Exploring the composition of the small airway lining fluid using breath analysis

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To Joel and Hampus

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ABSTRACT

The mechanisms underlying the pathogenesis of chronic obstructive pulmonary disease (COPD), asthma and COVID-19 are not completely understood. The small airways, which are lined with the respiratory tract lining fluid (RTLF), represent the major site of inflammation in these diseases, and changes in the composition of the RTLF reflect the underlying inflammatory process.

The aim of this thesis is to increase the knowledge on how smoking, asthma and SARS-CoV-2 infection affect the composition of the lining fluid in small airways, reflecting mechanisms of small airway inflammation, in samples obtained non-invasively through collection of exhaled particles.

The variability and repeatability of the method used for the collection of exhaled particles in this thesis, the PExA method, was comparable with other methods used for the collection of RTLF and was not affected by the time interval between the measurements. The Surfactant protein A and albumin contents of exhaled particles were influenced by diurnal rhythm, highlighting the importance of considering the time of the day when the RTLF is sampled. Active smoking has a strong impact on the protein profile in RTLF and it appears to affect women and men differently, as more pronounced alterations were seen in women. The complement system was identified as a biological pathway strongly affected by active smoking. Local activation of the complement and coagulation systems was also observed in asthma, where it was associated with small airway dysfunction and asthma control. A mild SARS-CoV-2 infection was shown to cause alterations in the surfactant phospholipids and these changes appear to persist over time. During acute infection, increased small airway resistance was observed, which

correlated with the abundance of several acute phase proteins, suggesting increased inflammation in small airways during mild COVID-19.

These findings improve the understanding of the mechanisms driving these diseases and open up for new possibilities for targeted drug development and precision medicine.

Keywords: small airways, exhaled particles, COPD, asthma, COVID-19, complement system, surfactant

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SAMMANFATTNING PÅ SVENSKA

Lungsjukdomar är en av de vanligast förekommande folksjukdomarna. Eftersom lungan hela tiden är exponerad mot den externa miljön när vi andas behöver den konstant immunologisk övervakning. Barriären som separerar blodet från stora mängder av inandad luft är väldigt tunn och första linjens försvar utgörs av det vätskeskiktet som täcker små luftvägar. Det innehåller proteiner och lipider vars huvudsakliga funktioner är att hålla luftvägarna öppna vid andning samt att skydda kroppen mot inandade bakterier, virus samt andra luftvägsirriterande ämnen. Material vi andas in kan orsaka förändringar i sammansättningen av vätskeskiktet som täcker små luftvägar och dessa förändringar kan bidra till utvecklingen av lungsjukdomar så som astma, KOL och COVID-19. Vid sjukdom som drabbar de små luftvägarna förändras sammansättningen också. På grund av storleken på de små luftvägarna (mindre än 2 mm i diameter), är de svåråtkomliga och svåra att provta. Därför är förståelsen av mekanismerna som bidrar till utvecklingen av lungsjukdomar bristfällig.

Denna avhandling syftade till att studera förändringarna i sammansättningen av vätskeskiktet som orsakas av rökning, astma och COVID-19, med målsättningen att öka kunskapen om olika mekanismer som bidrar till inflammation i de små luftvägarna. En icke-invasiv metod för att samla in små vätskedroppar (egentligen partiklar) i utandningsluft har använts. Dessa partiklar bildas från vätskeskiktet i de små luftvägarna vid andning och innehåller lipider och proteiner.

Resultaten visar att det är möjligt att studera protein- och lipid-förändringar i små luftvägar orsakade av olika exponeringar och sjukdomar med insamling och analys av utandade partiklar och förändringar i sammansättning över tid. Aktiv rökning påverkar proteinerna i vätskeskiktet och dessa förändringar skiljer sig mellan kvinnor och män. Komplementsystemet, en av de äldsta delarna av det medfödda immunförsvaret, var starkt påverkat av rökning, speciellt hos kvinnor. Komplement– och koagulationsaktivering verkar också spela en viktig roll i småluftvägsinflammation vid astma och är associerade med astmakontroll. Även mild COVID-19 kan påverka små luftvägar och orsakar förändringar i lipiderna som kan bestå under en lång tid.

Resultaten bidrar till ökad förståelse för olika mekanismer som driver dessa lungsjukdomar och öppnar upp för nya möjligheter för riktad läkemedelsutveckling och precisionsmedicin.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. **Kokelj S**, Kim J-L, Andersson M, Runström Eden G, Bake B, Olin A-C. Intra-individual variation of particles in exhaled air and of the contents of Surfactant protein A and albumin. PLoS ONE 2020; 15 (1), e0227980.

II. **Kokelj S**, Östling J, Georgi B, Fromell K, Nilsson Ekdahl K, Olsson HK, Olin A-C. Smoking induces sex-specific changes in the small airway proteome. Respiratory Research 2021; 22 (1), 234.

III. **Kokelj S**, Östling J, Fromell K, Vanfleteren L, Olsson HK, Nilsson Ekdahl K, Nilsson B, Olin A-C. Activation of the complement and coagulation systems in the small airways in asthma. Respiration 2023; 102 (8), 621-631.

IV. **Kokelj S**, Larsson P, Viklund E, Koca H, Slogén H, Vanfleteren L, Nilsson B, Fromell K, Westin J, Olin A-C. Changes in the pulmonary surfactant in patients with mild COVID-19. *Submitted.*

CONTENTS

Abbreviations iv
Definitions in short viii
1 INTRODUCTION 1
1.1 Airway morphology and small airways 2
1.2 Respiratory tract lining fluid 4
1.2.1 Small airway lining fluid 6
1.3 Small airway inflammation 7
1.3.1 Chronic obstructive pulmonary disease 8
1.3.2 Asthma 10
1.3.3 COVID-19 11
1.3.4 Risk factors for the development of small airway inflammation 12
1.4 The complement system and its role in lung disease 14
1.5 Methods to sample the respiratory tract lining fluid 17
1.5.1 Bronchoalveolar lavage 17
1.5.2 Induced sputum 17
1.5.3 Exhaled breath condensate 18
1.5.4 Nasal lavage 18
1.5.5 Exhaled particles 18
1.6 Physiological tests to assess small airway function 21
1.6.1 Spirometry 21
1.6.2 Forced oscillation technique 23
1.6.3 Inert gas washout 23
1.7 Gaps in knowledge 25
2 AIM 26
3 PARTICIPANTS AND METHODS 27
3.1 Study design 28
3.2 Study participants 28
3.3 Methods 31

3.3.1 Sampling of exhaled particles 31
3.3.2 Chemical analyses of exhaled particles 33
3.3.3 Lung function tests 37
3.3.4 Clinical information 38
3.3.5 Statistical analyses 39
4 RESULTS AND DISCUSSION 41
4.1 Variability and repeatability of exhaled particles and their contents (PAPER I) 41
4.2 Composition of the small airway lining fluid in smokers, asthma and COVID-19 (PAPERS II-IV) 44
4.2.1 Clinical characteristics of the cohorts 45
4.2.2 Proteomic changes 47
4.2.3 Complement system 54
4.2.4 Sex-specific differences 59
4.2.5 Lipidomic changes in COVID-19 61
5 STRENGTHS AND LIMITATIONS 65
6 CONCLUSION 68
7 FUTURE PERSPECTIVES 69
Acknowledgement 70
References 72

ABBREVIATIONS

ACE2	Angiotensin-converting enzyme 2
ACQ	Asthma control questionnaire
AOS	Airwave oscillometry
ARDS	Acute respiratory distress syndrome
AT1 cell	Type I alveolar cell
AT2 cell	Type II alveolar cell
AX	Area of reactance
BAL	Bronchoalveolar lavage
BMI	Body mass index
COPD	Chronic obstructive pulmonary disease
COVID-19	Coronavirus disease 2019
CR	Coefficient of repeatability
CRP	C-reactive protein
CV	Coefficient of variability
DPPC	Dipalmitoyl-phosphatidylcholine
EBC	Exhaled breath condensate
ELISA	Enzyme-linked immunosorbent assay
$FEF_{25-75\%}$	Forced expiratory flow between 25% and 75% of the forced vital capacity
$FEF_{75\%}$	Forced expiratory flow at 75% of the forced vital capacity

FENO	Fractional exhaled nitric oxide
FEV_1	Forced expiratory volume in 1s
FRC	Functional residual capacity
FVC	Forced vital capacity
HEPA	High efficiency particulate air
HexCer	Hexosylceramide
ICC	Intraclass correlation coefficient
ICS	Inhaled corticosteroids
IOS	Impulse oscillometry
LABA	Long-acting beta agonists
LCI	Lung clearance index
LC-MS/MS	Liquid chromatography–tandem mass spectrometry
LOD	Limit of detection
MBW	Multiple breath nitrogen washout
mMRC	modified Medical Research Council dyspnea scale
PASC	Post-acute sequelae of COVID-19
PC	Phosphatidylcholine
PCR	Polymerase chain reaction
PE	Phosphatidylethanolamine
PEF	Peak expiratory flow
PEx	Exhaled particles

PExA	Particles in Exhaled Air
PG	Phosphatidylglycerol
PI	Phosphatidylinositol
POPG	Palmitoyl-oleoyl-phosphatidylglycerol
PS	Phosphatidylserine
PTFE	Polytetrafluoroethylene
R^2	Coefficient of determination
R_{20}	Resistance at 20 Hz
R_5	Resistance at 5 Hz
RFU	Relative fluorescent units
RTLF	Respiratory tract lining fluid
RV	Residual volume
SABA	Short-acting beta agonists
S_{acin}	MBW derived index of ventilation inhomogeneity at the entrance to the acinar airways
SAD	Small airway dysfunction
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
SBW	Single breath nitrogen washout
S_{cond}	MBW derived index of ventilation inhomogeneity in the conductive airways
SD	Standard deviation
SM	Sphingomyelin

SP-A	Surfactant protein A
SP-B	Surfactant protein B
SP-C	Surfactant protein C
SP-D	Surfactant protein D
T_H2 cell	T helper 2 cell
TLC	Total lung capacity
UPR	Unfolded protein response
VC	Vital capacity
VT	Tidal volume
WBC	White blood cell

DEFINITIONS IN SHORT

Complement system	Part of the innate immune system comprised of more than 50 proteins.
Endotype	Distinct pathogenetic mechanisms at a cellular and molecular level that underlie a disease.
Exhaled particles	A matrix used to sample the respiratory tract lining fluid from the small airways.
Phenotype	A group of patients with common, usually clinical, characteristics that can be observed.
Small airway respiratory tract lining fluid	Lining fluid overlaying small airway epithelium, mainly consisting of the surfactant.
Small airways	Airways with an internal diameter of less than 2 mm. On average, they arise by the 8th airway generation, but this varies, as they can arise between 4th to the 13th generation. They have important structural and physiological differences from the large airways.
SOMAmers	Synthetic DNA Slow Off-rate Modified Aptamers used in the SOMAscan analysis.
Surfactant	Composed of phospholipids and surfactant proteins, it is lowering the surface tension and preventing alveolar collapse. It also represents the first line host defense.

1 INTRODUCTION

The lung is one of the most vulnerable organs to infection and injury due to the constant exposure to external environment, making lung diseases one of the top leading causes of death in the world. Chronic obstructive pulmonary disease (COPD) affects 4% of the world's population and represents the third leading cause of death worldwide[1]. Asthma is one of the most common non-communicable diseases affecting 262 million people worldwide in 2019[2], and the spread of the coronavirus disease 2019 (COVID-19), caused by the SARS-CoV-2 virus, has dramatically increased the mortality due to lower respiratory tract infection in the recent years. Altogether, lung diseases place a huge burden on the society, not only in terms of premature mortality, but also in terms of disability, and represent a major economic burden.

Although there has recently been a tremendous growth in our understanding of COPD, asthma and COVID-19 and their pathophysiology, the underlying mechanisms that contribute to the development and progression of these diseases are still not completely understood. COPD and asthma are considered very heterogeneous diseases and represent umbrella terms for multiple endotypes and phenotypes. Using a more personalized approach, by searching for biomarkers and biological processes and understanding different endotypes that drive these diseases, would facilitate the diagnosis and the development of a more targeted therapeutic approach[3]. A movement towards precision medicine would allow us to treat the underlying endotypes instead of focusing on clinical phenotypes[3].

The small airways represent the major site of inflammation in these lung diseases. They are lined with a lining fluid, consisting of proteins and lipids, which represents the first line of defense against inhaled particles and pathogens. Changes in the composition of the lining fluid reflect the underlying inflammatory processes. Due to the very distal position of the small airways in the airway tree as well as their small size, the collection of biological material from small airways is difficult, making the search for biomarkers and underlying mechanisms rather challenging. Therefore, an in-house developed method for the collection of small airway lining fluid, the PExA (Particles in Exhaled Air) method, was used for the studies in this thesis. It serves as a non-invasive alternative to the more traditional way of collecting material from the small airways with bronchoalveolar lavage (BAL)[4], giving us the opportunity to study larger cohorts and perform repeated measurements.

1.1 AIRWAY MORPHOLOGY AND SMALL AIRWAYS

The lungs are one of the largest organs in our body. They are made up of only about half a liter of tissue in a form of a very thin barrier which separates the blood from large amounts of inhaled air. As the main function of the lung is the gas exchange between air and blood, this tissue layer serves as a very large gas exchange surface, almost the size of a tennis court, that needs to be effectively ventilated and perfused with blood[5,6].

Due to the complex physiology and structure of the lungs, lung models are used to simplify the structure of the airways. Weibel's lung model, which is the simplest and most widely used lung model, divides the airway tree in approximately 23 generations of dichotomously branching airways (Figure 1). With each generation of the airways the airway lumen decreases, but the number of airways increase and therefore the total cross-sectional area of the airway lumen rapidly increases with every generation[6].

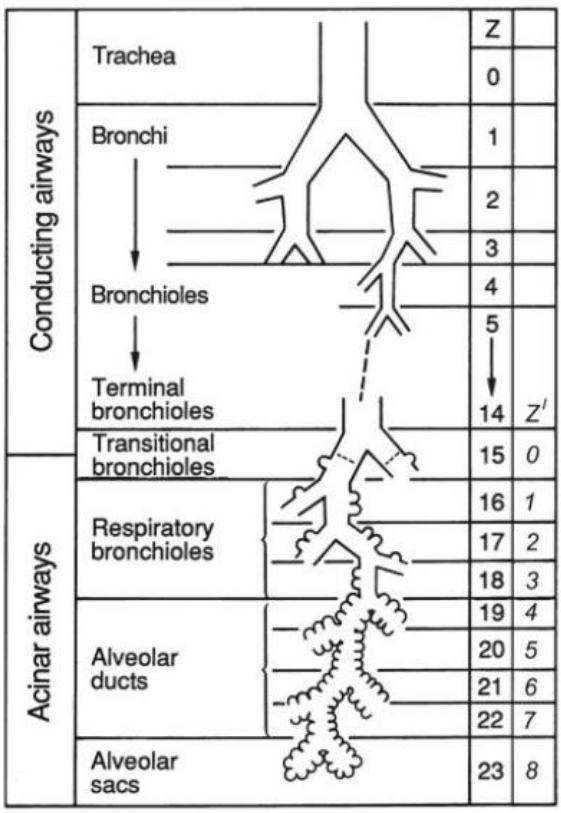

Figure 1. Weibel's lung model. A model of dichotomously branching airway tree from trachea (generation z 0) to alveolar ducts and sacs (generations z 20 to 23). Adapted with permission from Weibel ER. What makes a good lung? Swiss Med Wkly 2009;139(27-28):375-86. (In eng). DOI: 10.4414/smw.2009.12270.

The first 15 airway generations (0-14) make up the conductive zone and are lined with a bronchial epithelium. They do not take part in the gas exchange. In generation 15, on average, the gas-exchanging alveoli start appearing, forming the first order respiratory or transitional bronchiole[5]. By approximately generation 18, the alveolar ducts are formed, and the airway wall is completely decorated with alveoli. The alveolar ducts continue to branch until ending with alveolar sacs (generation 23). The generations 15-23 form the acinar zone that is ventilated by one transitional bronchiole and where gas exchange takes place[5]. The gas transport from the atmosphere to the blood depends on two fundamentally different processes in the conductive and acinar airway zones. In the conductive zone, the respiratory muscles create a pressure gradient, causing the gas to move by convection, or bulk transport. On contrast, in the acinar zone, the exchange of oxygen and carbon dioxide across the alveolar surface creates a concentration gradient, which is responsible for the movement of gas by diffusion in this zone[7].

The airway tree can also be divided into the central (large) and peripheral (small) airways. Small airways are, by definition, the airways with an internal diameter of less than 2 mm. On average, they arise by the 8th airway generation, but this varies, as they can arise between 4th to the 13th generation, even within the same lung[6]. They have important structural and physiological differences as compared to the large airways. First, the C-shaped cartilage supporting the trachea, or the irregular cartilage plates found in bronchi, are not present here. Second, they lack mucus glands and the inhaled particles are cleared by the airway macrophages and transcytosis across the epithelial cells instead[8]. Third, the total cross-sectional area of the small airways is much greater than that of large airways. The volume of gas passing through each airway generation is the same, but due to the large cross-sectional area of the small airways, the gas velocity is much lower, and the flow is laminar in the small airways as compared to the large, where the velocity is high and the flow turbulent. Consequently, the total resistance to the flow is low in small airways as compared to large, and they account for less than 10% of the total airway resistance below the larynx in the normal healthy lung[7]. Due to the low resistance in the small airways, disease can accumulate there over many years without causing symptoms or being detected. Therefore, small airways have been called the "quiet zone" of the lung[9]. It has been estimated that obstruction of 75% of all small airways is required before a decrease in forced expiratory volume in 1s (FEV_1) can be detected with spirometry[10]. Another important difference between the large and the small airways is that the lining fluid in the small airways has the characteristic of a surfactant with low surface tension, which helps keep the small airways from collapsing on expiration and at low lung volumes[11].

