contrast, SerpinB3 and Uteroglobin decreased exclusively in young non-susceptible individuals, and ELISA experiments confirmed this for SerpinB3. The differential SerpinB3 and Uteroglobin response on smoking between the two young groups suggests that these proteins might be crucial for the very first steps towards COPD, given its modulatory function in inflammation and fibrosis [27] and release of lysosomal proteinases from damaged epithelial cells [34]. SerpinB3 concentrations have been shown to be higher in bronchoalveolar lavage fluid of smokers than non-smokers [35]. It was therefore an unanticipated observation that the expression of this protective protein was not restored to baseline 24 hours after acute smoke exposure in non-susceptible individuals, in contrast to the susceptible individuals. Whether this finding in ELF is a negative mirror of what occurs in the airway wall after an attack of cigarette smoking needs to be determined in further studies. In that case a lower value in ELF in non-susceptible youngsters indicates an increased use in the lung tissue, whereas this does not occur in susceptible individuals. Uteroglobin or human Clara cell protein (CC16) is a 15.8-kDa homodimeric protein secreted in large amounts into the airways by the non-ciliated bronchiolar Clara cells. The exact physiological function in the lung is not known, but it likely plays a role in reducing airway inflammation and protecting against oxidative stress, in addition to immunosuppressive and anti-tumor qualities [31]. In an acute smoke model in rats a dose dependent increase in serum Uteroglobin was demonstrated with a peak level 2 hours after smoking and a return to normal levels after 24 hours [36]. Our results show a decrease of Uteroglobin only in young non-susceptible individuals 24 hours after smoking. Unfortunately, we have no information about its presence immediately after smoking, so future studies, using less invasive sampling techniques, are needed to understand its complete time-response. COPD patients demonstrated an opposite response to acute smoking compared with young susceptible and non-susceptible individuals, with higher expression of Peroxiredoxin I, SerpinB3, and AL-

DH3A1 after smoking. This finding supports our choice of studying young individuals for better understanding of the very first steps of COPD induction. Apparently, the bronchial tree in COPD patients has changed dramatically after many years of smoking and is able to up-regulate these mainly protective proteins for at least 24 hours after smoke inhalation.

To assess if the detected proteins in COPD reflect a nonspecific response to chronic smoking or rather are a disease-specific characteristic we compared healthy smokers and never-smokers (at baseline). The iTRAQ and immunohistochemistry results of ALDH3A1 clearly show that this protective protein is strongly up-regulated due to chronic smoking both in COPD and healthy smokers. Interestingly, one proteomic study demonstrated increased levels in BAL fluid from ex-smoking COPD patients [8]. Regarding Uteroglobin we expected to find a smoking-induced reduction as chronic smoking has been associated with a lower number of Clara cells in the bronchial tree [37] as well as with lower levels in BAL fluid [38–41]. Moreover, reduced Uteroglobin protein levels have been demonstrated in BAL [41] and serum [41,42] of COPD patients, whereas severe COPD patients demonstrated lower levels in sputum than moderate COPD patients [43]. In line, 2 proteomic studies demonstrated decreased levels in BAL fluid of asymptomatic smokers [40] and in induced sputum of smokers and COPD patients [12]. Our ELISA results indeed demonstrated reduced levels in ELF of COPD patients; a finding that did not match with the iTRAQ results in healthy smokers and never smokers.

A possible weakness of our study is that susceptibility at young age to develop COPD was based on family history. On the other hand, this strategy has been used in previous studies as well and provided clues for a genetic component of the disease [19,44–46]. A second limitation is that we included a relatively low number of participants, and the groups were not optimally balanced for age and gender, which poses questions regarding the generalization of the obtained results. Third, the iTRAQ samples of the different groups needed to be pooled which allowed only 5 comparisons. On the other hand ELISA was performed on individual samples from a larger group of participants and was not limited in the number of comparisons. Despite the above described methodological drawbacks, our study was able to show statistically significant differences, suggesting major changes. The observed differential proteomic profiles in susceptible and non-susceptible individuals open avenues for further biomarker development in larger studies.

In conclusion, we describe one of the first studies to assess proteins associated with susceptibility to develop COPD using an unbiased approach. We found statistically significant changes in expression of candidate proteins upon acute smoke exposure, by studying two young cohorts of individuals and a group of older COPD patients. Our data show that already at young age, subjects with a positive family history of COPD respond differently to cigarette smoke than those with a negative family history. Particularly SerpinB3 and Uteroglobin were found to be proteins that may play a role in the development of COPD.

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Chapter IV

Supplementary material

Table 1. Proteins identified in pooled ELF of young individuals (susceptible and non-susceptible) in the first and duplicate iTRAQ®-4plex experiments

	Both experiments
1.	AK1Adenylatekinase1
2.	AKR1A1Alcoholdehydrogenase[NADP+]
3.	ALDH1A1cDNAFLJ50286,highlysimilartoRetinaldehydrogenase1
4.	ALDH3A1Aldehydedehydrogenase3A1
5.	ANXA1AnnexinA1
6.	ANXA536kDaprotein
7.	ARHGDIA26kDaprotein
8.	AZGP1Zinc-alpha-2-glycoprotein
9.	BASP1Isoform1ofBrainacidsolubleprotein
10.	CAPSCalcyphosin
11.	CALM1;CALM3;CALM221kDaprotein
12.	CRIP1Cysteine-richprotein1
13.	CST3Cystatin-C
14.	CSTBCystatin-B
15.	CTSDCathepsinD
16.	CFL1Cofilin-1
17.	Diazepambindinginhibitor,spliceform1G
18.	Elongationfactor1-alpha
19.	ENO1Isoformalpha-enolaseofAlpha-enolase
20.	GAPDHGlyceraldehyde-3-phosphatedehydrogenase
21.	GSTA2GlutathioneS-transferaseA2
22.	GSTP1PutativeuncharacterizedproteinGSTP1
23.	HDGFHepatoma-derivedgrowthfactor
24.	Heatshock70kDaprotein1
25.	HMGB1HighmobilitygroupproteinB1
26.	HP42kDaprotein
27.	IGHG1
28.	IGHA1
29.	IGLV2-14
30.	KRT1549kDaprotein
31.	KRT8Keratin,typeIIcytoskeletal8
32.	Lactotransferrin
33.	LCN2PutativeuncharacterizedproteinLCN2
34.	LUZP6PutativeuncharacterizedproteinMTPN

Table 1. (continued)

35.	LYZLysozymeC
36.	NQO1NAD(P)Hdehydrogenase,quinone1(NQO1)
37.	PARK7ProteinDJ-1
38.	PDLIM1PDZandLIMdomainprotein1
39.	PEBP1Phosphatidylethanolamine-
40.	bindingprotein1
41.	PFN1Profilin-1
42.	PPIAPeptidyl-prolylcis-transisomeraseA
43.	PRDX1Peroxiredoxin-1
44.	PRDX5Peroxiredoxin-5
45.	PRSS128kDaprotein
46.	PSME1proteasomeactivatorcomplexsubunit1isoform2
47.	S100A11ProteinS100-A11
48.	S100A6ProteinS100-A6
49.	S100A8ProteinS100-A8
50.	S100A9ProteinS100-A9
51.	S100PProteinS100-P
52.	Serumalbumin
53.	SCGB1A1Uteroglobin
54.	SELENBP1Isoform1ofSelenium-bindingprotein1
55.	SERPINB1Leukocyteelastaseinhibitor
56.	SERPINB3Isoform1ofSerpinB3
57.	SH3BGRLSH3domain-bindingglutamicacid-rich-likeprotein
58.	SOD1Superoxidedismutase[Cu-Zn]
59.	TAGLN224kDaprotein
60.	TALDO1Transaldolase
61.	TFSerotransferrin
62.	TXNThioredoxin,isoformCRA_b
63.	UBB;RPS27A;UBCUbiquitin
64.	YWHAZ14-3-3proteinzeta/delta
	First experiment
1.	21kDaprotein
2.	ACTB14kDaprotein
3.	CST1Cystatin-SN
4.	CTSCDipeptidylpeptidase1
5.	EIF4HSimilartomKIAA0038protein
6.	ENSAIsoform4ofAlpha-endosulfine

Table 1. (continued)

7.	Glutathionreductasedelta8+9alternativesplicingvariant
8.	GOLM1Isoform1ofGolgimembraneprotein1
9.	HBA1;HBA2Hemoglobinalpha-2
10.	Hematologicalandneurologicalexpressed1-likeprotein
11.	HNRNPA2B1
12.	IGHA2
13.	IGJPutativeuncharacterizedproteinIGJ
14.	IGKCIgkappachainCregion
15.	KRT10Keratin,typeIcytoskeletal10
16.	KRT1Keratin,typeIIcytoskeletal1
17.	LASP1PutativeuncharacterizedproteinLASP1
18.	LCN1L1Putativelipocalin1-likeprotein1
19.	LOC100126583;IGHA2
20.	LOC100290309hypotheticalproteinXP_002348012
21.	LOC389842similartoRANbindingprotein1
22.	LTFcDNAFLJ58679,highlysimilartoLactotransferrin
23.	MB12kDaprotein
24.	MYL12Bmyosinregulatorylightchain12BisoformB
25.	Nuclearubiquitouscaseinandcyclin-dependentkinasessubstrate
26.	ORM1orosomucoid1precursor
27.	PIGRPolymericimmunoglobulinreceptor
28.	PRDX6Peroxiredoxin-6
29.	PTMAPutativeuncharacterizedproteinPTMA
30.	SERPINA1Isoform3ofAlpha-1-antitrypsin
31.	STIP1Stress-induced-phosphoprotein1
32.	SUMO3cDNAFLJ57440,Smallubiquitin-relatedmodifier3
33.	TIMP1TIMPmetallopeptidaseinhibitor1
34.	TPI1;TPI1P1triosephosphateisomerase1isoform2
35.	TPM3PutativeuncharacterizedproteinDKFZp686J1372
36.	TPPP3Tubulinpolymerization-promotingproteinfamilymember3
37.	TTR20kDaprotein
38.	TXNDC179kDaprotein
39.	YWHAE22kDaprotein
40.	ZG16BZymogengranuleprotein16homologB
	Second Experiment
1.	ACTG1Actin,cytoplasmic2
2.	ADH1CAlcoholdehydrogenase1C