1.2 RESPIRATORY TRACT LINING FLUID

Lungs are constantly exposed to the external environment and face pathogens, toxins, allergens, and other inhaled particles, all of which could have a detrimental effect on lung homeostasis. Despite this constant exposure during respiration, the frequency of lung injury and severe infections is relatively low in healthy individuals due to a constant immunological surveillance taking place in the lung[12]. The respiratory epithelium together with the respiratory tract lining fluid play an essential role in this host defense.

The airways are lined with ciliated, serous, goblet, basal, neuroendocrine and Club cells, that are joined together by tight junctions[7,13]. Ciliated cells are the most abundant cells in the large conducting airways, but towards the periphery, in the small airways, both ciliated and goblet cells decrease in number until they disappear and are replaced with the serous and Club cells[7]. In contrast to the diversity of cell types in the conducing airways, only two cell types are found in the alveoli: type I alveolar cells (AT1) and type II alveolar cells (AT2). They are present in similar numbers but are morphologically very different[14]. AT1 cells are large and have an elongated form and they cover approximately 90% of the alveolar surface. AT2 cells are cuboidal and reside in alveolar corners and are functionally distinct from AT1 cells[14] as they have an important role in the synthesis of surfactant lipids and proteins, but also act as progenitor cells for AT1 cells during the repair of the alveoli[13] (Figure 2).

The respiratory tract lining fluid (RTLF) overlays the underlying airway epithelial cells which significantly influence its local composition through their secretions. Due to very heterogeneous cell populations throughout the airway tree, the composition of the lining fluid differs dramatically from the nasal to the alveolar region. In the large airways, the RTLF mainly consists of mucus, which promotes the trapping and clearance of inhaled pathogens and particles through the mucociliary transport. In contrast, in the alveolar region, the RTLF consists mainly of the surfactant produced by AT2 cells[15].

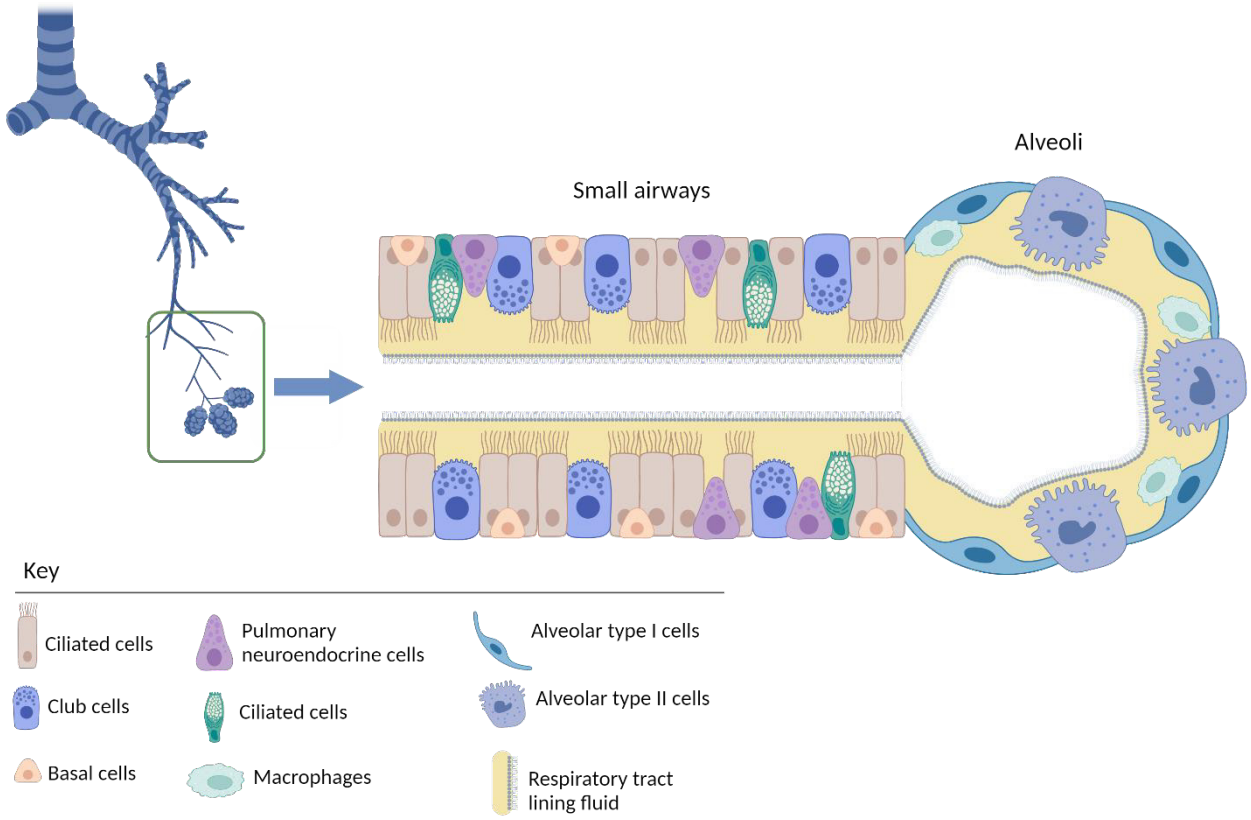

Figure 2. A schematic illustration of the respiratory epithelium and the respiratory tract lining fluid. Created with BioRender.com.

1.2.1 SMALL AIRWAY LINING FLUID

As mentioned previously, the major component of RTLF in small airways is the surfactant, which is composed of roughly 90% lipids, of which the majority are phospholipids, and 10% surfactant proteins. It also contains cholesterol, which is the major neutral lipid component[16,17], triglycerides, and fatty acids[18]. The main function of surfactant is lowering the surface tension and preventing alveolar collapse during expiration. However, it also plays a key role in innate immune response and represents the first line host defense[18].

Phospholipid classes in the lung surfactant are defined by their head group, i.e., phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), sphingomyelin (SM), phosphatidylinositol (PI), and phosphatidylserine (PS)[19]. They are primarily responsible for the surface activity of the surfactant film, but they also play an important role in altering the immune response[20]. PC is the major phospholipid, and approximately 60% of the PC class is made up of the dipalmitoyl-phosphatidylcholine (DPPC; PC 16:0/16:0), which is the major surface-active component[19]. The second most abundant class is the anionic PG class, constituting approximately 10% of the phospholipid pool, with the major species being palmitoyl-oleoyl-phosphatidylglycerol (POPG; PG 16:0/18:1), capable of modulating the innate immunity within the lung[21]. PG and PI have the ability to antagonize Toll-like receptor (TLR) activation, through which they can contribute to controlling viral infections and the associated inflammatory response. The clearance of surfactant lipids occurs either through degradation by alveolar macrophages or recycling by AT2 cells for reuse[22].

The most abundant proteins found in RTLF are Surfactant proteins A, B, C, D (SP-A, SP-B, SP-C, SP-D), which interact extensively with phospholipids in the surfactant. SP-B and SP-C are both hydrophobic proteins and are necessary for regulation of the surfactant film formation and stability. SP-A and SP-D, which belong to the family of collectins, are hydrophilic and play an important role in the inflammatory response and innate immunity[19]. In addition to the surfactant proteins, a variety of other proteins originating from different sources can be found in RTLF, which makes the composition of the RTLF proteome rather complex[4,23,24]. Proteins found in it can be synthetized locally, i.e. surfactant proteins, derived by leakage from plasma, e.g. albumin, or come from underlying epithelial and inflammatory cells[4]. Furthermore, the composition of the RTLF is affected by the clearance of these proteins as well. In the small airways, the proteins can be cleared by alveolar macrophages, degraded by peptidases or absorbed through the blood-gas barrier into the circulation[25]. Therefore, defect in one of these systems can cause marked alterations of the RTLF proteome.

1.3 SMALL AIRWAY INFLAMMATION

Small airways are the major site of airflow obstruction in most airway diseases[10,26,27]. They may be more prone to pathology due to their size as their narrow lumen makes them more vulnerable to complete obstruction[28]. Additionally, there is a transition between the gas being moved with convection to being moved with diffusion that occurs on the level of small airways, which makes them more vulnerable to the deposition of fine particles that remain suspended in the inhaled gas (Figure 3)[7].

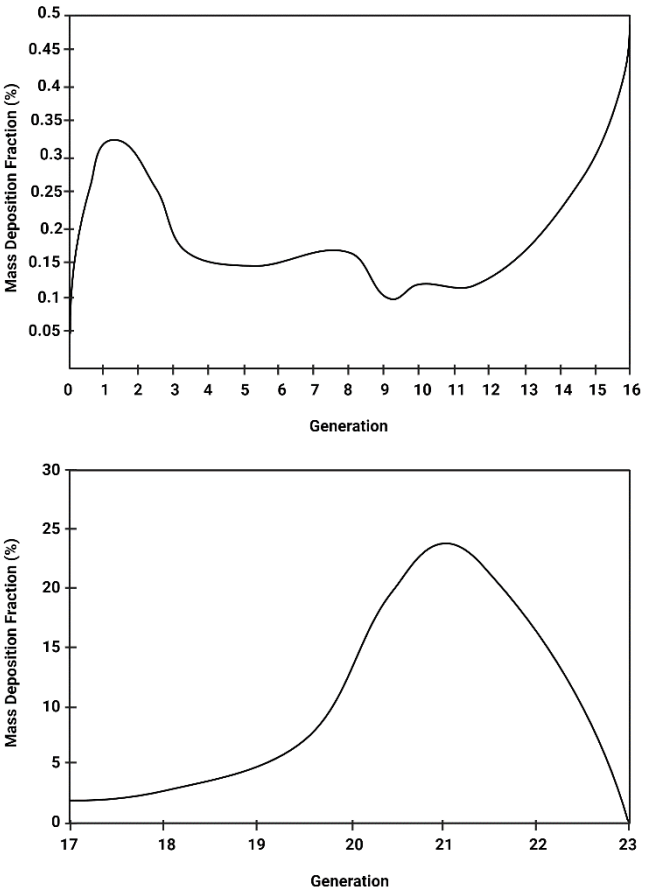

Figure 3. Pulmonary deposition of cigarette smoke particles for each airway generation (0-23). Mass deposition fraction on the y-axis shows the percentage of the inhaled total particle mass depositing in each airway generation. The deposition fraction is highest in the acinar zone (from generation 15 and on). Adapted and modified from R. J. Robinson & C. P. Yu (2001) Deposition of Cigarette Smoke Particles in the Human Respiratory Tract, Aerosol Science and Technology, 34:2, 202-215, DOI: 10.1080/027868201300034844. *Copyright © 2024 Taylor & Francis.*

The obstruction of small airways can be caused by various mechanisms, such as occlusion or reduction of the airway lumen with mucus or by inflammatory infiltrates, smooth muscle hypertrophy, airway wall thickening, or the loss of structural support which makes them more collapsible[28].

During inflammation and disease, not only structural changes occur in the small airways, but also changes in the composition of the RTLF, which has been shown to vary significantly in different airway diseases, e.g. asthma, COPD and respiratory infections[22,29-33].

1.3.1 CHRONIC OBSTRUCTIVE PULMONARY DISEASE

Chronic obstructive pulmonary disease (COPD) is a heterogeneous condition, characterized by chronic respiratory symptoms and persistent airflow obstruction[34]. It is the third leading cause of death worldwide[35] and its prevalence and burden are projected to increase even further in the next decades[34]. The most important risk factors for the development of COPD are cigarette smoking, occupational exposures, biomass exposure and air pollution[34].

The development of COPD is very complex and does not only depend on the exposure to risk factors, but instead is the end-result of dynamic interactions between genetic factors and the environment throughout the lifetime, which can cause lung damage and change the normal lung function trajectory[34,36]. Different pathological processes of varying degree and severity, such as emphysema, large and small airway inflammation, are driving COPD, which adds even further to the complexity of this heterogenous disease[37].

Airway remodeling occurs due to the injury caused by chronic irritants, as the lung tissue attempts to heal. However, the regenerative capacity of the lung tissue is limited and the process often results in scarring, which leads to permanent alterations of tissue architecture[38]. Chronic exposure to irritants also induces hypersecretion of mucus, resulting in mucus plugging, and immune cell infiltration. These changes lead to small airway obstruction and loss of small airways and occur already early in the process of COPD. However, an extensive small airway damage and loss usually occur before the changes are detectable with conventional spirometric tests, such as FEV_1 (Figure 4)[39,40]. Thus, spirometry is not a reliable method for early small airway dysfunction, and therefore, these early changes usually go undetected until the disease progresses.

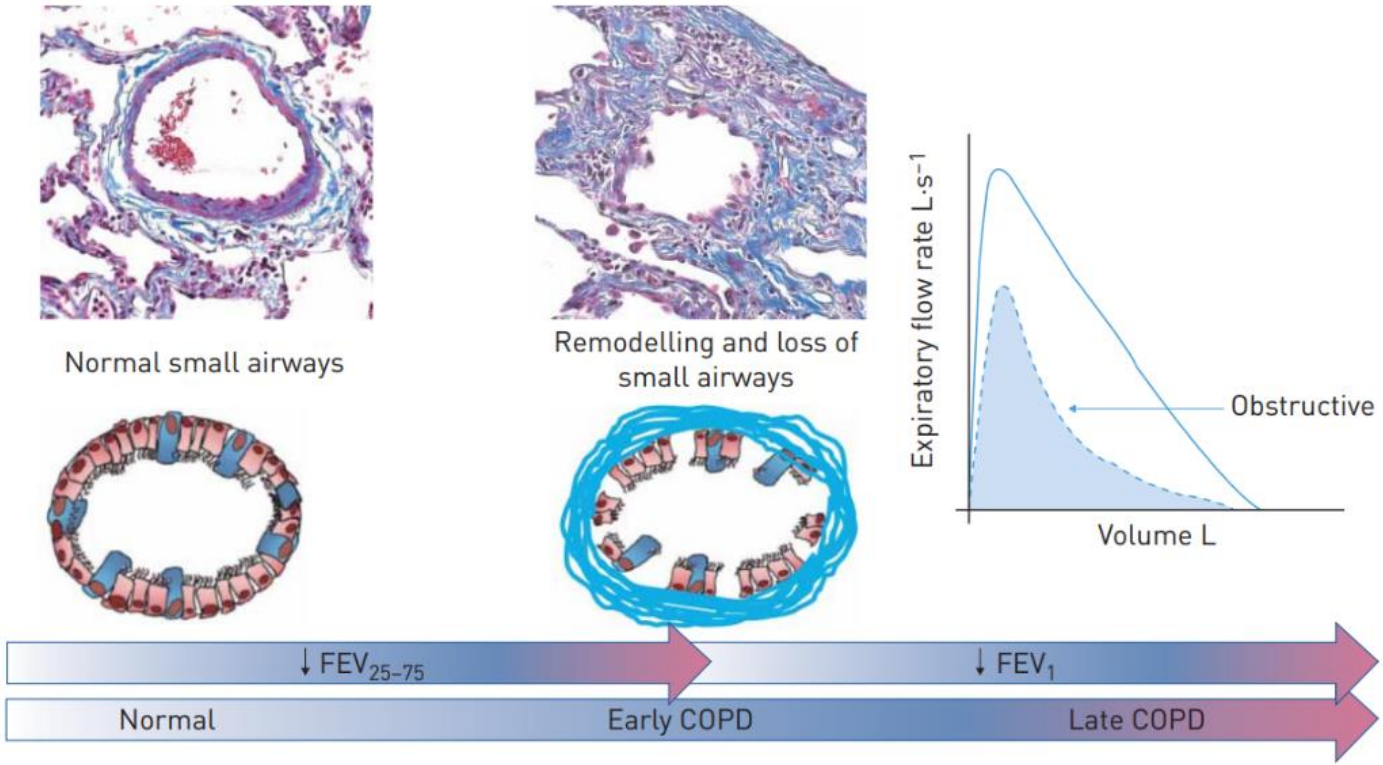

Figure 4. Healthy small airway of a never-smoker and a small airway of a smoker without COPD, where extensive airway remodeling can be observed. A decline in FEV_1 is not observed until the disease progresses and an extensive loss of small airways occurs. Reproduced with permission of the © ERS 2024: European Respiratory Journal 55 (3) 1902457; DOI: 10.1183/13993003.02457-2019. Published 5 March 2020.

As the RTLF represents a protective interface between the external environment and the respiratory epithelium, studying the changes in the composition of the small airway RTLF may clarify these early pathological changes that occur due to the exposure to irritants. Global surfactant dysregulation has been observed in the BAL fluid of patients with COPD, with lower levels of phosphatidylcholine, phosphatidylglycerol, phosphatidylinositol, SP-B, SP-A and SP-D as compared to controls[33]. Lower levels of SP-A have also been reported in exhaled particles collected from patients with COPD[41].

However, there is still a lot to be understood when it comes to the mechanisms that are underlying and driving the pathology of COPD, and especially regarding the changes that occur at an early stage before clinically evident COPD develops.

1.3.2 ASTHMA

Asthma is a heterogeneous disease, usually characterized by a chronic airway inflammation and defined by variable respiratory symptoms and variable airflow limitation[42].

It is considered an umbrella diagnosis for multiple phenotypes driven by different immunological mechanisms[43]. Asthma can be subdivided into two major groups – T_H2-high endotype and T_H2-low endotype – according to the level of type 2 inflammation. Type 2 inflammation is regulated by the $CD4^+$ T cell subpopulation known as T helper 2 (T_H2) cells that secrete cytokines IL-4, IL-5 and IL-13 and stimulate type 2 immunity. T_H2-high asthma is characterized by eosinophilia and responsiveness to glucocorticoids. And although T_H2-high asthma generally responds to treatment with corticosteroids, there is still a subgroup of patients with this endotype that has persistent symptoms and poor asthma control despite corticosteroid treatment[44]. Patients with T_H2-low asthma have levels of type 2 inflammation in the airways that are comparable with healthy controls and the mechanisms causing it are not well understood. Possible mechanisms of T_H2-low asthma could be intrinsic abnormalities in airway smooth muscles and/or effects of oxidative stress or neutrophils on the airway structure, causing hyperresponsiveness and airway obstruction[44].