Table 1. (continued)

3.	ALDOA45kDaprotein
4.	Alpha-amylase1
5.	ANXA2Isoform1ofAnnexinA2
6.	B2MBeta-2-microglobulin
7.	C6orf58UPF0762proteinC6orf58
8.	CASTIsoform6ofCalpastatin
9.	CST4Cystatin-S
10.	CST5Cystatin-D
11.	DEFA3Neutrophildefensin3
12.	DSTNDestrin
13.	EZR69kDaprotein
14.	FTH1Ferritinheavychain
15.	GSNcDNAFLJ53327,highlysimilartoGelsolin
16.	HBDBeta-globingenefromathalassemiapatient
17.	HeatshockproteinHSP90-alpha
18.	HeatshockproteinHSP90-beta
19.	HEBP2HEBP2protein(Fragment)
20.	HINT1Histidinetriadnucleotide-bindingprotein1
21.	HNRNPDIsoform3ofHeterogeneousnuclearribonucleoproteinD0
22.	IGHG2
23.	IGJImmunoglobulinJchain
24.	IGLV2-14
25.	KRT9Keratin,typeIcytoskeletal9
26.	LGALS3Galectin-3
27.	Longpalate,lungandnasalepitheliumcarcinoma-associatedprotein1
28.	MSMBIsoformPSP94ofBeta-microseminoprotein
29.	NCLNucleolin
30.	NEDD89kDaprotein
31.	PGK1Phosphoglyceratekinase1
32.	PIPProlactin-inducibleprotein
33.	Polymericimmunoglobulinreceptor
34.	PRDX2Peroxiredoxin-2
35.	PRDX6Peroxiredoxin-6
36.	Pre-Blymphocytegene2
37.	PSAPIsoformSap-mu-6ofProactivatorpolypeptide
38.	PSMA6Proteasomesubunitalphatype-6
39.	PTMAPutativeuncharacterizedproteinPTMA

Table 1. (continued)

40.	Putativeuncharacterizedprotein
41.	RibosomalproteinL29(RPL29),mRNA
42.	SLPIAntileukoproteinase
43.	SUMO4Smallubiquitin-relatedmodifier4
44.	TFF3trefoilfactor3precursor
45.	TMSL1Putativethymosinbeta-4-likeprotein1
46.	TPM3tropomyosinalpha-3chainisoform4
47.	triosephosphateisomerase1isoform2
48.	TUBA1CTUBA1Cprotein
49.	TUBB2CTubulinbeta-2Cchain
50.	Tubulinpolymerization-promotingproteinfamilymember3
51.	UBE2IUbiquitincarrierprotein
52.	WFDC2Isoform1ofWAPfour-disulfidecoredomainprotein2
53.	YWHAE14-3-3proteinepsilon

In the group of young subjects totally 157 proteins were identified in two experiments, 64 of them were overlapping in both experiments.

Table 2. Proteins identified in pooled ELF of the old individuals (COPD patients, healthy smokers and non-smokers) in the first and duplicate iTRAQ®-4plex experiments

	Both experiments
1.	AK1Adenylatekinaseisoenzyme1
2.	AKR1A1Alcoholdehydrogenase[NADP+]
3.	ALDH1A1Retinaldehydrogenase1
4.	ALDH3A1Aldehydedehydrogenase3A1
5.	ALDOA45kDaprotein
6.	ANXA1AnnexinA1
7.	ANXA536kDaprotein
8.	AZGP1Zinc-alpha-2-glycoprotein
9.	B2McDNAFLJ57067,highlysimilartoBeta-2-microglobulin
10.	C6orf58UPF0762proteinC6orf58
11.	CALM1;CALM3;CALM221kDaprotein
12.	CAPSCalcyphosin
13.	CBR1Carbonylreductase[NADPH]1
14.	CFL1Cofilin-1
15.	CRIP1Cysteine-richprotein1
16.	CST3Cystatin-C
17.	CSTBCystatin-B

Table 2. (continued)

18.	CTSDCathepsinD
19.	Diazepambindinginhibitor,spliceform1G
20.	DMBT1Isoform8ofDeletedinmalignantbraintumors1protein
21.	ENO1Isoformalpha-enolaseofAlpha-enolase
22.	FAM3DProteinFAM3D
23.	GAPDHGlyceraldehyde-3-phosphatedehydrogenase
24.	GSTP1PutativeuncharacterizedproteinGSTP1
25.	HBA1;HBA2Hemoglobinsubunitalpha
26.	Hematologicalandneurologicalexpressed1-likeprotein
27.	HMGB1HighmobilitygroupproteinB1
28.	HPXHemopexin
29.	IGHA1
30.	IGHG1
31.	IGHG2
32.	IGLV2-14
33.	KRT1Keratin,typeIIcytoskeletal1
34.	Lactotransferrin
35.	LASP1Isoform1ofLIMandSH3domainprotein1
36.	LCN1Lipocalin-1
37.	LCN2Lipocalin2
38.	LGALS3Galectin-3
39.	LYZLysozymeC
40.	NQO1NAD(P)Hdehydrogenase[quinone]1isoformb
41.	PARK7ProteinDJ-1
42.	PEBP1Phosphatidylethanolamine-bindingprotein1
43.	PFN1Profilin-1
44.	PIGRPolymericimmunoglobulinreceptor
45.	PPIAPeptidyl-prolylcis-transisomeraseA
46.	PRDX1Peroxiredoxin-1
47.	PRDX2Peroxiredoxin-2
48.	PRDX5Peroxiredoxin-5
49.	PRDX6Peroxiredoxin-6
50.	PRSS128kDaprotein
51.	PSAPIsoformSap-mu-6ofProactivatorpolypeptide
52.	PTMAPutativeuncharacterizedproteinPTMA
53.	Putativeuncharacterizedprotein
54.	S100A11ProteinS100-A11

Table 2. (continued)

55.	S100A6ProteinS100-A6
56.	S100A8ProteinS100-A8
57.	S100A9ProteinS100-A9
58.	S100PProteinS100-P
59.	SCGB1A1Uteroglobin
60.	SELENBP1Isoform1ofSelenium-bindingprotein1
61.	SERPINB3Isoform1ofSerpinB3
62.	Serumalbumin
63.	SH3BGRLSH3domain-bindingglutamicacid-rich-likeprotein
64.	SOD1Superoxidedismutase[Cu-Zn]
65.	SUMO3SMT3suppressorofmiftwo3homolog3(Yeast)
66.	TAGLN224kDaprotein
67.	TFSerotransferrin
68.	TPPP3Tubulinpolymerization-promotingproteinfamilymember3
69.	TXNThioredoxin
70.	YWHAZ14-3-3proteinzeta/delta
	First experiment
1.	Actin,alpha,cardiacmuscle(ACTC)
2.	AKR1B10Aldo-ketoreductasefamily1memberB10
3.	Anti-(ED-B)scFV(Fragment)
4.	BASP1Isoform1ofBrainacidsolubleprotein1
5.	C19orf33Isoform1ofImmortalizationup-regulatedprotein
6.	CASTIsoform6ofCalpastatin
7.	CLIC1Chlorideintracellularchannelprotein1
8.	CRISP3Cysteine-richsecretoryprotein3
9.	CSTAPutativeuncharacterizedproteinCSTA
10.	EEF1A1;EEF1A1P5Putativeelongationfactor1-alpha-like3
11.	EIF4HSimilartomKIAA0038protein
12.	EWSR1PutativeuncharacterizedproteinEWSR1
13.	GCIsoform2ofVitaminD-bindingprotein
14.	GOLM1Isoform1ofGolgimembraneprotein1
15.	GSTA2GlutathioneS-transferaseA2
16.	HBBHemoglobinsubunitbeta
17.	HDGFHepatoma-derivedgrowthfactor
18.	HeterogeneousnuclearribonucleoproteinA1-like2
19.	HNRNPA2B1PutativeuncharacterizedproteinH
20.	HP42kDaprotein

Table 2. (continued)

21.	HSPA6Heatshock70kDaprotein6
22.	IGHA2
23.	IGHV3OR16-14hypotheticalproteinXP_002343513
24.	IGJPutativeuncharacterizedproteinIGJ
25.	KRT10Keratin,typeIcytoskeletal10
26.	KRT1549kDaprotein
27.	KRT9Keratin,typeIcytoskeletal9
28.	LTFcDNAFLJ58679,highlysimilartoLactotransferrin
29.	NCLNucleolin
30.	NRNPA2B1
31.	Nuclearubiquitouscaseinandcyclin-dependentkinasessubstrate
32.	ORM1orosomucoid1precursor
33.	PDLIM1PDZandLIMdomainprotein1
34.	PRR4proline-richprotein4isoform1
35.	RPS6RibosomalproteinS6,isoformCRA_a
36.	S100A2proteinS100-A2
37.	SERPINA1Isoform1ofAlpha-1-antitrypsin
38.	TIMP1TIMPmetallopeptidaseinhibitor1
39.	TMSB10Thymosinbeta-10
40.	TPI1;TPI1P1triosephosphateisomerase1isoform2
41.	Tubulin-specificchaperoneA
42.	UBA52ubiquitinandribosomalproteinL40precursor
43.	YWHAE22kDaprotein
	Second experiment
1.	ACTG1Actin,cytoplasmic2
2.	CATHEPSINB
3.	cDNAFLJ59092
4.	CST1Cystatin-SN
5.	DEFA3Neutrophildefensin3
6.	FTH1Ferritinheavychain
7.	GSTA1GlutathioneS-transferaseA1
8.	GSTO1GlutathioneS-transferaseomega-1
9.	HBDBeta-globingenefromathalassemiapatient
10.	DSTNdestrinisoformb
11.	DYNLL1Dyneinlightchain1,cytoplasmic
12.	Heatshock70kDaprotein1
13.	HNRNPDPutativeuncharacterizedproteinHNRNPD

Table 2. (continued)

14.	IGJImmunoglobulinJchain
15.	IGLV2-14
16.	KRT8Keratin,typeIIcytoskeletal8
17.	LCN1L1Putativelipocalin1-likeprotein1
18.	Longpalate,lungandnasalepitheliumcarcinoma-associatedprotein1
19.	MSMBIsoformPSP94ofBeta-microseminoprotein
20.	ORM2Alpha-1-acidglycoprotein2
21.	PGK1Phosphoglyceratekinase
22.	PSME1Proteasomeactivatorcomplexsubunit1
23.	RDXRadixin
24.	RRBP1p180/ribosomereceptor
25.	SLPIAntileukoproteinase
26.	TALDO1Transaldolase
27.	TFF3trefoilfactor3precursor
28.	TMSL3Thymosinbeta-4-likeprotein3
29.	Triosephosphateisomerase1isoform2
30.	UBB;RPS27A;UBCUbiquitin
31.	WFDC2Isoform1ofWAPfour-disulfidecoredomainp rotein2

In the group of old subjects totally 144 proteins were identified in two experiments, 70 of them were overlapping in both experiments.

Table 3. Proteins differentially expressed at baseline

Young susceptible higher than non-susceptible	
FIRST	SECOND
Annexin A5	**Annexin A5**
Proteasome activator complex subunit 1 isoform 2	**Proteasome activator complex subunit 1 isoform 2**
Protein S100 A9	**Protein S100 A9**
Protein S100 A8	**Protein S100 A8**
Retinal dehydrogenase 1	**Retinal dehydrogenase 1**
Serpin B3	**Serpin B3**
14-3-3 protein zeta/delta	Adenylate kinase 1
Anti-folate binding protein	Alcohol dehydrogenase [NADP+]
Elongation factor 1-alpha	Annexin A1
Glyceraldehyde-3-phosphate dehydrogenase	Diazepam binding inhibitor, splice form 1G
IGHA2	Heat shock protein 70
LOC100290309 hypothetical protein	IGHA1
MB 12 kDa protein	Keratin type II cytoskeletal 8
Myosin regulatory light chain 12B isoform B	Lactotransferrin
NAD(P)H dehydrogenase	Peroxiredoxin V
Heat shock protein 70	Protein S100 A11
Protein LASP1	Serotransferrin
Serum albumin	
TPM3	
Young susceptible lower than non-susceptible	
FIRST	SECOND
Beta-2-microglobulin	Calcyphosin
Cystatin-SN	Cystatin S
Glutathione S-transferase A2	IGHG1
Lactotransferrin	Serum Albumin
Lipocalin-1	
Old healthy smoker higher than old never-smoker	
FIRST	SECOND
ALDH3A1	**ALDH3A1**
AZGP1 (zinc-alpha-2 glycoprotein)	**AZGP1**
C6orf 58	**C6orf 58**
CBR1 carbonyl reductase	**CBR1 carbonyl reductase**
Lactotransferrin	**Lactotransferrin**
Lysozyme C	**Lysozyme C**

Table 3. (continued)

NQO1 NAD(P)H dehydrogenase quinone1	**NQO1 NAD(P)H dehydrogenase quinone1**
Aldoketo reductase	IG alpha-1
GOLM1	IG J
MBT1	IGLV2-14
PRR4 proline-rich protein4	MSMB (Beta-microsemoniprotein)
SOD1 superoxide dismutase	PIGR (polymeric immunoglobulin receptor)
	PRDX1- PRDX2
	SLP1 antileukoproteinase
	TFF3
Old healthy smoker lower than old never-smoker	
FIRST	SECOND
HBB (hemoglobin subunit Beta)	GAPDH (Glyceraldehyde 3-phosphate dehydrogenase)
S100 A6	

In the group of young subjects totally 39 proteins were differentially expressed between young susceptible and non-susceptible individuals at baseline in two experiments; with 6 proteins showing overlap in both experiments. In the group of old subjects totally 23 proteins were differentially expressed in old healthy smokers and old never-smokers at baseline in two experiments; with 7 proteins showing overlap in both experiments. The proteins that overlap in both experiments are depicted in bold.

Table 4. Proteins increased or decreased after acute smoke exposure

Young susceptible increased	
FIRST	SECOND
Cystatin-SN	**Cystatin-SN**
Lactotransferrin	Alpha-amylase 1
Lipocalin 1	Cystatin-D
Unidentified protein (TGSGDIENNYND)	IGHG1
	Keratin 1 type II cytoskeletal 1
	Serum albumin
Young susceptible decreased	
FIRST	SECOND
Aldehyde dehydrogenase 3A1	Aldehyde dehydrogenase 3A1
Protein S100 A9	Protein S100 A9
Protein S100 A8	Protein S100 A8
Annexin A1	Annexin A1
Protein S100 P	Protein S100 P

Table 4. (continued)

NAD(P)H dehydrogenase quinone 1	NAD(P)H dehydrogenase quinone 1
Annexin A5	Annexin A5
Retinal dehydrogenase ALDH1A1	Retinal dehydrogenase ALDH1A1
ACTB 14 kDa protein	Alcohol Dehydrogenase 1C
Elongation factor 1-alpha	Diazepam binding inhibitor
Glutathione S-transferase P 1	Galectin-3
Glyceraldehyde 3-phosphate dehydrogenase	Glutathione S-transferase A2
Heat shock protein 70	Peroxiredoxin I
Keratin 1	Peroxiredoxin V
Keratin 10	Profilin-1
MYL12B myosin regulatory ligand	Protein S100 A11
Proteasome activator PSME1	Triosephosphate isomerase 1 isorform 2
Unidentified protein (FLIDGFPR)	
YWHAE 22 kDa protein	
Young non-susceptible increased	
FIRST	SECOND
Keratin 1 type II cytoskeletal 1	**Keratin, type II cytoskeletal 1**
Serum albumin	Alcohol dehydrogenase [NADP+]
Unidentified protein (TGSGDIENYND)	Alpha-amylase 1
	BASP1 Isoform 1 of Brain soluble acid soluble protein 1
	Cystatin-S
	Cystatin-D
	High Mobility Group Protein B1
	Keratin, type I cytoskeletal 9
Young non-susceptible decreased	
FIRST	SECOND
Aldehyde dehydrogenase 3A1	**Aldehyde dehydrogenase 3A1**
Polymeric immunoglobulin receptor	**Polymeric immunoglobulin receptor**
Alpha-enolase 1	Actin, cytoplasmatic 2
Unidentified protein (EIVMTQSPATD)	ALDH1A1 Retinal Dehydrogenase
	Aldolase A
	Annexin A1
	Calmodulin
	Galectin-3
	HBD Beta-globin
	Heat shock protein 90-alpha

Table 4. (continued)

	Heat shock protein 90-beta IGHA1
	Lactotransferrin
	Serotransferrin
	Serum albumin
	Peroxiredoxin I
	Peptidyl-prolyl cis-trans isomerase A
	PLUNC
	Profilin-1
	Protein S100 A8
	Proterin S100 A9
	Serpin B3
	Triosephosphate isomerase 1 isoform 2
	Uteroglobin
COPD increased	
FIRST	SECOND
Serum Albumin	**Serum albumin**
Aldehyde dehydrogenase 3A1	**Aldehyde dehydrogenase 3A1**
Calcyphosin	Annexin A5
Lipocalin 1	Carbonil Reductase [NADPH] 1
Peroxiredoxin I	Glutathione S-transferase P
PTMA	Heat shock protein 70
	Radixin
	Retinal Dehydrogenase ALDH1A1
	Serpin B3
COPD decreased	
FIRST	SECOND
Uteroglobin	**Uteroglobin**
Keratin 1	Trefoil factor 3 precursor
Lactotransferrin	
Lipocalin 2	
Lysozyme C	
GOLM1	
IGLV2-14	

In the group of young susceptible subjects the expression of totally 37 proteins increased or decreased after smoking in 2 experiments, showing overlap of 9 proteins in both experiments. In the group of young non-suceptible individuals this was 35 and 3 proteins respectively, in COPD 21 and 3 proteins. The proteins that overlap in both experiments are depicted in bold.