Asthma severity does not only depend on the underlying type of inflammation, but also varies greatly based on age, sex, atopy, lung function and body mass index (BMI). Female sex, older age, obesity, and atopy are usually associated with a more severe form of the disease[45-48].

Furthermore, small airway dysfunction (SAD) is a common feature in asthma and approximately 50-60% of patients with asthma display evidence of SAD[49].

Patients with asthma and impaired small airway function tend to have more severe asthma with suboptimal asthma control and higher risk of exacerbations. These patients may also have a relatively normal FEV_1, despite the presence of SAD, and are therefore difficult to diagnose with conventional measures of lung function[50].

Disturbed surfactant homeostasis also contributes to the pathogenesis of asthma and increased airway resistance. Surfactant with decreased surface activity has been observed in patients with asthma and several mechanisms for this have been proposed, e.g. inactivation caused by leakage of plasma proteins into the RTLF, hydrolysis of surfactant phospholipids and decreased synthesis of hydrophobic surfactant proteins[51]. Additionally, surfactant lipids and proteins have the ability to influence the inflammatory response in asthma by regulating the adaptive and innate immune response[52,53], highlighting the importance of the surfactant in host immune defense in asthma.

This shows that asthma is a complex disease caused by many different pathophysiological mechanisms and our understanding of these underlying mechanisms is lacking. The advantage of understanding asthma endotypes would be that each endotype could be treated by drugs specifically targeting the causative mechanisms[44], offering better disease control.

1.3.3 COVID-19

Coronavirus disease 2019 (COVID-19) is caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). COVID-19 has caused a global pandemic with over 774 million confirmed cases and over seven million deaths reported as of January 2024[54]. The clinical spectrum of the disease varies from asymptomatic to severe illness characterized by acute respiratory distress syndrome (ARDS). Most individuals infected with SARS-CoV-2 develop a mild form of the disease with fever, cough and shortness of breath, but in about 23% of cases the disease progresses to a severe form, with a mortality rate of about 6% in infected patients[55].

Furthermore, many individuals with COVID-19 have symptoms that persist beyond the acute phase and may be present for weeks or even years after the onset of the infection. The condition is called a post-acute sequelae of COVID-19 (PASC) and it affects around 10-30% of individuals infected with SARS-CoV-2[56]. PASC is highly heterogeneous and the underlying pathogenetic mechanisms are not completely understood[57], however persistent complement dysregulation has been proposed as one of the factors contributing to it[58].

Small airway dysfunction has been observed in patients with COVID-19, both during the acute phase[59], and up to a year after the acute infection[60]. SARS-CoV-2 uses the angiotensin-converting enzyme 2 (ACE2) receptor to enter the cells[61], which is highly expressed on AT2 cells[62]. This causes damage to the surfactant producing AT2 cells as well as to their ability to undergo full transition into AT1 cells, causing impaired lung regeneration[63]. As a result, SARS-CoV-2 causes alveolar damage, small airway dysfunction and disturbs surfactant homeostasis[22,62,64]. Furthermore, it induces endothelial injury in the lung and formation of microthrombi in the alveolar capillaries[65], further impairing the function of peripheral airways. Exploring the changes in the RTLF protein and lipid profiles induced by SARS-CoV-2 may lead to discovery of biomarkers that could be useful in disease management and development of new biological therapies.

1.3.4 RISK FACTORS FOR THE DEVELOPMENT OF SMALL AIRWAY INFLAMMATION

Cigarette smoke

The most important risk factor for the development of COPD is cigarette smoke, with approximately 15-36% of smokers developing COPD[66,67]. Cigarette smoke contains more than 4700 different toxic substances, and it has been estimated that a single puff contains more than 10^{14} oxidizing molecules[68,69]. Small airways are especially vulnerable to cigarette smoke due to the high rate of deposition of cigarette smoke particles in the transition zone and distal airways[70], as shown in Figure 3. These molecules can induce oxidative stress and trigger many cellular and signaling responses which contribute to the inflammation in the small airways[71]. As the RTLF presents the first line of defense against these toxic substances, alterations in the RTLF proteome occur with inhalation of cigarette smoke, especially in proteins involved in the response to oxidative stress, inflammation, and wound healing[31,72]. Injury and loss of AT2 cells due to smoking may also lead to altered surfactant synthesis, secretion or reuptake, which leads to disrupted surfactant homeostasis[73]. Smoking is associated with reduced surfactant phospholipids[74], and the surface tension ability of the surfactant is diminished due to decreased amount of DPPC in the lung[73]. Smokers also show decreased levels of SP-A and SP-D, which could contribute to increased risk for respiratory infections[33,75,76]. Dysregulation of surfactant is also associated to emphysema and airflow obstruction as measured by FEV_1 and FEV_1/FVC ratio[33].

Biomass exposure

Household air pollution due to cooking and heating using biomass fuels, such as wood and coal, is one of the largest causes of mortality worldwide. Exposure to indoor air pollution in children increases the risk for lower respiratory tract infections, and in adults it increases the risk for development of COPD and asthma. It has been estimated that the risk for development of COPD due to biomass smoke exposure is similar to that due to cigarette smoking[77].

Occupational exposure

Occupational exposure, including dusts, chemical agents, and fumes, is the most important risk factor for the development of COPD among never smokers in high income countries, accounting for 14% of the cases[78]. Workplace exposure is also an important risk factor for the development and exacerbation of asthma[78].

Air pollution

Exposure to air pollution has adverse effects on respiratory health and represents an important risk factor for small airway inflammation, as it is associated with decrease in pulmonary function, increased infection rate, respiratory symptoms, asthma onset, COPD exacerbations and increased respiratory mortality[79].

1.4 THE COMPLEMENT SYSTEM AND ITS ROLE IN LUNG DISEASE

During the past several years, more and more studies have been reporting on the key role of the complement system in lung diseases and it has been shown that there is an abundance of complement proteins in the respiratory tract lining fluid[24]. Several of these proteins are synthesized in the lungs by the AT2 cells and therefore provide a basis for local complement activation[80], which contributes to the defense against inhaled material and pathogens. However, the complement does not only have a protective role in the lungs as excess complement activation also can be the cause of lung injury and is involved in the development of lung diseases[81,82].

The complement system is one of the oldest innate immune systems comprising more than 50 soluble and membrane-bound proteins. Its activation leads to opsonization and lysis of the pathogen and the generation of the inflammatory response through production of potent proinflammatory molecules. The complement system also acts as a waste disposal system taking care of the clearance of apoptotic and damaged cells, immune complexes, and other products of tissue injury. Additionally, it does not only play a key role in the innate immune system, but also acts as a bridge between the innate and adaptive immune responses[83].

It is activated through three major pathways – the classical, lectin and alternative pathways (Figure 5). The classical pathway is initiated by the binding of C1q to the antigen-antibody-complexes; the lectin pathway is immunoglobulin-independent and is activated through the binding of soluble pattern-recognition molecules, such as mannose-binding lectin (MBL), to specific carbohydrate structures on microbial surface or damaged cells; the alternative pathway is mechanistically distinct from the other two as it is constitutively active at low levels under normal conditions due to spontaneous hydrolysis of C3 to C3(H_2O), which binds factor B and forms a complex that eventually leads to the formation of C3 convertase, that cleaves C3 into C3a and C3b. This creates a positive feedback loop for the alternative pathway activation and acts as a powerful amplification mechanism of complement activation during inflammation, when C3b is formed by the classical and lectin pathways[83,84].

Regardless of the initial trigger, all three pathways lead to the formation of C3 convertase that cleaves C3. The C3a anaphylatoxin formed has diverse immunomodulatory functions, and C3b is involved in the formation of C5

convertase, which in turn leads to the cleavage of C5 into C5a anaphylatoxin with potent immunomodulatory properties, and C5b, which initiates the formation of the membrane attack complex (MAC)[84]. Both anaphylatoxins act as chemoattractants for neutrophils, monocytes, and macrophages towards the site of injury.

Besides the three major pathways, complement system can also be activated through other routes, such as the kallikrein-kinin system, the coagulation cascade and cleavage by elastases released from neutrophils. Proteases such as plasmin, thrombin, kallikrein and several coagulation factors have the ability to cleave and activate C3, creating a crosstalk between the complement and coagulation pathways[83,84].

Complement system in lung disease

It is well known that the complement system plays an important role in the development of ARDS, but the exact mechanisms remain to be determined[81]. Elevated plasma C5a levels have been seen in patients with ARDS[85], and presence of C3 activation and C5a were seen in RTLF sampled with BAL from ARDS[86]. Complement activation also plays a role in the lung inflammation in COPD, where decreased serum C3 and C4[87,88], and increased sputum C3a and C5a[89,90] in a stable disease can be seen. In addition, during an exacerbation, further increase in sputum C5a has been observed[90]. It has also been reported that cigarette smoke can cause the activation of the alternative complement pathway[91]. Complement system may also act as a key regulator of the type 2 inflammation in asthma[92]. Increased C3a and C5a levels have been observed in BAL fluid after allergen challenge in patients with asthma[93], and high concentrations of plasma C3 have been associated with asthma severity[94].

The complement system has gotten a lot of attention in the recent years as it has been shown to play a key role in COVID-19[58]. During the acute stage of the disease complement overactivation has been observed[95]. Moreover, it has also been regarded as one of the major mechanisms for the development of PASC[58].

Despite the role of complement activation in lung disease being well established, the understanding of the mechanisms for the complement-mediated airway remodeling is still lacking.

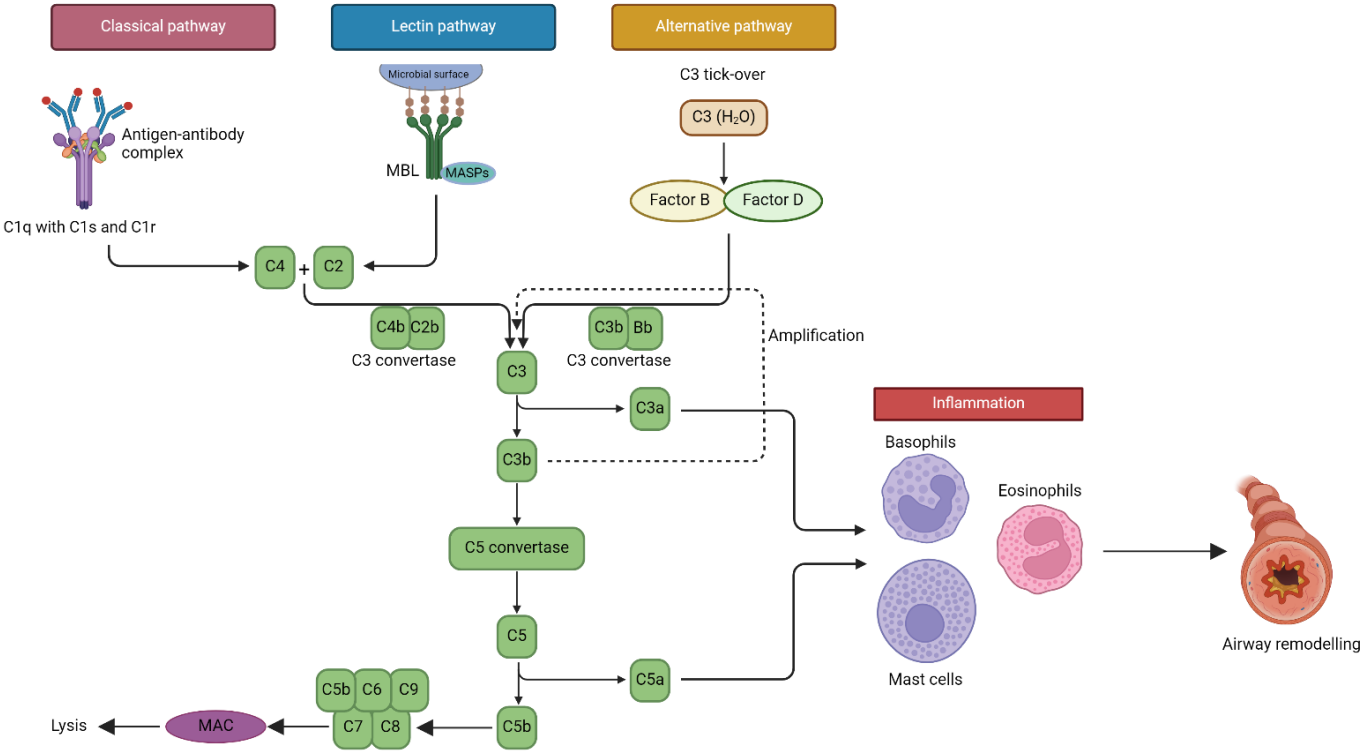

Figure 5. The complement activation pathways. Created with BioRender.com.

1.5 METHODS TO SAMPLE THE RESPIRATORY TRACT LINING FLUID

Several different methods for sampling the biological material from the airways can be used, such as bronchoalveolar lavage, induced sputum, exhaled breath condensate, nasal lavage, and exhaled particles[4,96,97]. The main differences between these methods are the invasiveness of the sampling (invasive vs non-invasive), the nature of the sample collected (liquid or exhaled breath) and the type of the sampled biological markers (cellular markers, soluble components, and volatile organic compounds). However, the underlying goal is the same, that is to provide insight into the pathogenetic mechanisms of respiratory diseases[96].

1.5.1 BRONCHOALVEOLAR LAVAGE

Bronchoalveolar lavage (BAL) fluid is obtained by instilling aliquots of saline during bronchoscopy and recovering it by gentle aspiration. It is used to study both the cellular and soluble material from the alveolar spaces[98]. Even though BAL is a safe procedure, it is still invasive in nature as it requires the use of a local anesthetic and sometimes conscious sedation[99]. Other than its invasiveness, there are other factors limiting the feasibility of the method. Although the repeatability of the method to study the cellular components is high, the measurements of the acellular components are of limited clinical value and mainly used as investigative tools due to the unknown dilution factor[96]. Additionally, sampling could be unspecific due to possible mixing of different lung compartments[100]. Furthermore, blood contamination can occur during sampling[100] and repeated or serial BAL can be a cause of airway inflammation in itself, further limiting its feasibility[96].

1.5.2 INDUCED SPUTUM

Induced sputum is a standardized technique used to study airway inflammation and is less invasive than BAL. Inhaling an isotonic or a hypertonic solution by nebulization induces a small amount of airway secretion that can be expectorated and analyzed[101]. Like BAL, induced sputum can also be used to sample both cellular and acellular components. The method shows good repeatability with high intraclass correlation coefficient (ICC), particularly for cellular components, i.e., neutrophils, eosinophils, and macrophages. However, like BAL, the use of induced sputum for studying acellular components is of limited clinical value due to the unknown dilution factor. Furthermore, it can induce an influx of neutrophils into the airway lumen and therefore cause changes in the composition of the RTLF[96]. Despite being considered a safe method, it can induce

bronchoconstriction and, therefore, subjects need to be closely monitored during the procedure[101]. The use of induced sputum for studying the composition of the small airway lining fluid is limited as it has been shown to mainly sample the central airways[101,102].

1.5.3 EXHALED BREATH CONDENSATE

Exhaled breath condensate (EBC) is obtained by cooling the warm exhaled breath in the cold EBC collector, causing condensation of the exhaled breath into liquid that can then be analyzed[103]. It is a non-invasive method that uses tidal breathing to collect both non-volatile and volatile compounds from the airways. Unlike BAL and induced sputum, EBC does not collect the cellular components from the airways[103]. The non-volatile constituents likely originate from the RTLF in the form of aerosols[104], but similar to induced sputum, EBC has been shown to predominantly sample the central airways[105]. The major limitation of the method is that the RTLF component of the EBC is highly diluted by water and there is a lack of a confident dilution marker[104]. Additionally, significant intra-individual variability in the concentrations of lipids and proteins has also been observed in EBC, likely due to the variability of dilution[104,106].

1.5.4 NASAL LAVAGE

Nasal lavage samples the nasal mucosa by irrigation with the isotonic saline and offers a non-invasive and easy alternative for sampling the RTLF. The method is, however, used to sample the upper airways, as opposed to BAL and exhaled particles. The major limitations of this method are the excessive sample dilution with saline, variability between subjects, and potential contamination with blood during sampling[107].

1.5.5 EXHALED PARTICLES

Exhaled particles are a matrix that contains non-volatile material originating from the RTLF. These particles are formed endogenously along the airways during breathing and can be sampled non-invasively through impaction with different particle sampling methods, such as Breath Explor® and the PExA® method[108,109].

Particles in exhaled air (PExA)

These particles are formed during the closure and re-opening of the airways by film bursting of the RTLF. This airway closure and re-opening has been shown to take place in the peripheral airways with an internal diameter of 0.4-0.6 mm in

dogs[110]. The exact location in humans is, however, not known, although some studies have indicated that the airway closure occurs in the small airways in humans as well[97,111,112]. There is a high degree of overlap between the proteomic composition of exhaled particles and BAL fluid[4], and phospholipid composition of exhaled particles is more closely related to that of BAL fluid than of induced sputum[97]. Comparison of albumin and SP-A concentrations between exhaled particles, BAL fluid, and bronchial wash also showed a high association between exhaled particles and BAL, while no association was found with bronchial wash[113]. These results support the peripheral origin of exhaled particles. Therefore, exhaled particles offer a non-invasive alternative to the sampling of the lining fluid from small airways.

In order to standardize the sampling and maximize the amount of particles sampled, a breathing maneuver has been developed[114]. The breathing maneuver used to sample exhaled particles with the PExA method enhances airway closure and re-opening, resulting in an increased production of particles. It starts with a deep exhalation and breath holding at the residual volume (RV), which causes closure of small airways. This is followed by an inspiration to the total lung capacity (TLC), causing small airways to re-open and the lining fluid to burst, creating the particles that are then exhaled through a normal exhalation and sampled in the instrument through impaction (Figure 6)[114]. However, despite using a standardized breathing maneuver, there is still a significant variation of these particles between subjects, as shown by Bake et al.[111].