Table 5. Subject characteristics for immunohistochemistry of ALDH3A1 in lung tissue

	COPD current smokers	COPD Ex-smokers	non-COPD current smokers	non-COPD never/ex smokers
Female / Male	0/5	0/5	2/3	2/3
Age	69 (44-71)	72 (65-76)	63 (51-68)	50 (36-73)
Pack-years	28 (14-50)	30 (8-50)	50 (25-75)	0 (0-50)

Values are medians (ranges), or numbers

Table 6. ELISA results of protein S100A8 of individual epithelial lining fluid (ELF) samples of young susceptible individuals, young non-susceptible individuals, healthy subjects and established COPD patients, before and after acute smoking. 5 Young susceptible, 5 Young non-susceptible, 7 COPD patients were investigated but only for few samples it was possible to determine the concentrations. Results are given in concentrations (ng/mL).

Young non susceptible before	Young non susceptible after	Young susceptible before	Young susceptible after	COPD before	COPD after	Healthy non smokers	Healthy smokers
0.132	NA	0.02657	0.03384	8.38E-03	0.03384	8.38E-03	0.06658
		0.08113		0.01929		1.11E-03	0.03748
		0.0302		1.11E-03		0.143	8.38E-03
				8.38E-03		0.01929	0.143
						0.0302	0.01202
						0.2484	0.02293
						1.11E-03	0.07749
						0.01929	
						0.2121	
						0.2375	
						0.263	

Table 7. ELISA results of protein ALDH3A1 of individual epithelial lining fluid (ELF) samples of young susceptible individuals, young non-susceptible individuals, healthy subjects and established COPD patients, before and after acute smoking. No significant differences were found comparing the different groups (p value >0.05). Results are given in concentrations (ng/mL).

Young non susceptible before	Young non susceptible after	Young susceptible before	Young susceptible after	COPD before	COPD after	Healthy non smokers	Healthy smokers
0.05029	0.04201	0.05242	0.05674	0.04404	0.1018	0.06115	0.04404
0.05457	0.06565	0.06115	0.05894	0.06339	0.082	0.06792	0.05457
0.05674	0.05029	0.05894	0.06115	0.05457	0.07488	0.07022	0.1147
0.0461	0.06339	0.07254	0.07488	0.07022	0.05674	0.07254	0.05894
0.05242	0.04404	0.05457	0.05674	0.08929	0.04819	0.04819	0.05894
				0.06565	0.08441	0.04819	0.07022
				0.05242	0.05894	0.05674	0.06339
						0.04819	0.05029
						0.07022	0.04819
						0.07488	
						0.06339	
						0.04819	
						0.07488	

Table 8. Proteins and relative peptides identified and quantified with confidence>95%. Each reporter ion area 114, 115, 116 and 117 represent the group of young non-susceptible after acute smoking; young non-susceptible at baseline, young susceptible after acute smoking and young susceptible at baseline, respectively. In the group of older subjects the area represent COPD patients after acute smoking; COPD at baseline; Healthy subjects never smokers; healthy subjects current smokers.

This table is uploaded separately as excel file in the *Online Repository*:
https://www.dropbox.com/s/zngj5tvr53gvryu/Chapter%204_Supplementary%20material_Table%20S8_Thesis%20LF%202015.xlsx?dl=0

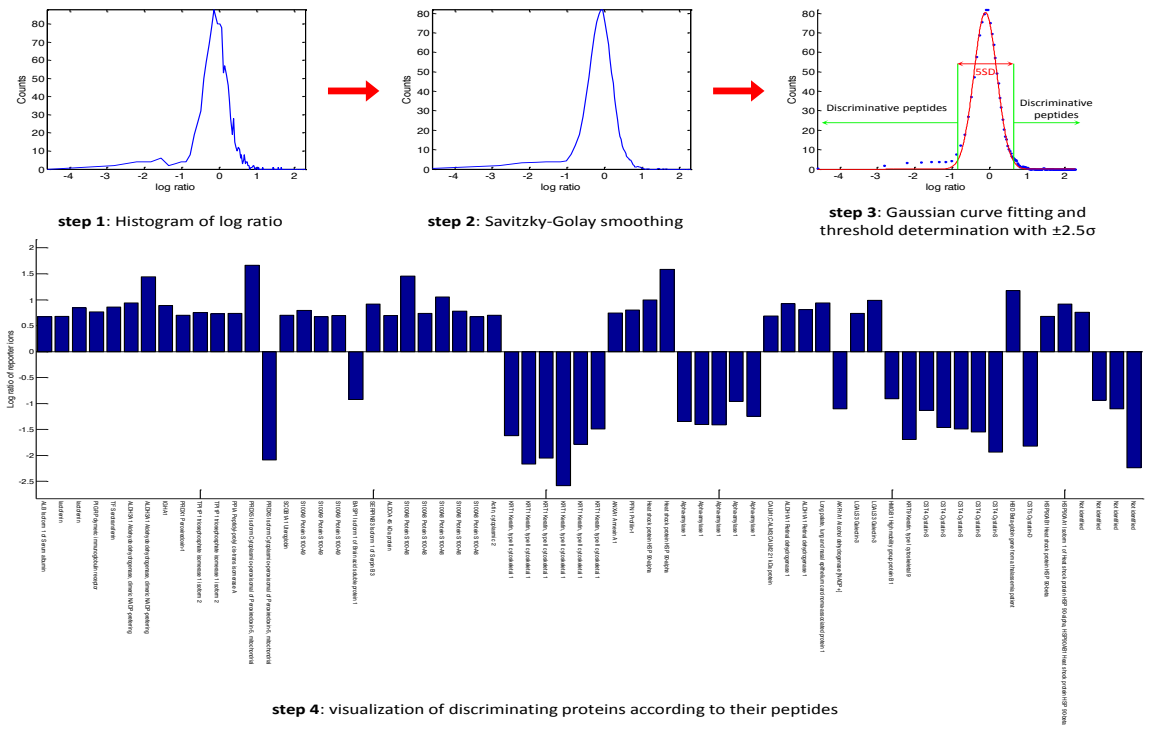

Figure 1. Main steps of statistical analysis to identify discriminative compounds from iTRAQ analysis data. The first step consists of calculating a histogram of natural logarithm reporter ions ratios of fragmented peptides between -4.6 (corresponding to ratio 0.01) and 2.3026 (corresponding to ratio 10) with step of 0.05. In the second step Savitzky-Golay smoothing with degree of 1 is applied. In the third step, Gaussian curve of normal distribution is fitted to the smoothed histogram. The parameters of the fitted curve were used to calculate the standard deviation (SD). The subplot for step 3 shows the data points of the smoothed curve of step 2 with blue dots, while the fitted Gaussian curve is shown with continuous red curve. For Gaussian curve fitting natural logarithm of ratios between -1 and 1 were used. Lower and upper threshold is selected at ±2.5 SD. The natural logarithm ratios of the reporter ions (in this example 114 and 115) of the selected discriminatory peptides with corresponding protein name are plotted in step 4. The method suppose that the majority of the protein do not change between the pooled samples, and therefore the central part of the Gaussian distribution can be used to fit a theoretical Gaussian curve. The peptides of discriminatory proteins are in the tails of the Gaussian. Gaussian curve reflect normal distribution of natural logarithm of reporter ion ratios and this curve can be used to setup a threshold based on type I error to select discriminatory peptides, and finally the list of discriminatory proteins.

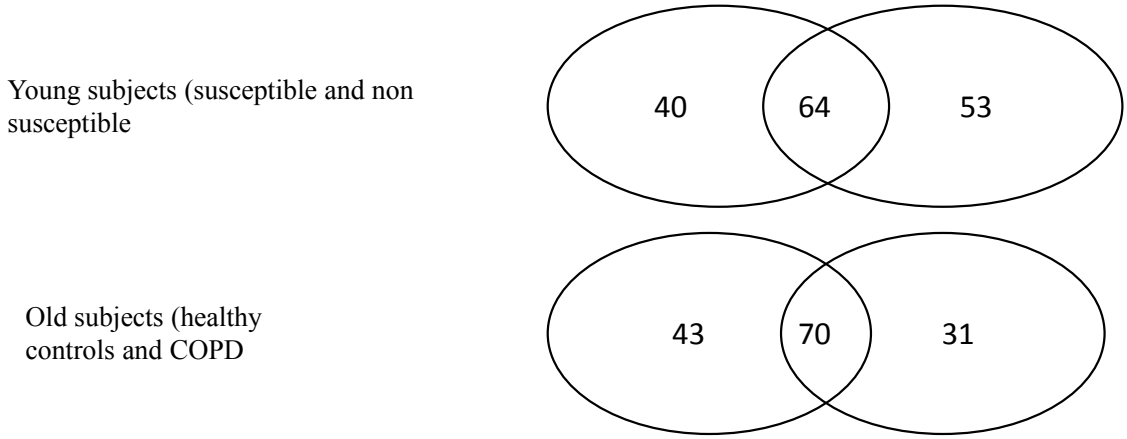

Figure 2. Overlap of the identified proteins between the first and second iTRAQ® experiment in the young subjects (susceptible and non-susceptible) and the old subjects (COPD and healthy controls)

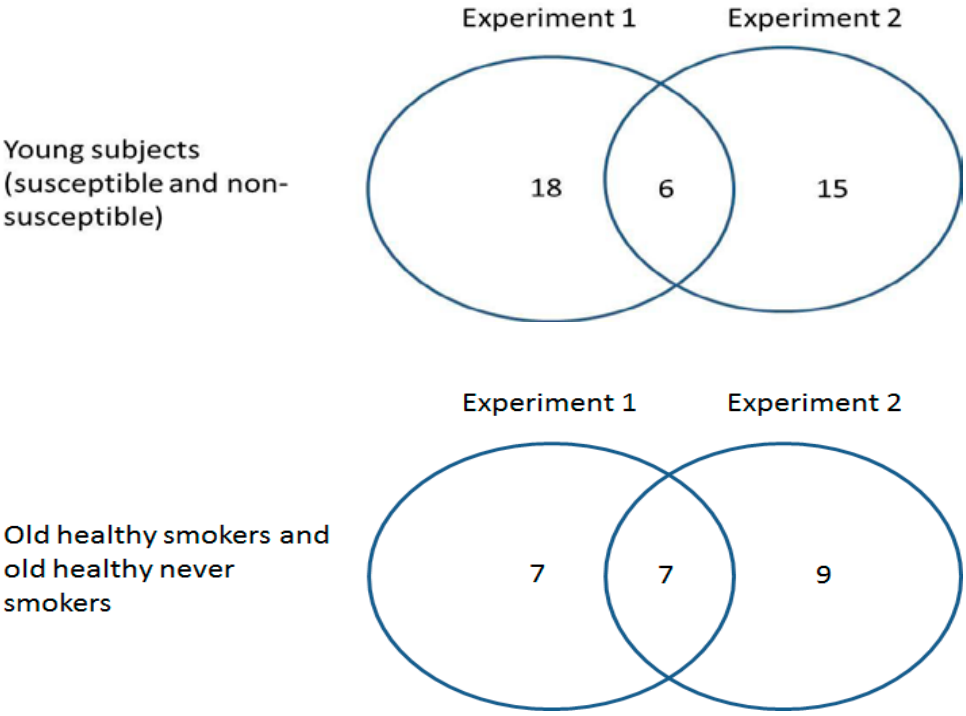

Figure 3. Overlap of the differentially expressed proteins in young subjects (both susceptible vs non-susceptible) and old subjects (old healthy smokers vs old never smokers) at baseline

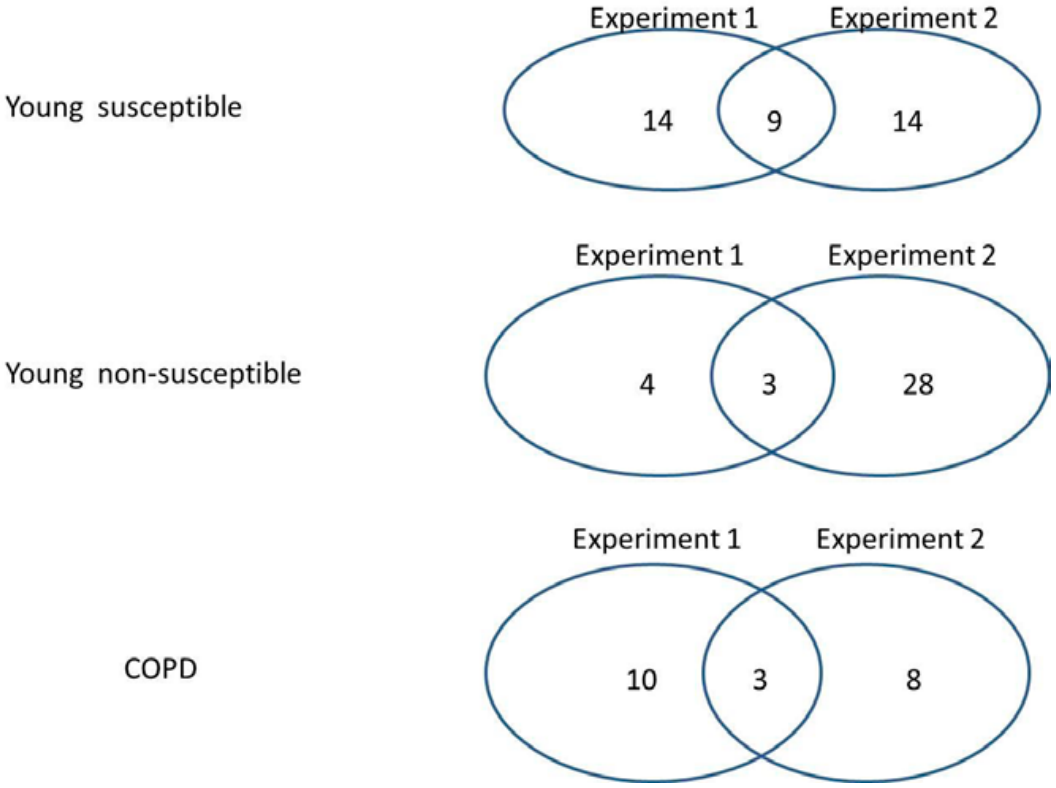

Figure 4. Overlap between the first and second iTRAQ® experiment in differentially expressed proteins before and after smoking in the young susceptible subjects, young non-susceptible and the COPD patients

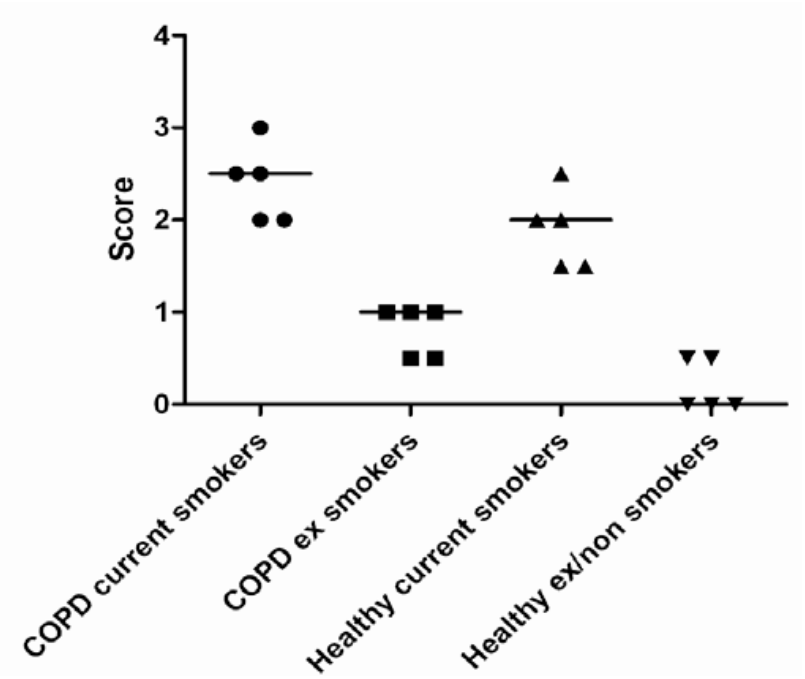

Figure 5. Semiquantitative analysis from immunohistochemistry of aldehyde dehydrogenase 3A1. Staining scoring: 0= negative; 1= weak; 2= positive; 3= strong positive

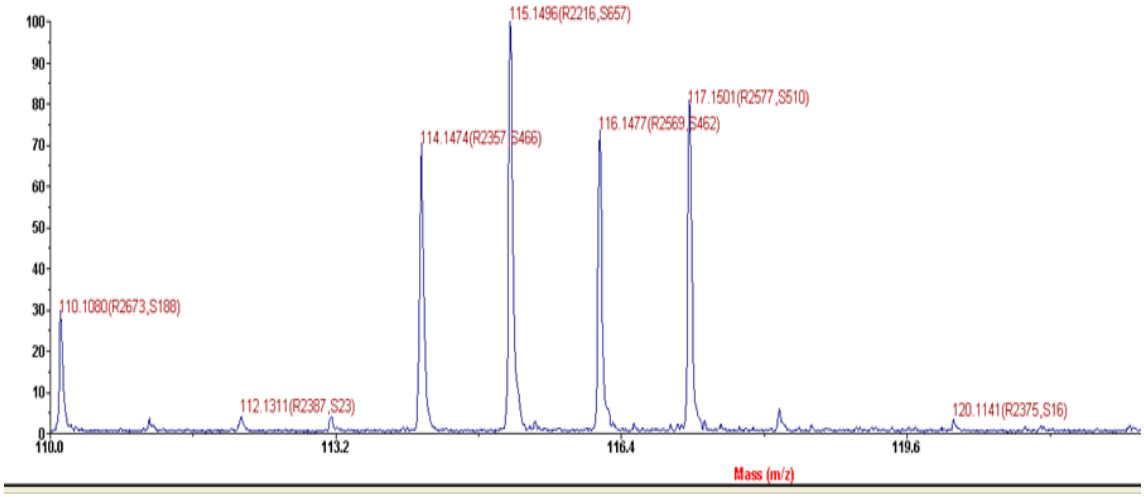

Figure 6. MS/MS spectra of peptide IGHPAPNFK of Peroxiredon I (precursor 1268.71). Peaks at 114, 115, 116 and 117 represent the group of young non-susceptible after acute smoking; young non-susceptible at baseline, young susceptible after acute smoking and young susceptible at baseline, respectively.