These particles are sampled on hydrophilic polytetrafluoroethylene (PTFE) membranes, which can then later be analyzed for proteins and lipids using different methods. As compared to BAL and induced sputum, the sampling of biological material with the PExA method does not deal with the issue of unknown dilution, as the particle number concentration and size of particles are characterized with the optical particle counter[108]. The optical particle counter measures the number of particles in eight size intervals within the total size range 0.4−4.6 μm. Assuming unit density of 1000 kg/m^3 and spherical shape, the mass of collected exhaled particles can be estimated[111].

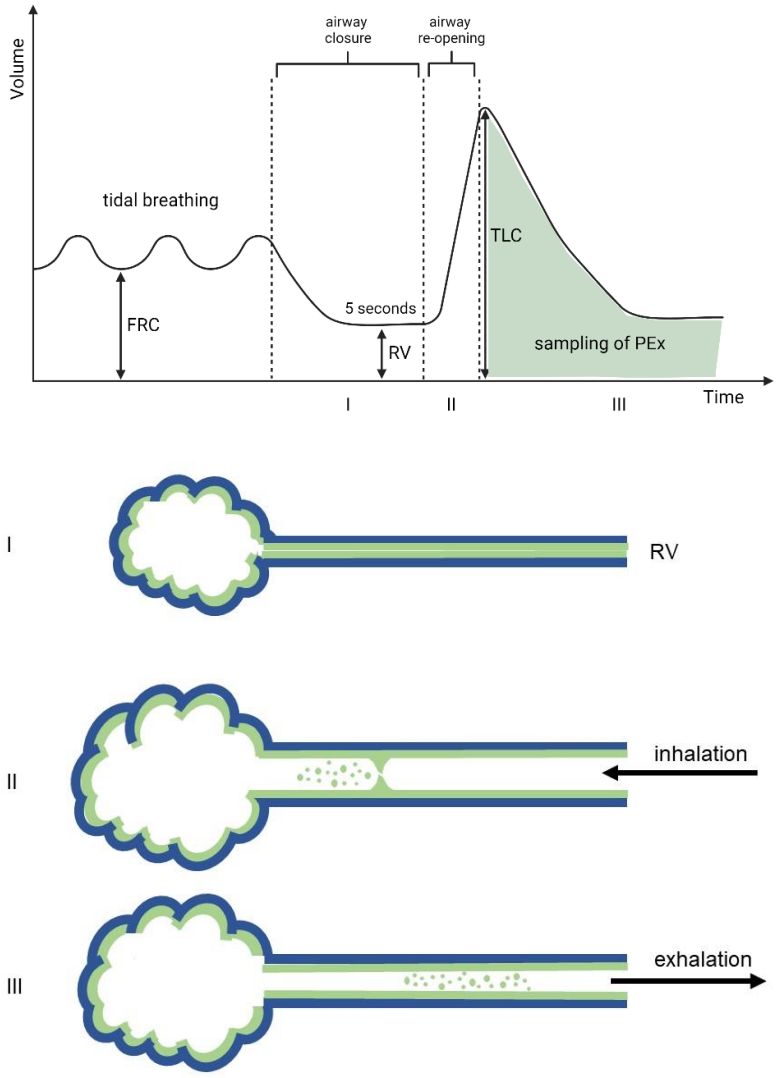

Figure 6. An illustration of the breathing maneuver and the corresponding airway closure and re-opening with the bursting of the RTLF film and creation of particles. During phase I, exhalation to residual volume (RV) and breath holding causes closure of small airways. This is followed by phase II, where inhalation to total lung capacity (TLC) causes the closed airways to re-open, creating a meniscus of RTLF between the airways walls which then bursts and particles are generated. These particles are then exhaled (phase III) and sampled in the PExA instrument. Created with BioRender.com.

1.6 PHYSIOLOGICAL TESTS TO ASSESS SMALL AIRWAY FUNCTION

The recognition that the small airways represent the silent zone of the lungs where the disease can start and progress over several years without being detected has led to the development of new physiological tests aimed to detected abnormalities in the small airways. So far, these methods have mostly been used in research settings, and are only now starting to be implemented for clinical use.

1.6.1 SPIROMETRY

Spirometry is the most widely used and accessible lung function test, with FEV_1 being the most common variable assessed. However, FEV_1 is of limited value in detecting small airway dysfunction as significant lung injury must occur before a decrease in FEV_1 can be observed[28], as shown in Figure 4.

The middle and end portions of the expiratory flow may offer additional information about the function of the small airways as compared to FEV_1. The flow volume curve is shown in Figure 7. The forced expiratory flow between 25% and 75% of the forced vital capacity (FVC), $FEF_{25-75\%}$, and forced expiratory flow at 75% of the FVC, $FEF_{75\%}$, are considered to reflect small airway function[28,115]. Low $FEF_{25-75\%}$ has been shown to be a useful predictor for the development of COPD[116], and has been associated with persistent and nocturnal symptoms and blood eosinophilia in patients with asthma[117]. On contrast, a large study by Quanjer et al. has shown that $FEF_{75\%}$ and $FEF_{25-75\%}$ do not add to the clinical decision making as compared to FEV_1, FVC, and FEV_1/FVC[118]. Furthermore, mid- and end-expiratory flows are highly dependent on the FVC[119], have poor reproducibility, and are often normal if the FEV_1/FVC ratio is above 75%[120]. Therefore, spirometry has limited potential in the assessment of small airway dysfunction.

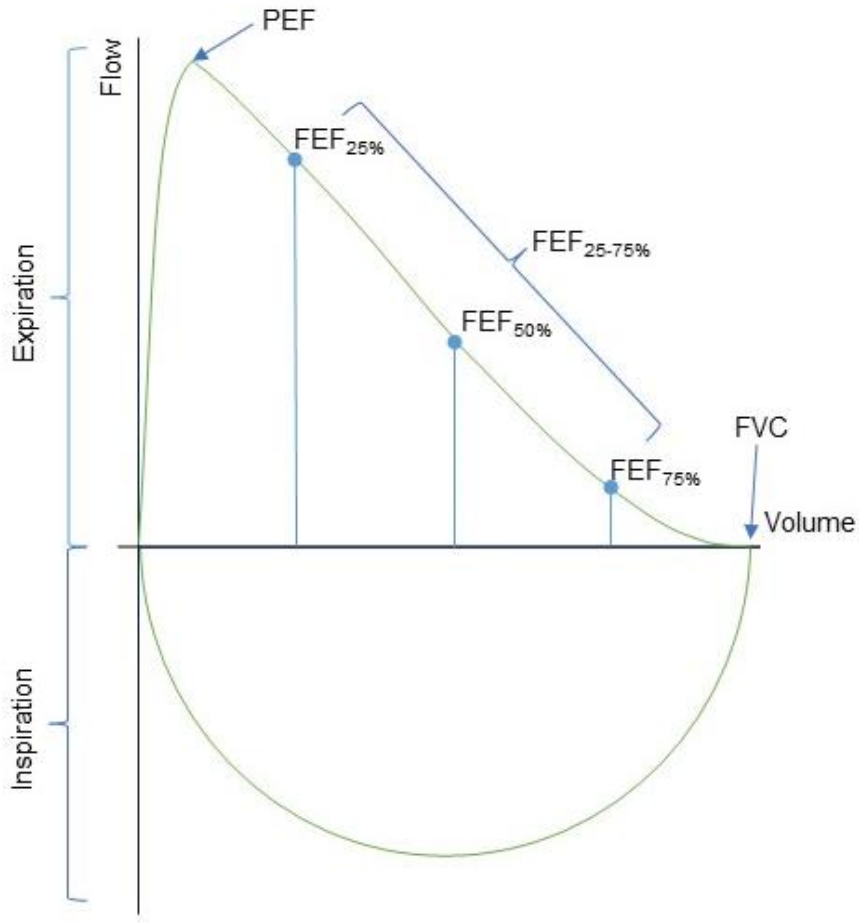

Figure 7. The flow volume curve. PEF, peak expiratory flow; FVC, forced vital capacity; $FEF_{25\%}$, forced expiratory flow at 25% of the FVC; $FEF_{25-75\%}$, forced expiratory flow between 25% and 75% of the FVC; $FEF_{75\%}$, forced expiratory flow at 75% of the FVC

1.6.2 FORCED OSCILLATION TECHNIQUE

The forced oscillation technique, or oscillometry, measures the mechanical properties of the lung, i.e. respiratory impedance (Z_{rs}), during normal tidal breathing. It includes impulse oscillometry (IOS) and airwave oscillometry (AOS).

The method applies oscillating pressure fluctuations, which can be in the form of sound waves, over a range of frequencies (usually between 5 and 35 Hz) to the respiratory system during normal tidal breathing[7,28]. This results in pressure and flow changes in the lungs which are then measured at the mouth and used to calculate impedance. Respiratory impedance measured by oscillometry consists of resistance of the airways and tissue to flow (R_{rs}), and reactance, which is the elastance or stiffness of the lung parenchyma and the chest wall in response to changes in the volume (X_{rs})[121].

These pressure fluctuations travel through the large and small airways. Higher frequencies (usually >20 Hz) travel shorter distances and do not propagate beyond the large airways, while lower frequencies (typically 5 Hz) travel deeper into the lung and reach the small airways. Therefore, the resistance at 5 Hz represents the total airway resistance (R_5) and the resistance at 20 Hz the resistance of the large airways (R_{20}). Subtracting R_{20} from R_5 thus gives us the resistance in the small airways (R_{5-20}). In healthy adults, the resistance is nearly the same at all frequencies. Obstruction increases the resistance; obstruction in the large airways increases resistance at all frequencies evenly, while the obstruction of small airways only increases the resistance at low frequencies (R_5), but not at high frequencies (R_{20}). Therefore, in small airway obstruction, the resistance becomes frequency dependent[28,122].

Other than respiratory resistance, another commonly reported outcome of oscillometry is the area of reactance (AX), which is a useful index of respiratory compliance and therefore small airway patency[122].

1.6.3 INERT GAS WASHOUT

The most commonly used inert gas washout techniques are single breath nitrogen washout (SBW) and the multiple breath nitrogen washout (MBW).

SBW is most often measured over a vital capacity (VC) by inhaling 100% oxygen from the RV to the TLC (a vital capacity breath) and then measuring the exhaled nitrogen (N_2) during a subsequent slow exhalation back to RV. During phase I, no N_2 is present in the exhaled air since the air from the airways proximal to the convection-diffusion interface (i.e. entrance to the alveolar zone) is being washed

out. During phase II (bronchiolar phase), the exhaled N_2 concentration rapidly starts increasing as the gas from the best ventilated regions now starts to contribute. Phase 3 (alveolar phase) follows, which represents the alveolar gas, and the expired N_2 concentration begins to plateau, but still slightly increases from the beginning to the end of the phase due to ventilation heterogeneity. During the final phase, phase IV, a steep rise in N_2 concentration can be seen as the most poorly ventilated areas of the lung empty (i.e. areas that were poorly diluted by the O_2 during a preceding inspiration). At this point the small airways start to close as well, and this is known as the closing volume. Analysis of the slope of phase III gives us information about the ventilation heterogeneity which can be affected in airway diseases[28]. Small airway dysfunction increases the slope of phase III and increases the closing volume.

MBW is a modification of the SBW technique and is performed by inhaling 100% oxygen from the functional residual capacity (FRC), with either a fixed tidal volume and respiratory rate, or a free tidal breathing pattern, to washout the nitrogen from the lungs. In contrast to SBW which if performed over a single breathing maneuver, the MBW is performed over several breaths and continues until the exhaled N_2 concentration is 2.5% of the original one. It gives us information on the efficiency of gas mixing in the whole lung through the lung clearance index (LCI), defined as the number of lung turnovers (the number of FRCs) that are required to wash out the nitrogen to $1/40^{th}$ (2.5%) of the original concentration[28]. Besides providing the information about the global ventilation inhomogeneity (LCI), a detailed analysis of the phase III slope of expirograms of the MBW also provides the information about the location in the lung where ventilation inhomogeneity occurs[123,124]. With that, we can distinguish between ventilation inhomogeneities originating at the entrance to the acinar compartment (S_{acin}) and the ones originating in the more proximal conducting airways (S_{cond}).

1.7 GAPS IN KNOWLEDGE

The method for collecting exhaled particles, the so called PExA method, is a novel method for collecting biological material from small airways and the knowledge about the repeatability and intra-individual variation of the method is still lacking. As all the papers included in this doctoral thesis are based on this method, it was important that the variability of the method and the effects of circadian rhythm on the contents of exhaled particles were studied. The methodological *paper I* therefore provides a basis for further studies using the PExA method.

In *papers II-IV*, the proteomic and lipidomic changes in the small airway lining fluid in smokers, asthma, and COVID-19 were studied. Smoking is the most important risk factor for COPD, which is a very heterogeneous disease with many different endotypes driving the disease, and similar is true for asthma and COVID-19. The knowledge about these endotypes and underlying mechanisms is still lacking. Most in vivo studies have been exploring systemic proteomic changes in blood in these diseases and the knowledge about the local changes in the RTLF is limited. Furthermore, as the complement system represents the central component of the innate immune system and plays a key role in inflammation, it is essential to study its role in these diseases. Increasing the understanding of how the complement activation contributes to the development of COPD, asthma, and what role it plays in COVID-19, could potentially lead to the discovery and development of new therapeutic targets.

The sex differences between women and men in how smoking affects the small airway proteome are addressed in *paper II*. Very little is known about the mechanisms for increased susceptibility of females for COPD. It is imperative to consider sex when studying the pathological mechanisms of airway diseases and searching for biomarkers in order to achieve precision medicine, as there are important genetic, hormonal, and dimensional differences between females and males.

Paper IV explores the phospholipid composition of the RTLF and how a mild SARS-CoV-2 infection alters it. There is a lack of knowledge about the role of surfactant lipids in COVID-19, and especially in mild infections. Most studies have reported changes on patients with a very severe infection and ARDS, while the knowledge about the phospholipid changes in a mild COVID-19 is limited.

2 AIM

The overall aim of this thesis was to explore the composition of the respiratory tract lining fluid, sampled non-invasively from small airways. More specifically, we aimed to study how smoking, asthma, and COVID-19 affect its composition, in order to increase the understanding of the underlying pathogenesis that contributes to the development of airway inflammation in these diseases.

Paper I

The primary aim of this study was to study the within-subject variability and repeatability of exhaled particles, sampled with the PExA method, a method used to sample biological material from small airways in this thesis. The secondary aim was to investigate whether these markers are subjected to diurnal variation.

Paper II

The aim of this study was to explore the effects of smoking on the protein profile of the small airway lining fluid in samples obtained by the collection of exhaled particles in order to identify dysregulation of proteins associated with smoking.

Paper III

The primary aim of this study was to explore whether the clinical characteristics of asthma are associated with the activation of the complement and coagulation systems. The secondary aim was to test the hypothesis whether we can identify different asthma sub-groups based on the levels of complement factor C3 in the small airway lining fluid.

Paper IV

The primary aim of this study was to explore the composition of the respiratory tract lining fluid, with regards to both phospholipids and proteins, in patients with an ongoing mild SARS-CoV-2 infection. The secondary aim was to investigate whether these changes are associated with small airway dysfunction in COVID-19 and whether these alterations change over time.

3 PARTICIPANTS AND METHODS

The overview of the studies, their design, participants, methods, and outcomes are shown in Table 1. The details are presented in the following sections.

Table 1. Overview of the studies included in the thesis.

	PAPER I	**PAPER II**	**PAPER III**	**PAPER IV**
Study design	Study with repeated measurements	Cross-sectional study	Cross-sectional study	Prospective cohort study
Study participants	16 healthy subjects	38 current smokers, 47 former smokers, 22 never smokers	20 subjects with asthma, 10 healthy controls	29 subjects with COVID-19, 17 healthy controls
Primary outcomes	Particle number concentration, SP-A, albumin	Small airway proteome	Small airway proteome	Small airway proteome, surfactant lipids
Lung function tests, sampling of RTLF	Spirometry PExA method	Spirometry PExA method	Spirometry, MBW PExA method	Spirometry, AOS PExA method
Clinical information	Medication use	Medical history, medication use, CRP, WBC count	Medical history, medication use, ACQ-6, CRP, WBC count, skin prick tests, FENO	Medical history, medication use
Chemical analysis of exhaled particles	ELISA	SOMAscan	SOMAscan	SOMAscan, LC-MS/MS

3.1 STUDY DESIGN

Paper I

Paper I is based on a study with repeated measurements, in which each study participant was examined on eleven occasions on five non-consecutive days. On three days, the measurements were made in the morning, at noon and during the afternoon. On the other two days, only one measurement per day was made, at any time during the day.

Paper II

Paper II is based on a cross-sectional study where study participants were examined on one occasion and separated into three groups based on their smoking habits.

Paper III

Paper III is based on a cross-sectional study where two groups of study participants (patients with asthma and healthy controls) where examined on one occasion.

Paper IV

Paper IV is a prospective cohort study where the study participants with COVID-19 were examined on two occasions months apart (baseline and follow-up) and the control subjects only on one occasion at baseline.

3.2 STUDY PARTICIPANTS

Paper I

Sixteen healthy individuals, eight men and eight women, aged 22-69 years were included in the study. All participants were recruited using posted notices. Eligible for inclusion in the study were never or former smokers with no reported hay fever and no history of respiratory diseases. Former smokers were defined as those who smoked regularly but quitted more than six months prior to the study. Exclusion criteria were an ongoing respiratory tract infection and pregnancy.

All participants provided a written informed consent prior to the measurements. The study was approved by the Regional Ethics Committee at the University of Gothenburg (application number 030-18).

Paper II

One hundred and seven individuals with a median age of 61 years, of which 51% were women, were included in the study. All participants were recruited either from our previous studies on smokers or by an advertisement in a daily paper.