Chapter V

Summary and Future Prospectives

Summary

The scope of this thesis was to identify potential biomarkers for Chronic Obstructive Pulmonary Disease (COPD) via a proteomic approach.

COPD is the fourth leading cause of morbidity and mortality worldwide. It is characterized by chronic airflow limitation and associated with inflammatory responses in the airways. Smoking is the main causative agent for the onset and development of COPD, despite the fact that only 15-20% of current smokers develop the disease later on in their life.

In order to identify the first processes involved in the onset of COPD, we aimed to compare not only confirmed (older) COPD patients with healthy controls, but also younger subjects susceptible or non-susceptible to develop the disease, based on their family history.

After assessing the feasibility of our proteomic approach in the investigation of biomarkers in Epithelial Lining Fluid (ELF), we firstly compared the differential proteomic profiles in COPD patients and healthy controls. The proteins that resulted as statistically significantly different between these groups were subsequently investigated via other approaches, namely immunohistochemistry and ELISA.

To obtain a deeper understanding of the first processes responsible of the disease, we set up the acute smoking model in young susceptible and non-susceptible individuals, COPD patients and controls.

Proteomic analysis of Epithelial Lining Fluid: practical aspects

In chapter 2 we describe the practical aspects of sampling ELF followed by a detailed description of proteomic analysis by LC-MS/MS after protein separation by SDS PAGE and in-gel digestion. As an example, we apply this proteomic platform to the identification and quantification of proteins in ELF from COPD patients and healthy subjects.

Bronchoscopic microprobe (BMP) sampling of ELF is a recently developed technique that uses small probes containing an absorptive tip that are introduced bronchoscopically. Epithelial lining fluid forms a thin fluid layer that covers the mucosa of the large airways, small airways and alveoli. In the field of proteomic studies focusing on lung diseases, it represents an interesting specimen since it constitutes the first barrier between the lung and the outer world.

The main advantage of this technique is the possibility to collect undiluted ELF directly from the mucosa of the airways, in contrast to other methods used so far for the collection of biofluids from the lung (e.g. bronchoalveolar lavage fluid, induced sputum). Therefore ELF contains high concentrations of biomolecules. In addition, the investigated location in the tracheobronchial tree is well defined and there is no contamination with oropharyngeal bacteria or saliva. Despite occasional blood contamination of the probes by scratching the

mucosa of the airways, the proteomic analysis of microprobe-sampled ELF opens new possibilities for research in lung diseases. The identified proteins in the present study belong to families that are involved in various aspects of COPD pathogenesis, such as inflammation, oxidative stress, bacterial infection and the protease-antiprotease balance. This feasibility study on the ELF proteome focuses particularly on the induction and progression of cigarette smoke-induced COPD. Nevertheless, the study of ELF still represents a challenge: first of all due to the invasiveness of the brochoscopic methodology and secondly due to the difficulty of recruiting "healthy subjects" willing to undergo such a procedure for the collection of ELF.

Our work is thus a first approach to develop a method for the analysis of this biofluid from human candidates and still more development is needed in terms of standardization of the procedure and its further validation.

Proteomic analysis of Epithelial Lining Fluid: feasibility study

Chapter 3 shows the feasibility of performing chipLC-MS/MS and quantitative proteomic in human ELF.

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 COPD patients and healthy controls using microprobes.

A comparative study of ELF from COPD patients and healthy controls using chemical stable isotope labeling (iTRAQ®-8Plex) showed that the levels of four proteins (lactotransferrin, high-mobility group protein B1 (HMGB-1), alpha 1-antichymotrypsin and cofilin-1) differed significantly between ELF from COPD patients and healthy controls (p-values < 0.05). These results were reproduced in another, independent set of ELF samples from COPD patients and healthy controls and further validated by immunohistochemistry. Lactotransferrin and alpha 1-antichymotrypsin were found to be increased in the COPD group while, surprisingly, HMGB-1 was down-regulated in the cohort of patients. Interestingly, cofilin-1 revealed to be significantly increased in smokers, irrespective of the presence or absence of COPD.

These proteins are directly involved in two of the key processes responsible for the onset and development COPD, namely inflammation and oxidative stress. Therefore, despite the limited number of patients and controls, our results show the possibility to identify proteins related to biological processes occurring in COPD.

Proteomic analysis in young susceptible and non-susceptible individuals

Chapter 4 shows that smoking induces a differential protein response in ELF of susceptible and non-susceptible young individuals, which differs from patients with established COPD. This is the first study applying unbiased proteomic profiling to unravel the underlying mechanisms that induce COPD. Our data suggest that SerpinB3 and Uteroglobin could be interesting proteins in understanding the processes leading to COPD.

Cigarette smoking is the main risk factor for COPD, yet only a subset of smokers develops COPD. Family members of patients with severe early-onset COPD have an increased risk to develop COPD and are therefore defined as "susceptible individuals". Therefore we performed unbiased analyses of proteomic profiles to assess how "susceptible individuals" differ from age-matched "non-susceptible individuals" in their response to cigarette smoking.

Epithelial lining fluid was collected at baseline and 24 hours after smoking of 3 cigarettes in young individuals susceptible or non-susceptible to develop COPD and older subjects with established COPD. Controls at baseline were older healthy smoking and non-smoking individuals. Five samples per group were pooled and analysed by stable isotope labelling (iTRAQ®) in duplicate. Six proteins were selected and validated by ELISA or immunohistochemistry.

Interestingly, several proteins showed a differential expression pattern between the groups. After smoking, 23 proteins increased or decreased in young susceptible individuals, 7 in young non-susceptible individuals, and 13 in COPD patients in the first stable isotope labelling experiment ; 23 proteins increased or decreased in young susceptible individuals, 32 in young non-susceptible individuals, and 11 in COPD patients in the second iTRAQ® experiment. SerpinB3 and Uteroglobin decreased after acute smoke exposure in young non-susceptible individuals exclusively, whereas Peroxiredoxin I, S100A9, S100A8 and ALDH3A1 decreased both in young susceptible and non-susceptible individuals. Significant changes were observed between the groups of young individuals for Uteroglobin with iTRAQ and for Serpin B3 with iTRAQ and ELISA measurements. Peroxiredoxin I, SerpinB3 and ALDH3A1 increased in COPD patients after smoking, and ALDH3A1 was found strongly up-regulated both in COPD and healthy smokers.

At baseline, old healthy smokers showed higher levels of ALDH3A1 and Peroxiredoxin I than old healthy non-smokers.

Our data show that already at young age, subjects with a positive family history of COPD respond differently to cigarette smoke than those with a negative family history. Particularly SerpinB3 and Uteroglobin were found to be proteins that may play a role in the onset of COPD.

SerpinB3 and Uteroglobin decreased exclusively in young non-susceptible individuals after acute smoking exposure, and ELISA experiments confirmed this for SerpinB3. The dif-

ferential SerpinB3 and Uteroglobin response upon smoking between the two young groups suggests that these proteins might be crucial for the very first steps towards COPD.

Discussion and future perspectives

In the present studies, we aimed to investigate the possibility to discover specific protein markers for COPD via a proteomic approach.

Epithelial Lining Fluid was chosen as specimen of preference for the analyses due to its unique characteristics of "undiluted" sample, high content of biomolecules and specific location of collection in the airways. The protein composition of ELF reflects the effects of factors such as cigarette smoke, air pollutants, microbial agents that are the main causative agents of lung diseases. Therefore, the outcomes of proteomic investigations in this biofluid can reveal potential candidate markers specific for the disease and give more insights in the mechanisms responsible for the onset of COPD.

Despite the interesting outcomes of our investigations, some limitations still influence the choice of this methodology for "large" clinical/validation studies.

First of all the invasiveness of the procedure (via a bronchoscope) for the collection of ELF; secondly the possible occasional blood contamination of the probe by the contact with the mucosa and scratching of the inner wall of the airways during collection.

Third the limited number of patients and controls available to undergo such a procedure.

Proteomic research for biomarker(s) for a specific disease still presents certain hurdles with respect to the lack of standardization of the pre-analytical and analytical phases, to generate solid and reproducible data and the lack of validation of the biomarker candidates via other approaches and their (sometimes) difficult correlation with clinical parameters.

Despite the limitations of our studies (e.g. relatively low number of recruited subjects), we were able to reproduce and confirm our results with other methodologies (ELISA, immunohistochemistry). Our work opens the route for further investigations (in a larger clinical setting) on markers specific for COPD, considering that, up to date, only a few studies based on ELF proteomic analysis have been performed. New integrated and multidisciplinary approaches (as the combination of nanoproteomic techniques with bioinformatics platforms) can lead to target a narrowed window of the proteome for the discovery of specific markers. Furthermore the use of labelling strategies, the exploration of post-translational modifications etc, can indicate new directions to explore routes for a better understanding and diagnosis of COPD.

To note, we are first to provide an insight into some potential protein candidates responsible for the onset and development of COPD. We design our studies not only for investigation in COPD and healthy controls but we developed the "acute smoking model"

recruiting young subjects susceptible or non-susceptible to develop the disease later on, based on their family history.

In the samples collected from all the recruited groups, we were able to identify (via proteomic) and further validate with different other techniques (immunohistochemistry, ELISA), biomarkers involved in key pathways of the inflammatory processes occurring in COPD. Most importantly we were able to link the pattern of these markers with the acute smoking model of the recruited subjects. Despite these interesting outcomes, yet improvements and modifications in the analyses need to be performed.

Firstly, it is necessary to translate the investigations into a more suitable and easy-to-collect specimen (for example blood or urine).

Secondly, it is important to focus on the criteria for recruitment of the participants: as example, in our investigation, the susceptibility of the young subjects was based only on family history. In order to avoid a "generalization" of the outcomes, the balance age/gender combined with the genetic background should be taken into consideration. It would be important, but difficult and time-consuming, to design a follow-up study in the young susceptible subjects, because of the nature of COPD (a disease that develops at older age), to follow and monitor the clinical responses from the same subjects later on in life in order to have a better understanding of the early phases of the onset and further development of COPD.

Due to the complexity and heterogeneity of a disease such as COPD, with an increasing clinical burden in the Western world, there is an urgent need to understand the mechanisms underlying its pathobiology and all the possible phenotypes. Complex diseases are mostly the result of gene-environment interactions that determine the clinical presentation of the disease.

Several genome-wide association studies (GWAS) have highlighted loci associated with disease susceptibility. Despite the importance of genomic studies, a single gene can be associated with several different proteins and peptides. Biofluids such ELF, BALF, plasma, despite their low amount of DNA or RNA, contain large amount of proteins that could be important specific biomarkers for the disease. Therefore analysis of proteomic profile detectable in biological samples could lead to the identification of biomarkers specific for the disease and molecular pathways that could provide pharmacological targets for new therapeutic agents.

Additionally, the investigation of lung tissue can provide high valuable information on the lung pathology. However, this is a very invasive procedure that is not applicable in daily clinical routine.

These "omics" techniques (proteomics, peptidomics, genomics, metabolomics, transcriptomics, etc.) can be combined into systems biology assessments as to pinpoint the causal chain of gene-environment interactions on gene expression proteins and metabolites that ultimately lead to COPD in its various forms.

All the data generated by these "omics" platforms should additionally be correlated with the characterization of the patients, into specific subtypes like chronic bronchitis, small airway dysfunction and emphysema, thus providing translational bench-to-bedside (and back again) approaches.

COPD is recognized by major changes in spirometry, however this technique measures only one feature of the disease, it poorly correlates with emphysema and it is not linear in time. Therefore, other methodologies have been applied to diagnose the COPD population, despite spirometry remains the "gold standard".

More recently, high-resolution computed tomography (CT) scans have been the methodology of choice for identifying the presence of emphysema. At the moment, CT scanning is recognized as the most sensitive technique to evaluate the patients affected by emphysema, since it is more sensitive than spirometry. With the advantage of clearly identifying the pathology and the change in the architecture of the lung, it possesses however some drawbacks, as the exposure of the patient to radiation and the limitation in repeating measurements.

Taking all these considerations together, it is still not possible to define a unique approach to diagnose and treat such a complex disease as COPD. Nonetheless, over the past decade there has been an increasing awareness and research in COPD that led to a better management of the disease. A deeper understanding of the genetic background, the recruitment of patients with rigorous selection criteria and the application of "omics" techniques could give in the nearby future a clearer indication of the mechanisms responsible for the disease, and therefore developing an early diagnosis and treatment for COPD.

Hoofdstuk V

NEDERLANDSE SAMENVATTING

De doelstelling van dit proefschrift was om potentiële biomarkers voor Chronic Obstructive Pulmonary Disease (COPD) te identificeren met behulp van een proteomics benadering.

COPD is de vierde voornaamste oorzaak van morbiditeit en mortaliteit wereldwijd. Deze invaliderende aandoening wordt gekenmerkt door hoesten, slijm opgeven, en kortademigheid. In het longfunctieonderzoek is een chronische luchtwegobstructie aanwijsbaar die geassocieerd is met ontstekingsreacties in de luchtwegen. In de westerse wereld is roken de belangrijkste oorzaak voor het ontstaan van COPD, ondanks het feit dat slechts 15-20% van de rokers de ziekte uiteindelijk ontwikkelt.

Om de allereerste processen bij het ontstaan van COPD te identificeren, hebben we niet alleen oudere personen met een aangetoond COPD vergeleken met gezonde leeftijdsgenoten, maar ook jongere personen die nog geen COPD hebben, maar het mogelijk wel kunnen ontwikkelen op latere leeftijd. De (on)gevoeligheid om COPD te ontwikkelen op latere leeftijd werd daarbij afgeleid uit het voorkomen van COPD bij familieleden die voldoende aantal sigaretten per dag gedurende een aantal jaren gerookt hadden. Om een krachtige COPD-specifieke respons te detecteren hebben wij onze proefpersonen onderzocht voor en na het roken van 3 sigaretten.

Voordat wij onze proefpersonen blootstelden aan het roken van 3 sigaretten hebben wij voorbereidend onderzoek gedaan naar de haalbaarheid van proteomics analyse in de vloeistoffilm die de luchtwegen bedekt (de zogenaamde Epithelial Lining Fluid of ELF). Daarbij hebben we aandacht geschonken aan zowel de bemonstering als proteomics analyse. Tevens is een eerste 'proof of principle' vergelijking gemaakt tussen COPD en gezond.

Proteomics analyse van ELF: praktische aspecten

In hoofdstuk 2 beschrijven we de praktische aspecten van de bemonstering van ELF gevolgd door een gedetailleerde beschrijving van de proteomics analyse door LC-MS / MS na scheiding van eiwitten door SDS-PAGE en in-gel-digestion. Een proteomics platform werd gevormd ter identificatie en kwantificering van eiwitten in ELF van COPD-patiënten en gezonde proefpersonen.

Bronchoscopische microprobe (BMP) bemonstering van ELF is een recent ontwikkelde techniek waarbij kleine sondes met een absorberend tip via de bronchoscoop contact maken met ELF. ELF is de dunne vloeistoffilm die het slijmvlies van de grote luchtwegen, kleine luchtwegen en longblaasjes bedekt. Het is een interessante techniek voor proteomics onderzoek van longaandoeningen omdat ELF de eerste barrière tussen de longen en de buitenwereld vormt.

Een belangrijk voordeel van deze techniek is de mogelijkheid om onverdunde ELF rechtstreeks van de mucosa van de luchtwegen te bemonsteren, in tegenstelling tot andere werkwijzen zoals broncho-alveolaire lavage vloeistof of geïnduceerd sputum. Aangetoond is dan

ook dat ELF hoge concentraties biomoleculen bevat. Een ander voordeel is dat exact duidelijk is waar bemonsterd wordt. Ten slotte treedt er geen verontreiniging met bacteriën of speeksel uit de mondkeelholte op zoals bij geïnduceerd sputum het geval is. Een nadeel is dat er soms verontreiniging met bloed optreedt doordat de probes onder invloed van de ademhaling over het slijmvlies van de luchtwegen schuren. Een ander nadeel is dat de bronchoscopische procedure zeer belastend is voor de proefpersonen, dus voorlopig alleen toepasbaar is in onderzoeksverband. De eiwitten die met deze techniek werden geïdentificeerd blijken eerder in relatie gebracht te zijn met verschillende aspecten van de pathogenese van COPD, zoals ontsteking, oxidatieve stress, bacteriële infecties en de protease-antiprotease balans. Samenvattend vormt bovenstaande aanpak een eerste stap om met proteomics analyse van ELF uit de luchtwegen biomarkers te identificeren die een rol spelen bij COPD. Echter er moeten nog een aantal belangrijke stappen genomen worden wat betreft standaardisatie en validatie.

Proteomics analyse van ELF: haalbaarheidsstudie

Hoofdstuk 3 toont de haalbaarheid van chipLC-MS / MS en kwantitatieve proteomics in menselijke ELF.

Microfluidics gebaseerde nanoLC-MS / MS (chipLC-MS / MS) technieken werden gebruikt om eiwitten in ELF te identificeren en kwantificeren. De ELF werd verkregen met behulp van bronchoscopische microprobe bemonstering (BMP) uit de grote luchtwegen van COPD-patiënten en gezonde controles.

Met behulp van chemische stable isotope labeling (iTRAQ®-8Plex) werden significante verschillen in de aanwezigheid van vier eiwitten aangetoond tussen ELF van COPD-patiënten en gezonde controles (p-value <0,05). Het betrof lactotransferrin, hoge mobiliteit groep eiwit B1 (HMGB-1), alfa 1-antichymotrypsine en cofilin -1. Deze resultaten werden gereproduceerd in een andere, onafhankelijke set van ELF monsters van COPD-patiënten en gezonde controles, en verder gevalideerd met immunohistochemie van longweefsel. Lactotransferrin en alfa 1-antichymotrypsine waren verhoogd in de COPD groep, terwijl HMGB-1 tot onze verrassing verlaagd bleek. Een interessante bevinding was de hogere expressie van cofilin bij rokers, ongeacht de aan- of afwezigheid van COPD.