The cohort consisted of 38 current smokers, 47 former smokers and 22 healthy never smokers. Current smoking was defined as smoking on a regular daily basis for at least one year at the time of inclusion in the study. Former smoking was defined as a history of smoking on a daily basis, but no smoking in the last twelve months. The inclusion criteria for healthy never smoking individuals were that they have never smoked on a daily basis and post-bronchodilation FEV_1/FVC ratio >0.70.

All participants provided a written informed consent prior to the measurements. The study was approved by the Regional Ethics Committee at the University of Gothenburg (application numbers 442-17 and 390-06).

Paper III

Thirty individuals were included in the study; 20 individuals with a physician-diagnosed asthma and 10 healthy controls. All participants were recruited from our previous studies on asthma or by an advertisement in a daily paper.

All individuals were screened with the nitrogen multiple breath washout test (MBW) prior to the inclusion in the study. Lung clearance index (LCI) was assessed. The asthma cohort therefore consisted of ten individuals with the LCI z-score <2 and ten individuals with LCI ≥2.9 (individuals with asthma and the highest LCI z-score were recruited from previous studies, which is how a cut-off of 2.9 was selected). The exclusion criteria for the asthma group were current smoking, smoking within the last ten years, smoking history of >10 pack-years and diagnosis of systemic inflammatory disease, cardiovascular disease, or pregnancy.

The inclusion criteria for the control group were the absence of respiratory symptoms and the history of respiratory disease, and a LCI z-score <2.

All participants provided a written informed consent prior to the measurements. The study was approved by the Regional Ethics Committee at the University of Gothenburg (application number 390-06).

Paper IV

Twenty-nine individuals with an ongoing SARS-CoV-2 infection and 17 healthy controls were included in the study. Individuals with COVID-19 were recruited through two different channels; those who tested positive for SARS-CoV-2 infection with a PCR test of oro/nasopharyngeal sample at primary health centers in the Gothenburg area were invited to participate in the study, as well as hospital health care workers with a positive PCR test during routine testing for hospital staff. Healthy controls were recruited by posted notices among hospital staff who have never had a confirmed COVID-19, did not have any ongoing symptoms related to COVID-19, and had a negative rapid SARS-CoV-2 antigen test (Boson Biotech, Xiamen Boson 113 Biotech Co., Ltd, Xiamen, China) at the time of inclusion in the study.

Inclusion criteria for the COVID-19 group were a positive PCR of oro/nasopharyngeal sample and ongoing symptoms related to COVID-19 at the time of inclusion in the study, but no need for hospital treatment and oxygen supplementation.

Exclusion criteria in this study was current smoking, defined as smoking on a daily basis in the last year prior to the study.

All individuals were examined at baseline (visit one), which for the COVID-19 group was during the acute SARS-CoV-2 infection. Those with COVID-19 were invited once more for a follow-up examination (visit two). Seven participants dropped-out at the time of follow-up and consequently, twenty-two individuals were re-examined at follow-up.

All participants provided a written informed consent prior to the measurements. The study was approved by the Regional Ethics Committee at the University of Gothenburg (application number 2020-07184).

3.3 METHODS

3.3.1 SAMPLING OF EXHALED PARTICLES

Exhaled particles were sampled with the PExA instrument (PExA AB, Gothenburg, Sweden) in all four papers.

The PExA instrument set-up

Sampling of exhaled particles with the PExA method was originally described by Almstrand et al.[108]. The set-up of the PExA instrument used in papers I, II and IV is shown in Figure 8. The instrument used in paper III was PExA 1.0, which

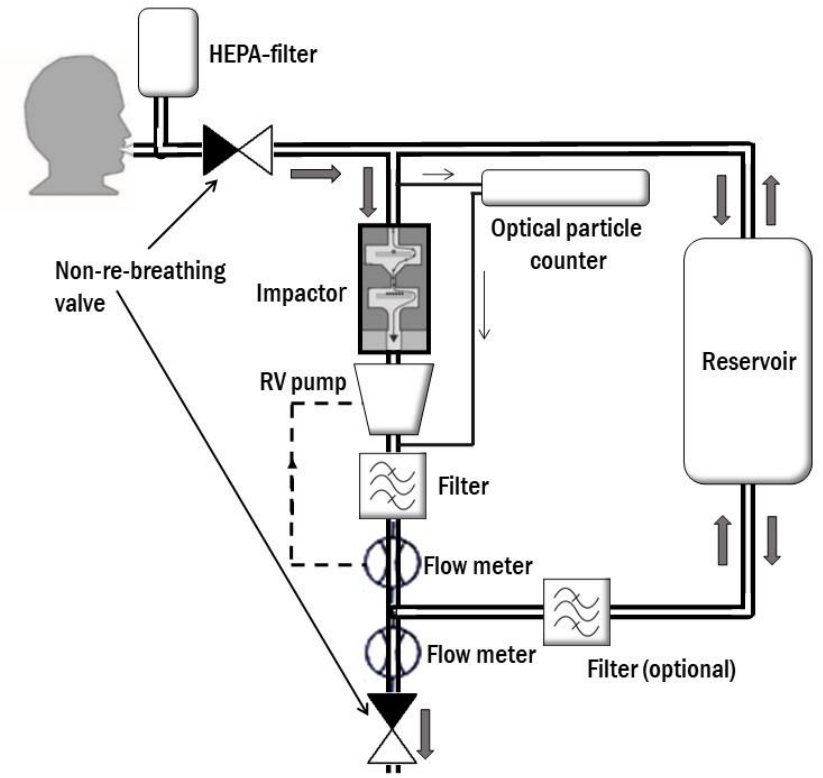

Figure 8. The set-up of the PExA 2.0 instrument. Illustration adapted and modified with permission from © PExA AB.

is an in-house built version of the instrument before the commercially available version of the instrument, PExA 2.0, was released.

In short, particle-free air is inhaled through a HEPA-filter (high-efficiency particulate absorbing filter). The subject breaths through a mouthpiece via a two-way, non-rebreathing valve into the instrument. A fraction of the air is characterized by an optical particle counter (Grimm model 1.108; Grimm Aerosol Technik GmbH & Co., Ainring, Germany) that operates at flow of 20 mL/s, while the remainder is drawn through the impactor. Particles are sampled from the aerosol with a two-stage inertial impactor (Dekati Ltd, Tampere, Finland) that is set up with a constant volumetric flow of 230 mL/s (measured using an ultrasonic flow meter) using a RV pump (rotary vane pump). To handle exhalations exceeding the flow rate through the impactor, a reservoir that can buffer the exhaled air is used. A flow meter (OEM flow sensor; Spiroson-AS, Medical Technologies, Zürich, Switzerland) also measures the exhalation flow into the reservoir. All parts of the PExA instrument, except the mouthpiece, are in a box thermostated at 36 °C.

The optical particle counter measures the number concentrations and particle size within the range 0.41-4.55 μm classified into eight size intervals. In papers II and III, the last size interval was not measured (2.98-4.55 μm) and therefore particles in seven size intervals from 0.41-2.98 μm were counted.

Particles are sampled on a thin hydrophilic polytetrafluoroethylene (PTFE) membrane (FHLC02500, Millipore, Billerica, MA, USA) covering the lower plate of the impactor.

Particle number concentration and particle mean mass

As described previously, the optical particle counter counts exhaled particles in different size intervals. Since the PExA instrument provides the information about the exhaled volume, the particle number concentration can be calculated as the number of particles per liter of exhaled air (n/L). Another outcome commonly reported with the PExA method is the number of particles per breath (n/breath). To assess the particle size distribution, the particle mean mass (pg) is reported. It is calculated by dividing the particle mass concentration (ng/L) by particle number concentration.

The sampling procedure

The sampling of exhaled particles was performed after bronchodilation in papers II and III. No bronchodilation was used in papers I and IV. All subjects wore a nose clip throughout the whole sampling procedure. At the start of the procedure, subjects inhaled through a HEPA-filter for a minimum of three breaths in order to remove particles from the ambient air, before the breathing maneuver and sampling of the particles took place. A standardized breathing maneuver was used (Figure 6), which starts with an exhalation at a normal flow rate to residual volume (RV), followed by breath holding at RV for 5 seconds. After breath holding, subjects were instructed to inhale rapidly to total lung capacity (TLC) and then finally exhale normally to RV or functional residual capacity (FRC). During this final exhalation, exhaled particles were sampled. Before they were instructed to perform the next breathing maneuver, subjects breathed in a relaxed manner through the mouthpiece for at least three breaths.

In papers I, II and IV, the sampling continued until 120 ng of exhaled particles were collected. In paper IV, the sampling was stopped before the 120 ng of material was collected if the participants were unable to continue with the sampling. In paper III, the sampling consisted of two consecutive sampling sessions with a short break in between, and a total of 240 ng of exhaled particles was collected from each subject.

In papers I, II and III, the sample holder was transferred to a clean air room after collection and the substrate was cut out with a scalpel from the sample holder area and placed in Millipore Ultrafree-MC LH Centrifugal Filter insert (FC30LH25) and stored at -80 °C until the extraction. In paper IV, where both lipid and protein profiles were analyzed, the PTFE membranes were cut in half and placed in separate, 1.5 mL SC microtube PC-PT cryotubes (Sarsteds, Nümbrecht, Germany) and stored at -80 °C until extraction and analysis.

3.3.2 CHEMICAL ANALYSES OF EXHALED PARTICLES

3.3.2.1 ELISA

Enzyme-linked immunosorbent assay (ELISA) was used to quantify SP-A and albumin in paper I.

Before analysis, samples were extracted from PTFE membranes using 140 μL of extraction buffer with a composition of 10 mM PBS containing 1% BSA w/v and 0.05% TWEEN-20 v/v (Thermo Scientific, Rockford, IL, USA). Extraction was

conducted for 60 min using a thermomixer (Comfort, Eppendorf AG, Hamburg, Germany) set at 37˚C and 400 rpm. The extracted sample volume was split: 40 μL for SP-A, 40 μL for albumin, and the rest was saved as a backup. SP-A and albumin were analyzed according to the manufacturers' instructions (SP-A: BioVendor, Brno, Czech Republic; albumin: E-80AL; Immunology Consultant Laboratories, Portland, OR, USA) with minor modifications. Buffer (i.e., extraction buffer:assay diluent buffer, 1:2 ratio) was prepared for standards and controls to match the sample buffer composition. Before analysis, 80 μL of assay diluent buffer was added to the samples. The first incubation time for SP-A was two hours at 37˚C with agitation at 300 rpm. Albumin was incubated for one hour at room temperature with agitation at 300 rpm. The reaction time was nine minutes. The precision of the assays was monitored by using three sample replicates in each run. The CVs of the replicates for SP-A and albumin were 0–10% with median CVs of 3% for SP-A and 4% for albumin and no significant difference among the three assays. SP-A and albumin results were expressed in weight percent (wt%), i.e., mass divided by the total particle mass and expressed as a percentage.

3.3.2.2 SOMASCAN ANALYSIS

SOMAscan proteomics platform (SomaLogic Inc, Boulder, USA) was used to analyze the proteins in PExA samples in papers II, III and IV.

SOMAscan is an aptamer-based assay optimized for protein biomarker discovery in blood. The method simultaneously measures a broad range of protein targets. In papers II and III, a panel to analyze 1305 human protein analytes was used, and in paper IV a panel with 7000 analytes was used. SOMAmers, the name for the aptamers used in SOMAscan analyses, are synthetic DNA Slow Off-rate Modified Aptamers (SOMAmers), constructed with chemically modified nucleotides, that bind to proteins with high affinity and specificity in a similar fashion to antibodies[125]. It transforms each individual protein concentration into a corresponding SOMAmer reagent concentration, which is then quantified by standard DNA hybridization[126].

The sample preparation differed slightly between papers II and III and paper IV. In papers II and III, proteins were extracted from the PEx substrate by adding sample buffer (proprietary SomaLogic SB17 buffer with addition of Tween-20 to 0.1% final concentration) to the centrifugal filter insert (Millipore Ultrafree-MC LH Centrifugal Filter) containing the PTFE membrane. In paper IV, the halves of the PTFE membranes were transferred to protein LoBind Eppendorf tubes, followed by the addition of the sample buffer (SomaLogic SB17 buffer with addition of Tween-20 to 0.8% final concentration).

In papers II and III, the volume of sample buffer was adjusted to reach a final concentration of PEx at 1 μg/ml, and in paper IV at 0.33 μg/mL, to normalize for the differences in the collected amount of exhaled particles. After addition of sample buffer, the sample tubes were briefly vortexed and placed in a thermal rotary shaker for 1 hour at 30°C and 400 rpm in papers II and III, and for 45 minutes at 30°C and 1400 rpm in paper IV. In papers II and III, after incubation on the thermal shaker, protein extracts were retrieved in the lower part of the centrifugal filter unit by centrifugation at 10,000 ×g for 10 min and then stored at -80°C before they were sent to SomaLogic. In paper IV, 112 μL of protein extract was transferred to Matrix™ 500μL ScrewTop Tubes (ThermoFisher 196 Scientific, Waltham, Massachusetts, US) and stored at -20°C prior to being sent to SomaLogic.

Blank samples in all three papers were made by extraction of the material on PTFE membranes subjected to the same sampling and sample preparation procedure as all other samples but omitting the breathing maneuver. Dilution samples in paper IV were made by extracting the material on a PTFE membrane sampled from one individual and then diluting the extracted sample with different buffer volumes to obtain different concentrations of PEx.

SOMAscan readout is reported in relative fluorescent units (RFU), which is directly proportional to the amount of target protein in the initial sample, as informed by a standard curve generated for each protein-SOMAmer pair[126].

3.3.2.3 LC-MS/MS METHOD

The phospholipids in exhaled particles were analyzed with the liquid chromatography–tandem mass spectrometry (LC-MS/MS) method in paper IV.

The lipid panel used to analyze the samples in paper IV was selected during method development and is based on the screening of PExA samples for lipids abundant in small airways. The most abundant lipids in each class were selected and further verified based on the chromatographic retention times, molecular mass, and fragmentation pattern in the mass spectrometer.

The first step in the sample preparation process was spiking each PTFE membrane with a diluted internal standard (SPLASH ® LIPIDOMIX 330707-1EA, Avanti Polar lipids, Alabaster, AL, 150 USA) and allowing it to dry. Lipids were extracted from the PTFE membrane in a thermomixer using two solvents: first isopropanol and then methanol. The eluate of each solvent was combined into a LC-vial. The solvent was evaporated to dryness under nitrogen and the lipid film was solvated in 70 μL of acetonitrile and isopropanol (ratio 2:1). The

extracted lipids were analyzed with a targeted LC-MS/MS method using the Waters ACQUITY UPLC I-Class chromatography system coupled to an electrospray ionization source on a Waters Xevo TQ-XS triple quadrupole mass spectrometer detector (Milford, MA, USA). The mass detector was using the Multiple Reaction Monitoring (MRM) mode for data acquisition.

PC lipids are the major class of lipids in the sample. To minimize the interferences within this major lipid class, PC lipids were separated with reverse phase chromatography before the mass spectrometer. With reverse phase chromatography, PC species are separated based on the fatty acyl chain length, number of double bonds and their position. The low abundant lipid classes were separated by a HILIC amide chromatography where the lipid classes are separated by the head group that defines the class. With the HILIC amide method, interferences between lipid classes can be avoided as the lipid classes are fully separated by the elution time.

Quantification was based on the peak area of the analyte divided by the peak area of the internal standard. The analyte-to-standard ratio was multiplied with the mol of internal standard spiked to the sample. One internal standard per lipid class was used to adjust for the extraction variability. Good linearity and precision were evaluated during method development and confirmed by the QC samples included in the run. Each lipid fragment can have different response factors and without a pure standard using the same fragment this response factor is not known. For a large lipid panel in a screening method, it is not feasible to purchase a standard for each compound, but good selectivity, precision and linearity are sufficient for comparing samples analyzed in the same run with the same method. The amount of collected PExA sample was approximately the same but has a larger uncertainty than the chemical analysis (typically CV <20%). For this reason, each lipid was expressed as a relative amount expressed as a mol% (mol%=analyte_mol/lipid_sum_mol) rather than normalized to the sampled PEx mass. Type II isotope correction (+2 isotope of one lipid can be isobaric with another lipid species with one double bond less) was done for the PE/PG/PI classes of lipids as this effect varies with sample composition, and correction factors are calculated for each sample. Type II isotope correction factors were calculated with the online correction tool LICAR, which is described by Gao et al.[127].

3.3.3 LUNG FUNCTION TESTS

3.3.3.1 SPIROMETRY

Dynamic spirometry was used to assess lung function in papers I, II and III. In all three studies spirometry was performed using a Spirare spirometer (Spirare, Stockholm, Sweden) in accordance with the ATS/ERS criteria[128]. In paper I spirometry was performed only once, before bronchodilation, and in papers II and III it was performed both before and after bronchodilation with 400 μg of salbutamol.

The values were expressed as a percentage of the reference value (% predicted) according to Brisman et al.[129] in papers I and II, and as z-scores according to the ERS Global Lung Function Initiative[130] in paper III.

3.3.3.2 NITROGEN MULTIPLE BREATH WASHOUT

Nitrogen multiple breath washout test in paper III was performed using the Exhalyzer® D device (Eco Medics AG, Dürnten, Switzerland) and software (Spiroware 3.1) in accordance with the current international consensus standards[131]. Test was performed until end-tidal nitrogen concentration was below $1/40^{th}$ of the starting concentration for three consecutive breaths.

Indices assessed were $S_{cond} \times VT$ and $S_{acin} \times VT$, and lung clearance index (LCI), and were expressed as z-scores according to Houltz et al.[132].