Al deze eiwitten spelen een rol bij twee belangrijke processen die bijdragen aan het ontstaan van COPD, namelijk ontsteking en oxidatieve stress. Dat ondanks het geringe aantal proefpersonen deze verschillen aantoonbaar waren was voor ons extra reden om door te gaan met de proteomics analyses van ELF in het kader van gevoeligheidonderzoek voor COPD.

Proteomics analyse bij jonge gevoelige en niet-gevoelige personen

In hoofdstuk 4 blijkt dat roken een verschillende eiwit respons oproept in de ELF van gevoelige en niet-gevoelige jonge mensen, die verschilt van oudere patiënten met een bewezen COPD. Dit is de eerste studie die op een onpartijdige (unbiased) manier met behulp van proteomics analyses de eiwitten in kaart brengt die mogelijk betrokken zijn bij het ontstaan van roken-geïnduceerde COPD. Onze gegevens suggereren dat vooral SerpinB3 en Uteroglobine in dat opzicht interessante eiwitten zijn.

Zoals eerder aangegeven vormt roken (in de westerse wereld) de belangrijkste risicofactor voor COPD, terwijl slechts een gedeelte van de rokers COPD ontwikkelt. In de literatuur is beschreven dat familieleden van patiënten met ernstig COPD op jonge leeftijd een verhoogd risico hebben op het ontwikkelen van COPD. Deze genetische predispositie was voor ons de directe aanleiding van het onderzoek, en mede de reden om de gevoeligheid voor COPD op jonge leeftijd in te schatten op basis van de familieanamnese. ELF werd verzameld 24 uur na het roken van 3 sigaretten, en 6 weken later zonder vooraf gerookt te hebben (uitgangswaarde). Vergeleken werden jonge proefpersonen die wel of niet gevoelig waren voor COPD, oudere patiënten met een vastgesteld COPD, oudere personen die jarenlang gerookt hadden maar geen COPD ontwikkeld hadden, en oudere personen zonder COPD die nooit gerookt hadden. Vijf monsters per groep werden samengevoegd en met stable isotope labelling (iTRAQ®) in duplo geanalyseerd. Zes eiwitten werden uiteindelijk geselecteerd en gevalideerd met ELISA of immuun-histochemie.

Verschillende eiwitten lieten een verschillend patroon zien tussen de groepen. Na roken veranderde in het eerste iTRAQ® experiment de aanwezigheid van 23 eiwitten bij jonge gevoelige personen, van 7 eiwitten bij jonge niet-gevoelige individuen, en van 13 eiwitten bij COPD-patiënten. In het tweede iTRAQ® experiment veranderde de aanwezigheid van 23 eiwitten bij jonge gevoelige personen, van 32 eiwitten in jonge niet-gevoelige individuen, en van 11 eiwitten bij COPD-patiënten. De aanwezigheid van SerpinB3 en Uteroglobine nam exclusief af na acute rook blootstelling bij jonge niet-gevoelige proefpersonen, terwijl Peroxiredoxine I, S100A9, S100A8 en ALDH3A1 afnamen bij zowel jonge gevoelige als niet-gevoelige proefpersonen. Significante veranderingen werden waargenomen tussen de twee groepen van jonge mensen voor Uteroglobine met iTRAQ en voor Serpine B3 met zowel iTRAQ alsook ELISA metingen. Peroxiredoxine I, Serpine B3 en ALDH3A1 namen na roken toe bij COPD-patiënten.

Samenvattend toont onze studie aan dat personen met een positieve familiegeschiedenis voor COPD op jonge leeftijd anders reageren op sigarettenrook dan jonge personen met een negatieve familiegeschiedenis. Vooral Serpine B3 en Uteroglobine bleken eiwitten die een cruciale rol zouden kunnen spelen in de ontwikkeling van COPD.

Discussie en toekomst perspectieven

In de huidige studie hebben we geprobeerd om eiwit markers te ontdekken die een rol spelen bij de ontwikkeling van COPD met gebruikmaking van proteomics.

Bronchoscopische microprobe bemonstering van ELF werd gekozen als onderzoeksmethode omdat deze techniek niet de ELF verdunt, er op deze manier een hoge concentratie van biomoleculen aanwezig blijft, en exact duidelijk is waar ELF in de long verzameld wordt. Daarnaast was de aanname dat de eiwitsamenstelling van ELF de respons van de luchtwegen op prikkels als sigarettenrook, luchtverontreiniging, en microbiële producten weerspiegelt. Daarom kan proteomics onderzoek op ELF potentiële biomarkers voor COPD onthullen, alsmede inzicht geven in de mechanismen die verantwoordelijk zijn voor het ontstaan van COPD. Om een krachtige COPD-specifieke respons te detecteren hebben wij onze proefpersonen onderzocht voor en na het roken van 3 sigaretten. En om de allereerste stappen in de ontwikkeling naar COPD te detecteren hebben wij dit acute rookmodel ook toegepast op jonge proefpersonen.

Ondanks de interessante uitkomsten van ons onderzoek heeft boven beschreven aanpak een aantal nadelen waardoor toepassing in grote klinische studies niet snel zal plaats vinden. Allereerst is bronchoscopisch onderzoek voor het verzamelen van ELF een dure, tijdrovende, logistiek lastige en voor de patiënt belastende procedure. Het invasieve karakter schrikt ook patiënten af, wat negatieve gevolgen heeft voor de rekrutering van grote studies. Op de tweede plaats kan de betrouwbaarheid van de proteomics bepalingen in de analytische en pre-analytische fasen verbeterd worden. Tevens is een snellere (high throughput) bepalingsmethode hard nodig. Op de derde plaats is onderzoek naar alleen eiwitten in ELF een te simpel model om een complexe ziekte als COPD te begrijpen. Er dient ook aandacht te zijn voor de relatie met genen, genexpressie, genexpressie modulerende factoren, pathologische veranderingen, verschillen tussen long/luchtwegcompartimenten, en sub(feno)types van COPD. Ten slotte is met het huidige onderzoek niet zeker of jonge personen wel/niet na jarenlang roken COPD ontwikkelen; een betere karakterisering bijvoorbeeld op basis van een genetisch profiel of een uitgebreide karakterisering van familieleden zou hierin kunnen helpen.

Rekening houdend met boven beschreven voor en nadelen kunnen een aantal suggesties gedaan worden voor toekomstige studies.

- Vanwege de invasiviteit van bronchoscopisch onderzoek is het noodzakelijk om te kunnen volstaan met makkelijk te verzamelen lichaamsmateriaal zoals bloed of urine. Oftewel zouden de huidige onderzoeksresultaten gevalideerd moeten worden in deze materialen.
- Meer zekerheid zou verkregen moeten worden of jonge personen wel of niet COPD ontwikkelen op oudere leeftijd. De huidige strategie was gebaseerd op de familieanamnese

zonder longfunctionele controle van familieleden. Wellicht dat in de toekomst metingen van kleine luchtwegbetrokkenheid de kans op ontwikkelen van COPD beter of vroeger kunnen inschatten. Echter de ideale benadering is om daadwerkelijk te bewijzen dat een jonge roker wel/of niet COPD ontwikkelt; wat dus een zeer langdurig longitudinaal onderzoek behoeft.

- COPD is een complexe ziekte die het gevolg is van een ingewikkelde gen-omgeving interactie, die zich ontwikkelt na jarenlang roken, in meerdere lichaam- en longcompartimenten, met verschillende klinische expressies. Om deze complexiteit beter in kaart te brengen is het noodzakelijk om:
    - o Grote studies te creëren met voldoende power, mede om de verschillende sub(feno) types te kunnen herkennen.
    - o Zorg te dragen voor extensieve gedetailleerde fenotypering. Dus niet alleen spirometrie maar bijvoorbeeld ook nieuwe CTscan technieken ter bepaling van luchtwegwanddikte en longparenchymdichtheid.
    - o Combinatie met andere "omics" technieken (peptidomics, genomics, metabolomics, transcriptomics, epigenetische technieken etc.) na te streven. Oftewel te kiezen voor een systeembiologische evaluatie.
    - o Deze omics niet alleen in ELF bepalen maar in verschillende compartimenten van de long of lichaam (sputum, BAL, centraal luchtwegweefsel, perifeer luchtwegweefsel, longparenchym, bloed, neus).
    - o Deze omics relateren aan pathologische veranderingen op weefsel, cel, en subcellulair niveau.
    - o Deze omics technieken uiteindelijk toe te passen op eenvoudig te benaderen lichaamsmateriaal zoals bloed en/of urine.

Al deze overwegingen beschouwend moge het duidelijk zijn dat op dit moment er nog onvoldoende houvast is om een eenvoudige studie te ontwerpen die kansrijk is om de ontwikkeling van een complexe ziekte als COPD in kaart te brengen. Dit zal meerdere studies vergen met verschillende invalshoeken. Toch hopen wij dat een stapsgewijze benadering langs de hierboven beschreven paden hieraan kan bijdragen.

LIST OF PUBLICATIONS

List of publications

Proteomics of epithelial lining fluid obtained by bronchoscopic microprobe sampling.
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