3.3.3.3 FORCED OSCILLATION TECHNIQUE

Forced oscillation technique in paper IV was performed using the airwave oscillometry device, Tremoflo C-100 (Thorasys, Montreal, Canada) in accordance with the current European Respiratory Society standards[133]. Indices assessed were resistance at 5 Hz (R_5), frequency dependence of resistance (R_{5-20}) and area of reactance (AX), and were expressed as z-scores according to Oostveen et al.[134].

3.3.4 CLINICAL INFORMATION

In papers II and III, all individuals were instructed to withdraw from corticosteroids, long-lasting bronchodilators, and short-acting bronchodilators at least 3 days, 24 hours and 6 hours prior to the examination, respectively. All filled out a detailed questionnaire on symptoms, medical history, use of medication, and smoking habits, and blood samples were obtained for the analysis of CRP and white blood cell (WBC) differential count.

In paper III, all individuals with asthma filled out the Asthma Control Questionnaire (ACQ-6)[135,136]. Based on their ACQ score, they were divided into two groups as well-controlled asthma (ACQ ≤1) and inadequately controlled asthma (ACQ >1). The ACQ score cut-off of 1 was chosen instead of the more optimal cut-offs of ≤0.75 for well-controlled asthma and >1.5 for inadequately controlled asthma[137] due to a small group size and in order to be able to balance groups. Allergic sensitization was tested using skin prick tests to common allergens in Sweden and a positive histamine control and a negative control were used. A positive result was defined as a wheal diameter ≥3 mm. Fractional exhaled nitric oxide (FENO) was measured to assess airway inflammation. A single measurement at an expiratory flow of 50 mL/s was performed using the NIOX MINO device (Aerocrine AB, Stockholm, Sweden).

In paper IV, a questionnaire on symptoms and their duration, COVID-19 vaccination status, smoking habits, medical history, and use of medication was obtained both at baseline and follow-up.

In papers II, III and IV, the modified Medical Research Council (mMRC) dyspnea scale was used to assess dyspnea.

3.3.5 STATISTICAL ANALYSES

Paper I

Statistical analyses in paper I were performed using IBM SPSS Statistics for Windows, version 25 (IBM Corp., Armonk, NY, USA). The significance level was set to p-value <0.05. Coefficient of repeatability (CR), intraclass correlation coefficient (ICC), and coefficient of variation (CV) were calculated to assess the intra-individual variability and repeatability of the method.

CR was calculated based on the difference between the measurements, i.e. 1.96 × SD (standard deviation of the differences by subtracting a later from an earlier measurement). As the CR calculation assumes normal distribution of the differences, the distribution was assessed by using histograms and the comparison of mean and median values. SP-A and albumin differences were normally distributed, but the distribution of the particle number concentration differences was skewed, so they were log transformed (lnPEx).

The two-way repeated ANOVA was tested for differences between different times of the day and between the days.

Within-day CR was calculated based on the differences between the three measurements for each day for each individual. Within-day ICC was calculated for each of the three days with several measurements separately and the average of the three was presented. Within-day CV was calculated for each individual using three days with three measurements each and the average within-day CV and SD are used for the calculation of CV (SD/mean).

Between-days variation was calculated based on all five days, but only noon measurements were used for the days with multiple measurements.

Papers II, III and IV

The limit of detection (LOD) was calculated for each protein analyte as the mean plus 3 standard deviations based on the blank samples. In paper II, all proteins with RFU values >LOD in more than 50% of the samples were included in further analyses. In paper III, proteins with RFU vales >LOD in more than 80% of the samples were selected and among them 53 coagulation and complement proteins were picked out and included in the further analyses. They were also subjected to group median based normalization in paper III to account for systematic differences. In paper IV, the correlation of the coefficient of

determination (R^2) was calculated first for each protein analyte based on the three dilution samples. Proteins with R^2 >80% and RFU values >LOD in more than 50% of samples were considered for further analyses.

Descriptive statistics on clinical and demographic data were analyzed using IBM SPSS Statistics for Windows, version 29 (IBM Corp., Armonk, N.Y., USA) with the significance level set to $p < 0.05$.

The statistical analyses of the protein and lipid data were performed with Qlucore Omics Explorer, using general linear model-based statistics (Qlucore AB, Lund, Sweden), and taking account for the repeated measurements in paper IV. The software version 3.6 was used in paper II, 3.7 in paper III and 3.8 in paper IV.

Protein and lipid data was $\log_2$ transformed before the analysis due to the skewness of the data, in order to achieve normal distribution. All the analyses in paper II were adjusted for the investigator performing the PExA sampling and the age of the subjects, and in paper III just for the age of the subjects.

Correction for multiple testing (q-value) was performed using the Benjamini-Hochberg method. Group comparisons of the protein and lipid data were considered hypothesis free and results with p-value <0.05 and q-value <0.20 (paper II) and q-value <0.30 (papers III and IV) were considered statistically significant.

In paper III, individuals with asthma were divided into tertiles based on the abundance of complement C3 and the differences in clinical data among the tertiles were assessed using the Kruskal-Wallis test with p-values adjusted by the Bonferroni correction for multiple tests.

4 RESULTS AND DISCUSSION

In this section, the results are presented and discussed in order to give a clearer overview and facilitate the comprehensive understanding of the results, especially in papers II-IV. For further details, please see the individual papers.

4.1 VARIABILITY AND REPEATABILITY OF EXHALED PARTICLES AND THEIR CONTENTS (PAPER I)

No significant differences between the morning, noon and afternoon measurements of particle number concentration, number of particles per breath and particle mean mass were observed (Table 2). This shows that the amount of particles exhaled appears to be unaffected by the time of day.

Table 2. Results of the within-day measurements. Particle number concentrations are expressed as n x 1000 per litre of exhaled air (kn/L) and per breath (kn/breath).

	Mean (95% CI) or median* (interquartile range)		
	Morning	**Noon**	**Afternoon**
PEx (kn/L)*	12.8 (9.3–18.0)	14.7 (9.6–18.1)	14.7 (10.0–19.7)
lnPEx (kn/L)	2.6 (2.3–2.8)	2.6 (2.4–2.8)	2.7 (2.4–3.0)
PEx (kn/breath)*	40.7 (30.4–75.2)	49.8 (28.8–65.3)	54.9 (30.1–78.7)
lnPEx (kn/breath)	3.8 (3.5–4.1)	3.8 (3.6–4.1)	3.9 (3.6–4.2)
Particle mean mass (pg)*	0.46 (0.37–0.63)	0.42 (0.33–0.52)	0.42 (0.32–0.50)
Surfactant protein A (wt%)	4.5 (3.8–5.2)	5.1 (4.4–5.8)	5.1 (4.3–5.9)
Albumin (wt%)	5.4 (4.7–6.0)	6.7 (5.8–7.6)	6.7 (5.9–7.4)

Significant within-day variation was observed for SP-A and albumin. Levels of both proteins were lowest in the morning and increased towards noon (Figure 9). The increase in SP-A from morning to noon was small, 13%, and the magnitude of increase in albumin was slightly larger, 25%.

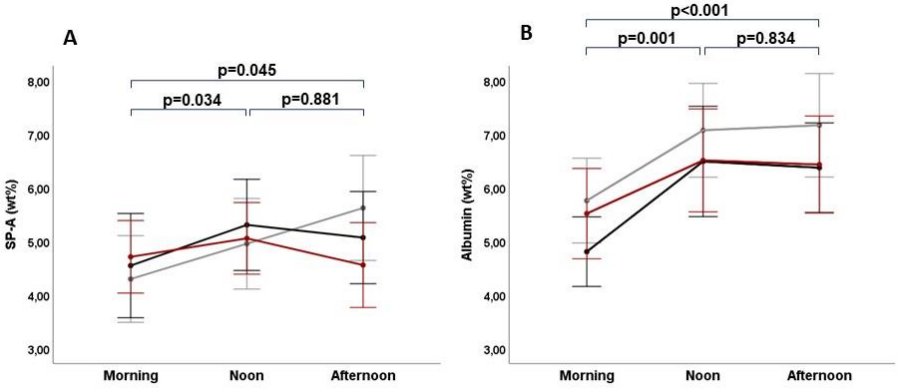

Figure 9. The within-day variation of SP-A and albumin. Each of the lines represents one of the three days with multiple measurements. Mean value and the corresponding 95% confidence intervals are displayed for each time point. P-values are based on the two-way ANOVA.

This shows that albumin, and to a smaller extent SP-A, exhibit diurnal variation. Other proteins that are secreted by respiratory epithelial cells that have been measured in serum, such as Surfactant protein D (SP-D) and Club cell protein (CC-16), also display diurnal variation[138,139]. These diurnal differences may, to some extent, be explained by the diurnal variation in the production and secretion of these proteins by the AT2 cells. Regarding albumin, it may be due to circadian regulation of epithelial tight junctions' permeability, which affects the transepithelial leakage and the amount of albumin in the RTLF[140,141]. The circadian rhythm is likely to affect all tissues and organs in the body and relates to the natural variations of wakefulness, blood pressure, body temperature, and hormone levels over a 24-hour cycle[142], all of which possibly affecting the composition of the RTLF. Time of the day should therefore be considered when dealing with repeated measurements as well as comparing differences in the composition of RTLF between groups.

The within- and between-days coefficients of repeatability, intraclass correlation coefficients and coefficient of variation are presented in Table 3.

Table 3. Coefficient of repeatability (CR) intraclass correlation coefficient (ICC) and coefficients of variation (CV) for the particle number concentrations, particle mean mass, SP-A, and albumin. CRs for particle number concentrations are calculated based on the logged values.

	Within-day			Between-days		
	CR	**ICC**	**CV (SD)**	**CR**	**ICC**	**CV (SD)**
Particle number concentration (per liter)	0.63	0.75	29% (14%)	0.85	0.5	27% (11%)
Particle number concentration (per breath)	0.72	0.75	33% (16%)	0.96	0.68	30% (12%)
SP-A (wt%)	2.5	0.64	18% (5%)	2.6	0.66	17% (7%)
Albumin (wt%)	2.8	0.55	19% (6%)	2.9	0.59	16% (6%)

The CR is useful when interpreting results from case-control studies or longitudinal studies with repeated measurements or following an intervention. Differences between the cases and controls or between different occasions should be larger than the reported CR. The CRs for SP-A and albumin are rather high with values higher than the differences observed in cross-sectional studies between healthy and COPD/asthma[41,143]. However, the CRs in our study were calculated based on sixteen individuals only and are, therefore, likely overestimating the real variation.

Differences between the within-day and between-day CR were small, which suggests that the levels of these variables remain somewhat stable over a longer period of time (up to two months). Furthermore, no important effect of the time interval between the measurements (up to 60 days) on the differences was seen. This shows that the variation of repeated measurements of exhaled particles and their contents does not depend on the time interval between the measurements. Similar has been observed for SP-D and CC-16 in serum, both shown to remain stable over a longer period of time[138,144,145].

Intraclass correlation coefficient ≥0.60 is generally considered clinically useful and indicates good reproducibility[146]. Particle number concentration, particle mean mass, and SP-A all showed good reproducibility, while within-day reproducibility

for albumin was lower, which could be explained by the diurnal variation of albumin mentioned above. Furthermore, particle number concentration per breath showed better long-term reproducibility than particle number concentration per liter. By assessing the ICC, the reproducibility of the exhaled particles as a method sampling the small airway RTLF was compared with other methods, such as BAL and sputum. The previously reported ICCs for SP-D and albumin measured in BAL and sputum[147,148] are similar as to the ICCs for exhaled particles, which suggests the reproducibility of exhaled particles is comparable to BAL and sputum.

By calculating the coefficient of variability, the variability of the method for collection of exhaled particles can be compared with other similar methods, i.e. the method used by Schwarz et al.[149], showing the intra-individual variability of the PExA method is comparable to other methods.

4.2 COMPOSITION OF THE SMALL AIRWAY LINING FLUID IN SMOKERS, ASTHMA AND COVID-19 (PAPERS II-IV)

In paper II, 203 proteins fulfilled inclusion criteria and were included in the analyses. Comparing our list of detected proteins with a previously published SOMAscan BAL proteomic data[150], a high degree of overlap was observed, with 89% of the proteins detected in exhaled particles being previously detected in BAL with SOMAscan. In order to study the potential effect of smoking on the detection rates, separate lists of proteins detected in current-, former- and never smokers were compared, and a significant overlap was observed among the groups. This also shows that it is unlikely that cigarette smoke causes changes in the protein structure that compromise the affinity of the SOMAmers.

In paper III, the specific 53 proteins that take part in the coagulation and complement pathways were selected to study local activation of the coagulation and complement cascades in small airways. The results on the differential abundance of all the proteins detected in exhaled particles in this asthma cohort have been presented previously by Östling et al.[151].

In paper IV, 46 proteins fulfilled inclusion criteria and were included in analyses. Proteins fulfilling inclusion criteria in each group (COVID-19 cases at baseline and follow-up, healthy controls) were compared to assess the effect of the viral infection on the detection rate. A major overlap in the proteins detected was seen and no major differences in protein detection rates were found between the

groups. Unfortunately, due to the lab error at SomaLogic, approximately one third of the samples (17 samples) were lost during the SOMAscan analysis. Proteins were therefore only analyzed in ten samples from controls, twenty-two samples from cases at baseline and seventeen from cases at follow-up. In individuals where enough material from small airways was collected (>80 ng of exhaled particles), samples were also analyzed with LC-MS/MS to study lipids. Lipid profiles were analyzed altogether in sixty samples: fifteen controls, twenty-three cases at baseline and twenty-two cases at follow-up. Ninety-five lipid species belonging to eight lipid classes were identified and included in the analyses.

4.2.1 CLINICAL CHARACTERISTICS OF THE COHORTS

Paper II

The cohort consisted of 22 never smokers (13 females), 38 current smokers (17 females) and 47 former smokers (25 females). Former and current smokers were significantly older than never smokers. Median (range) pack-years among current smokers was 39 (7-84), and 22 (1-64) among former smokers. Seventeen former smokers had post-FEV_1/FVC ratio <0.7, and among them twelve GOLD 1 grade of airflow obstruction and five GOLD 2. Among nine current smokers with post-FEV_1/FVC ratio <0.7, three were classified as GOLD 1 and six as GOLD 2. This shows former smokers had more airflow obstruction than current smokers, despite having less tobacco exposure than current smokers. Current smokers had significantly higher blood neutrophils count than former smokers, but no significant differences in plasma CRP levels were observed between the groups.

Paper III

The cohort consisted of 20 individuals with asthma (7 females) and 10 healthy controls. There were 11 former smokers in the asthma group and 3 among healthy controls. No significant difference in age was seen between the asthma group and controls. Individuals with asthma had significantly lower FEV_1 z-score, FEV_1/FVC ratio, $FEF_{75\%}$ z-score and $FEF_{25-75\%}$ z-score than healthy controls.

Eleven patients with asthma were receiving a combination of inhaled corticosteroids (ICS) and long-acting beta agonists (LABA), and five patients a combination of ICS and short-acting beta agonists (SABA). Four patients were receiving oral corticosteroids and six leukotriene receptor antagonists (montelukast).

Twelve individuals (4 females) were classified as having well-controlled asthma (ACQ ≤1), and eight (3 females) as having inadequately controlled asthma (ACQ >1). No significant differences in any of the clinical outcomes, e.g. lung function measured with spirometry and MBW, FENO, blood eosinophils, and use of medication, was observed between patients with well-controlled asthma as compared to inadequately controlled asthma.

Paper IV

The cohort consisted of 29 cases (17 females) with an ongoing SARS-CoV-2 infection at baseline and a median age of 39 years, of which 22 were re-examined at follow-up, as well as 17 sex- and age-matched controls. Among cases, 10 were former smokers, while only one control reported a history of smoking. There were two individuals with asthma among cases and one among controls.

Median days from symptom onset to first examination was 8, and 5 days from a positive PCR-test to examination. Median number of days between baseline and follow-up was 192. Dyspnea, defined as mMRC ≥2, was reported by twelve cases at baseline and only one at follow-up. Cases as baseline had significantly higher R_5, R_{5-20} and AX than the control group. This shows that even a mild SARS-CoV-2 infection can affect the function of small airways. The AX (both absolute value and z-score) decreased significantly from baseline to follow-up in cases, but the absolute values still remained higher at follow-up as compared to controls.

4.2.2 PROTEOMIC CHANGES

Significant differences were observed in the abundances of 120 proteins among current-, former- and never smokers, and a clustering analysis based on smoking status revealed that these proteins mainly distinguished current smokers from never smokers (Figure 10).

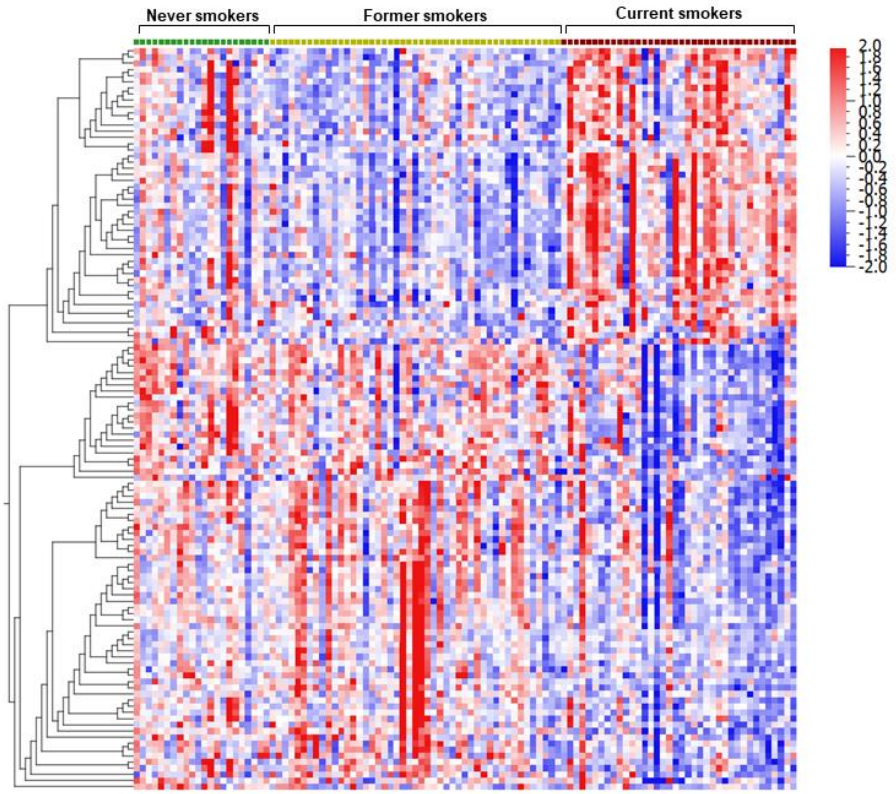

Figure 10. Clustering analysis of proteins based on smoking status in paper II.

Comparison of current smokers and never smokers revealed differential abundance of 81 proteins between the groups, with the majority of proteins being less abundant in current smokers. The proteins most clearly distinguishing current from never smokers were soluble receptor for advanced glycation end products (sRAGE), follistatin-related protein 3 (FSTL3), testican-2 (SPOCK2) and protein S (PROS1), all being less abundant in current smokers (Figure 11). sRAGE has anti-inflammatory properties and decreased levels are suggestive of deficient inflammatory control[152]. Decrease in sRAGE is associated not only with smoking

and COPD[153-155], but also with various other diseases, i.e. cardiovascular diseases and diabetes[152,156]. FSTL3 is also a protein with anti-inflammatory properties[157], while SPOCK2 can modulate the expression and activation of matrix metalloproteinases[158], which are known to play a role in the development of COPD[159], but it also protects epithelial cells against influenza virus infection[160]. PROS1 has the ability to inhibit coagulation and inflammation and takes part in the clearance of apoptotic cells[161,162]. Evidence suggests that increased apoptosis accompanied by diminished clearance of apoptotic cells play an important role in many lung diseases, including COPD and asthma[163]. Decrease in levels of PROS1 in current smokers may lead to decreased clearance of apoptotic cells, resulting in airway inflammation. Together with the downregulation of proteins with anti-inflammatory properties in the RTLF that protect against tissue damage, these changes may promote inflammation in the small airways in relation to smoking, resulting in small airway dysfunction.

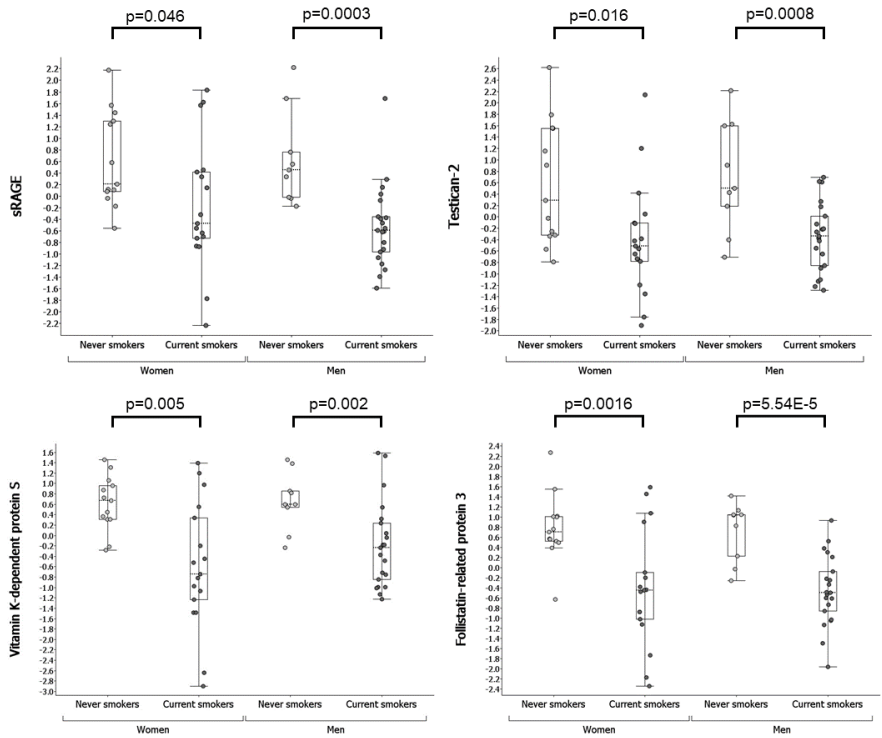

Figure 11. Abundance of sRAGE, testican-2, follistatin-related protein 3 and protein S in never and current smokers. Y-axis shows normalized abundance of protein levels (log_2 transformation and normalization to mean 0 and variance 1). Box ranges from the 25th to the 75th percentile and median value is marked with dotted line. p-values from pairwise comparisons are shown over each box plot.

Among the few proteins that were upregulated in current smokers were heat shock protein 90 (HSP90) and translationally-controlled tumor protein (TCTP). Both proteins participate in the "unfolded protein response" (UPR)[164,165], of which main function is to reduce accumulation of aberrantly folded proteins due to oxidative stress that could cause cell damage[166]. This supports the notion that the oxidative stress caused by smoking leads to structural changes in the proteins and causes abnormal folding, which in turn leads to increased expression of UPR-proteins in order to decrease tissue damage.

Comparison of former smokers and never smokers revealed differential abundance of 31 proteins, with all proteins less abundant in former smokers. After correction for multiple testing ($q < 0.20$), fourteen proteins were significantly abundant in former smokers. Proteins differentially abundant in current smokers were also compared to those differentially abundant in former smokers to study whether the changes caused by active smoking persist after smoking cessation. Four proteins (Macrophage mannose receptor 1, MRC1; Complement-decay accelerating factor, CD55; Interleukin-1 receptor-like 1, ST2; Adenylate kinase isoenzyme 1, AK1) remained significantly downregulated in former smokers, and twelve proteins that were upregulated in current smokers were downregulated in former as compared to never smokers. Several of the proteins that were downregulated in current smokers also positively correlated with the time since smoking cessation in former smokers. This indicates that the majority of changes that are caused by active smoking are reversible after smoking cessation, which has been observed previously as well[31]. However, former smokers remain at increased risk for the development of COPD even after smoking cessation. The persistent changes in MRC1, CD55, ST2, AK1 might play a role in the pathogenesis of COPD.

Based on the observed proteomic changes in current smokers, the complement system was identified as the pathway most affected by active smoking, particularly in women, and a model of smoking-induced small airway inflammation was developed, as shown in Figure 12.

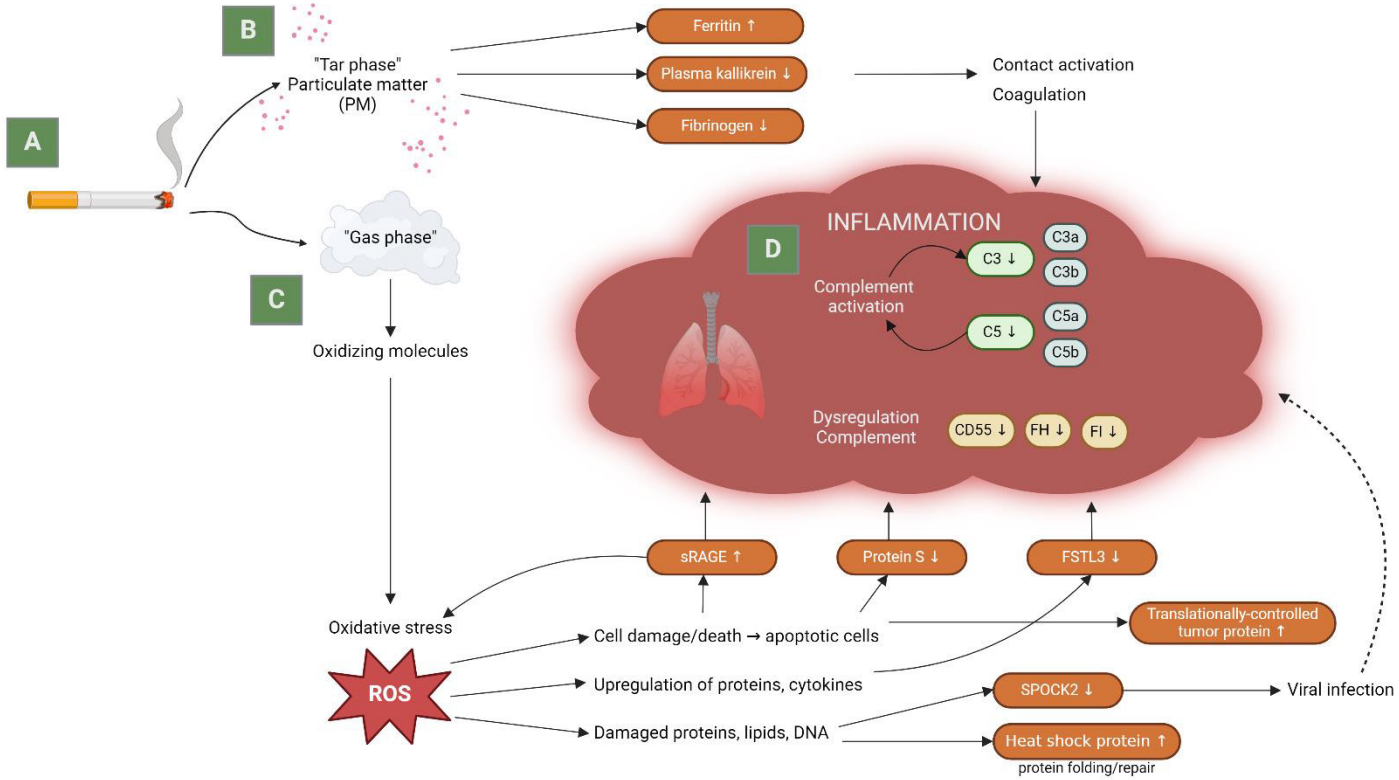

Figure 12. A model of the effects of cigarette smoke on the small airway protein profile. Created with BioRender.com.

In patients with asthma (paper III), several proteins were identified as differentially abundant, indicating activation of complement and coagulation systems. Individuals with asthma had higher levels of alpha-2-antiplasmin (SERPINF2), protein kinase C alpha type (PRKCA), cAMP-dependent protein kinase catalytic subunit alpha (PRKACA), coagulation factors IX and X (FIX, FX) and angiostatin (PLG) than healthy controls, as shown in Figure 13. Among these proteins, only SERPINF2 and FIX were analyzed in paper II, however, no significant effect of smoking status on these two proteins was seen.

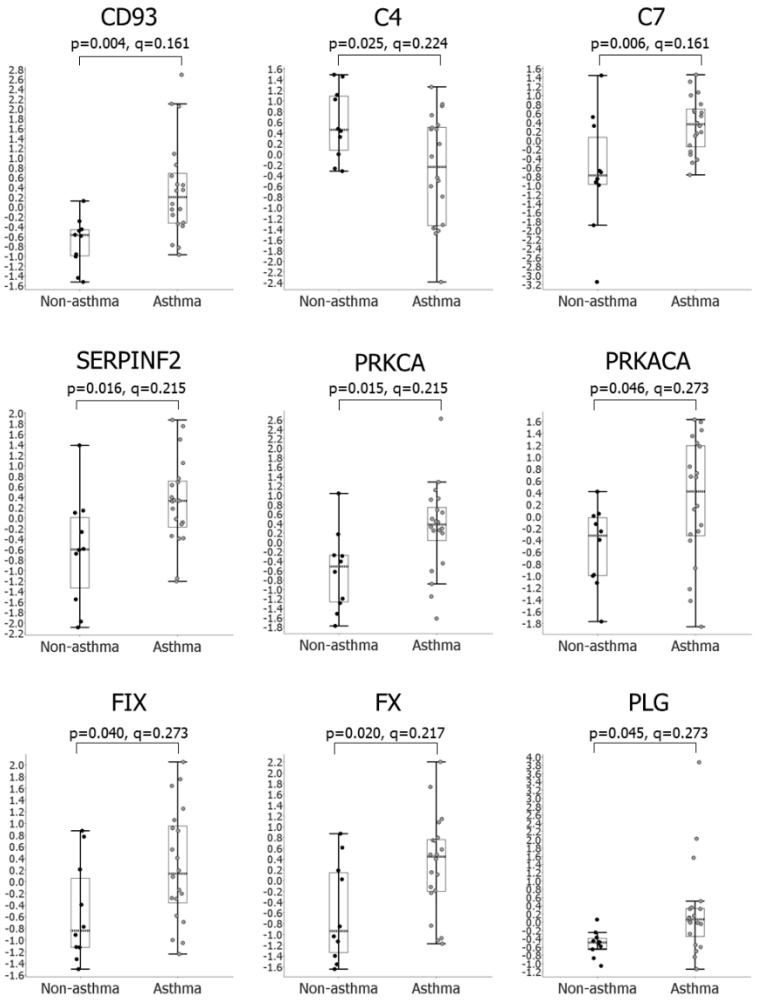

Figure 13. Differentially abundant proteins in asthma. y-axis shows normalized abundance of protein levels (log_2 transformation and normalization to mean 0 and variance 1). Box ranges from the 25th to the 75th percentile, and median value is marked with dotted line.

In paper IV, no differentially abundant proteins were found between patients with COVID-19, both at the acute stage and also at follow-up, and controls. However, levels of several proteins changed during the course of the acute disease, i.e. ephrin-A1, which plays a role in inflammation and regulates endothelial permeability, was highest at the beginning of COVID-19. This may partly explain why no differences between cases and controls were seen, as disease duration at the time of sampling varied among the cases.

Several coagulation proteins, i.e. protein S (PROS1), apolipoprotein E (APOE) and von Willebrand factor (VWF), were also associated with asthma control. Interestingly, patients with poorer asthma control had higher levels of PROS1 than controls, while current smokers had lower levels of PROS1 than healthy never smoking controls. PROS1, a protein with anticoagulant, anti-inflammatory and anti-apoptotic properties, is believed to have a protective role in asthma[167], acute lung injury[168] and pulmonary fibrosis[169]. PROS1 deficiency in RTLF of smokers suggests decreased protective mechanisms in smokers which could contribute to the development of COPD, and therefore PROS1 may represent a potential therapeutic target for COPD.

Proteomic changes and small airway function

Significant correlation between proteins alpha-1-antitrypsin (SERPINA1) and angiostatin (PLG) and LCI, S_{cond}, S_{acin} and $FEF_{25-75\%}$ z-scores was observed in paper III. Levels of coagulation factor FX, von Willebrand factor and immunoglobulin M also correlated with LCI, S_{acin} and $FEF_{25-75\%}$, but not with S_{cond}. In patients with an ongoing mild SARS-CoV-2 infection (paper IV), levels of serotransferrin (TF), prothrombin (F2), complement factor I (CFI), hemopexin (HPX), complement C4b (C4b), vitamin D-binding protein (GC), thyroxine-binding globulin (SERPINA7), complement factor B (CFB), beta-2-glycoprotein 1 (APOH) and neurotrimin (NTM) increased significantly with increasing R_{5-20} z-score, as shown in Figure 14.

Despite the use of different methods to assess lung function in patients with asthma and COVID-19, quite a few coagulation factors were associated with different indices of small airway dysfunction in both studies, suggesting the activation of the coagulation cascade plays an important role in the small airway remodeling. Procoagulant activity leads to fibrin deposition which in turn can lead to alveolar epithelial injury[170]. The coagulation system is also tightly linked with the complement system, as they are capable of activating each other, which causes simultaneous activation of both cascades in most pathophysiological situations[171].

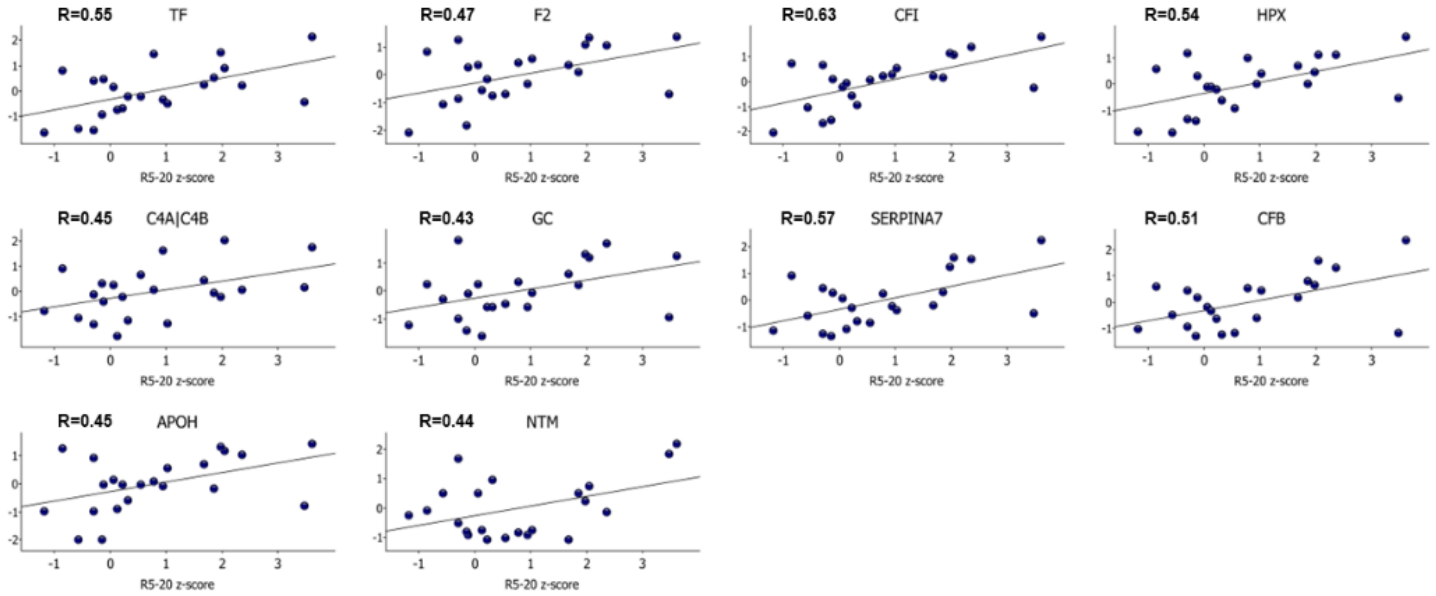

Figure 14. Correlation between protein levels and the $R_{5\text{-}20}$ z-score among patients with an ongoing mild SARS-CoV-2 infection. (TF, Serotransferrin; F2, Prothrombin; CFI, Complement factor I; HPX, Hemopexin; C4A/C4B, Complement C4b; GC, Vitamin D-binding protein; SERPINA7, Thyroxine-binding globulin; CFB, Complement factor B; APOH, Beta-2-glycoprotein 1; NTM, Neurotrimin)

4.2.3 COMPLEMENT SYSTEM

Exhaled particles originating from small airways are abundant with proteins associated with the innate immunity, and especially the complement and coagulation systems, as shown by Östling et al.[24]. Similar has been observed in paper II, where many of the proteins that have been detected in exhaled particles were associated with the complement system. The complement system was also identified as one of the biological pathways most affected by active smoking, especially in women. Several complement factors were differentially abundant in female current smokers as compared to never smokers, but only a few of these differences were observed in male current smokers. Downregulation of C3, C5, C2, C6, C7 and C9 as well as factor D, H, I and complement decay-accelerating factor (CD55) were observed in female current smokers, as shown in Figure 15. CD55 was less abundant in male current smokers as well (Figure 15).

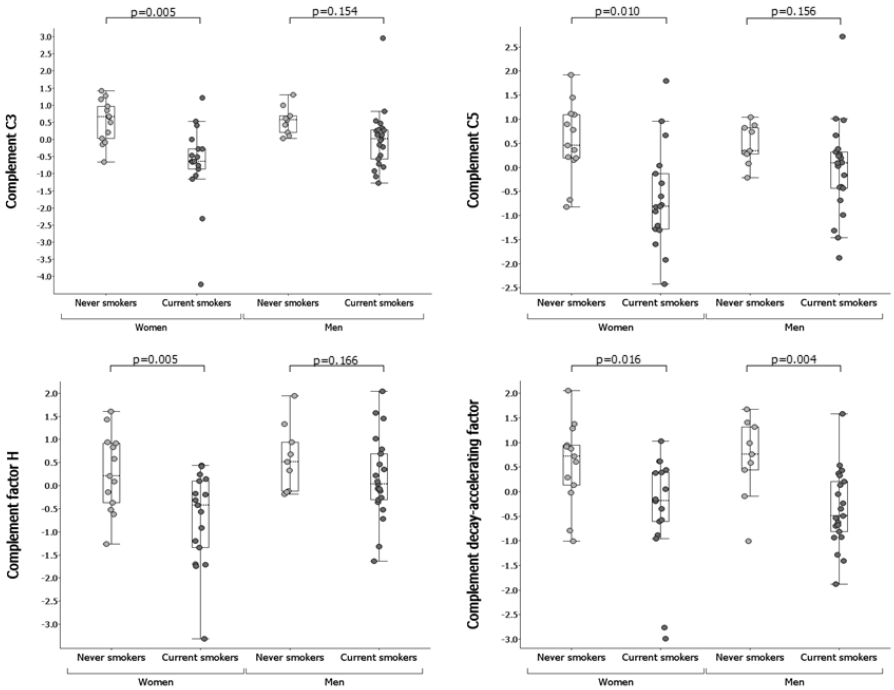

Figure 15. Abundance of complement factors C3 and C5, factor H and decay-accelerating factor (CD55) in never and current smokers. Y-axis shows normalized abundance of protein levels (log_2 transformation and normalization to mean 0 and variance 1). Box ranges from the 25th to the 75th percentile and median value is marked with dotted line.

Changes in several proteins that promote inflammation were observed in current smokers and based on these findings, it seems that the complement system might play a central role in the inflammatory response to smoking, as proposed in the model in Figure 12. Downregulation of regulatory proteins could lead to excessive complement activation, resulting in depletion of complement factors C3 and C5, which may represent an important initiating step in the pathogenesis of small airway inflammation.

An important role of local complement activation in small airways was observed in asthma as well and was associated with asthma control. Upregulation of CD93 (C1q receptor) and complement component C7, and downregulation of C4 was seen in patients with asthma (Figure 13). Furthermore, patients with inadequately controlled asthma had higher levels of C3 than patients with well-controlled asthma (Figure 16). Significant positive correlation was also observed between the ACQ score and levels of C3, C3b and C4b.

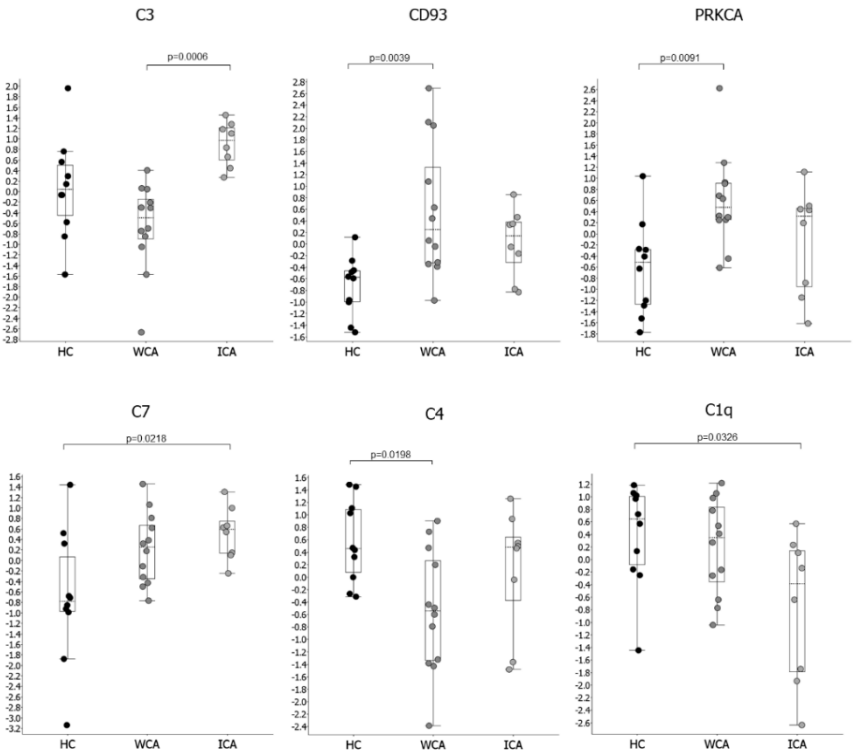

Figure 16. Differentially abundant proteins in well-controlled asthma (WCA) and inadequately controlled asthma (ICA). y-axis shows normalized abundance of protein levels (log_2 transformation and normalization to mean 0 and variance 1). Box ranges from the 25th to the 75th percentile and median value is marked with dotted line. Tukey's post-hoc test was used for pairwise comparisons.

Complement activation and small airway function

Complement components C4, C5b-C6 complex and CD93 (C1q receptor) correlated significantly with the physiological tests to measure small airway function in paper II. Small airway dysfunction, assessed by the increase in LCI, S_{cond}, S_{acin} z-scores and the decrease in $FEF_{25-75\%}$ z-score, was associated with lower levels of C4 and higher levels of CD93 and C5b-C6 complex. These results suggest that there is a local activation of the complement system in the small airways, causing consumption of C4 and production of the C5b-C6 complex, and that this leads to the small airway dysfunction.

During inflammation, levels of the complement factor C3 can change in two directions. C3 is an acute phase reactant, and its levels can increase during inflammation. Given that the cleavage of C3 leads to the production of C3a and C3b, increased levels of C3 may be fueling the complement activation and promoting inflammation, since C3a is a potent pro-inflammatory mediator. Patients with asthma and high levels of C3 in the RTLF appear to have a higher degree of small airway dysfunction, less asthma control, and higher levels of plasma CRP (Figure 17). Additionally, there was a correlation between C3b and asthma control. These findings further strengthen the hypothesis about the importance of complement activation in small airway inflammation.

However, since the complement activation leads to the cleavage of the C3 component, during inflammation, levels of C3 can also decrease due to over-activation and consumption of the complement system, and even patients with low levels of C3 appear to have a higher degree of small airway inflammation (Figure 17). Similarly, decreased levels of C3 were observed in current smokers as well. This suggests there may be different underlying mechanisms that lead to complement activation and inflammation in asthma and smokers.

In patients with an ongoing SARS-CoV-2 infection, levels of complement factor B and I and factor C4b positively correlated with the R_{5-20} z-score. Complement factor B is a key component of the alternative pathway and has been previously associated with COVID-19 severity[172], and factor I is an important inhibitor of the complement activation. An increase in C4b in association with increasing small airway resistance in patients with COVID-19 would also suggest that the complement activation caused by the SARS-CoV-2 infection leads to the inflammation in small airways.

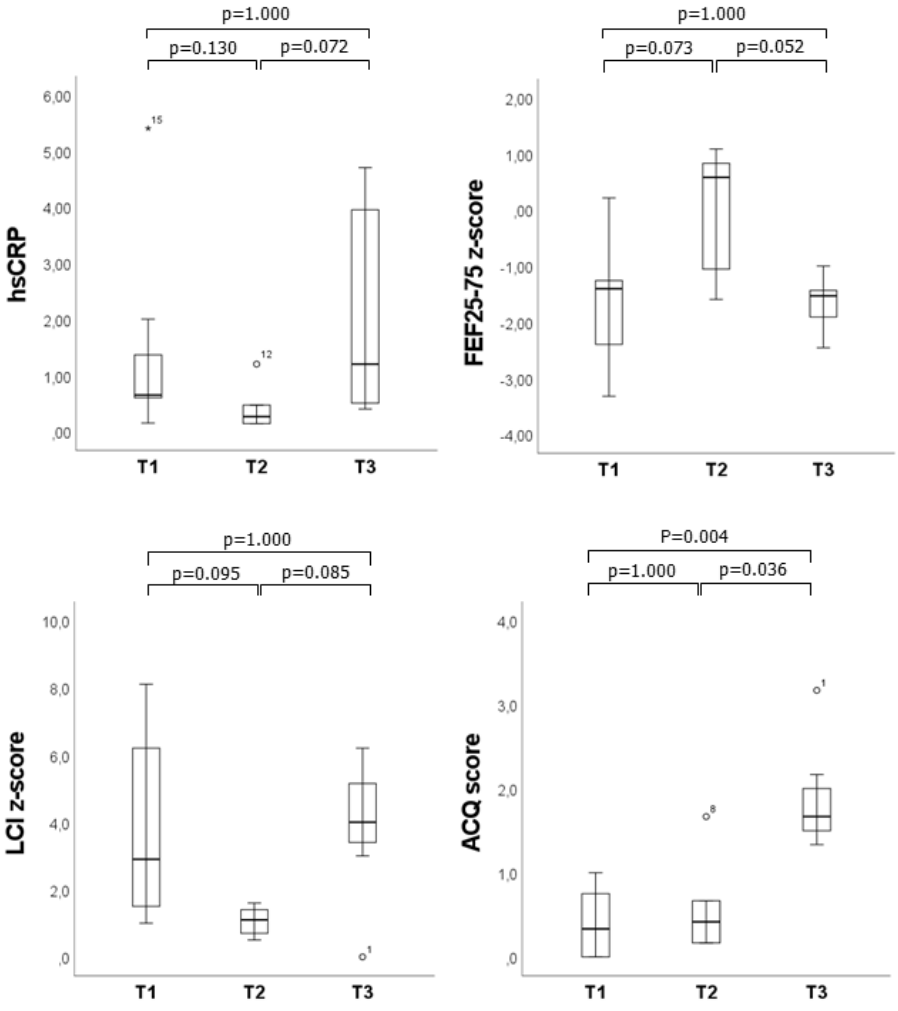

Figure 17. Boxplots of hsCRP (mg/L), $FEF_{25–75\%}$ z-score (post-bronchodilation), LCI z-score, ACQ score in subjects with asthma divided into tertiles based on the levels of complement C3 in the small airway lining fluid (T1, lowest; T2 median; T3, highest levels). p-values have been adjusted by the Bonferroni correction for multiple tests.

Effects of circadian rhythm

As sampling time was shown to influence the levels of albumin and SP-A, the effect of sampling time was studied in papers II-IV. It was separated into morning (8:00-10:30), noon (11:00-14:00) and afternoon (14:30-18:00) measurements. Protein abundances were compared between these times of the day and the analyses were adjusted for either the presence of asthma or COVID-19 and smoking status in respective papers. In paper II and III, no significant differences were seen between morning, noon, and afternoon measurements. In paper IV, significant differences in low affinity immunoglobulin gamma Fc region receptor III-B (FCGR3B) and serotransferrin between noon and afternoon measurements were observed. However, adjusting the initial analyses, i.e. the differences between the groups (cases at baseline/follow-up vs controls), for the time of sampling did not change the results, as still no significant differences in protein profiles between the groups could be seen.

4.2.4 SEX-SPECIFIC DIFFERENCES

The underlying mechanisms by which smoking leads to the development of COPD may differ between women and men[173-176], and women might be more susceptible to the damaging effects of tobacco smoke[177]. The notion that women are more susceptible for COPD is supported by the evidence that several studies have reported over the years, i.e. there is a higher prevalence of women among patients with severe, early-onset COPD[178], female smokers have an accelerated annual decline in FEV_1 % predicted as they age as compared to men[179], as well a greater reduction in FEV_1 per pack-year[180]. The reason for this is, however, still unknown, but several different explanations have been proposed. Hormonal differences between women and men could be one of the reasons for the increased susceptibility in women, as sex hormones can inflect oxidative stress and alveolar regeneration[181]. Furthermore, it has been proposed that the smaller size of the female lung could lead to a greater exposure of cigarette smoke from each cigarette[182]. Silverman and colleagues have also shown that a genetic susceptibility plays an important role in these sex-related differences[178].

In paper II, the study was not designed to investigate the differences between men and women and the primary aim of the study was only to look at the effects of smoking on the composition of RTLF. However, when analyzing the data, it became clear that sex is an important determinant which needs to be accounted for. Significant differences in the effect of smoking on the RTLF proteome were observed between male and female current smokers (Figure 18). In an analysis stratified for sex, significant differences were found in the abundance of 58 proteins between female current and never smokers, while only 8 proteins were differentially abundant between male current and never smokers. Proteins belongings to the complement system were among the ones most strongly affected by smoking in women, but this was not observed in men. As over-activation of the complement system is known to contribute to inflammation, this may be one of the reasons that could explain the increased susceptibility to COPD in women.

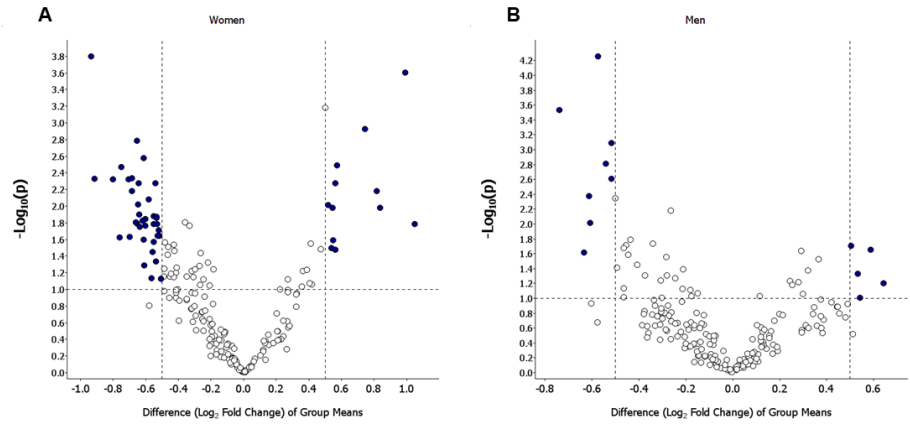

Figure 18. Volcano plots showing differentially abundant proteins in female (A) and male (B) current smokers as compared to never smokers.

COPD is not the only lung disease where sex is an important determinant that needs to be considered. There are significant differences in asthma prevalence and severity between women and men as well[47]. In children, asthma is more common among boys, but this turns in adulthood when asthma becomes more common in women[47]. These differences cannot be explained just by sex as a biological determinant, but can also be gender-related, as sociocultural and environmental determinants plays an important role, just like in COPD[47,183]. Moreover, in asthma, just like in COPD, women have a higher likelihood to develop a more severe form of the disease[184]. Women with asthma also tend to be less atopic than men, respond poorer to corticosteroid treatment and are more obese[47]. Opposite to COPD and asthma, in COVID-19 men are the ones that tend to develop a more severe form of the disease and have a higher mortality as compared to women[185]. Important differences in the immune responses during the early stage of the SARS-CoV-2 infection have been found between men and women[185].

Our results together with the previous studies emphasize the importance of considering sex-differences when searching for biomarkers and biological pathways in lung diseases, such as COPD, asthma, and COVID-19. Unfortunately, due to a small sample size sex-specific differences in papers III and IV were not investigated. A larger study designed to explore the sex-specific differences in the composition of the RTLF and how these differences contribute to the development of various diseases in men and women would be of interest.

4.2.5 LIPIDOMIC CHANGES IN COVID-19

In patients with an ongoing SARS-CoV-2 infection (baseline, visit one), hexosylceramide (HexCer) and phosphatidylcholine (PC) classes showed a trend to be more abundant as compared to healthy controls. Differences in lipid classes became more prominent in these individuals during follow-up, where a significantly higher abundance of PC and lower abundance of phosphatidylethanolamines (PE) was seen as compared to controls. Additionally, PE classes decreased significantly from baseline to follow-up in these individuals.

When comparing lipid species, thirteen were found to be differentially abundant between COVID-19 cases at baseline and controls. Similarly, as with the lipid classes, the differences became more prominent at follow-up, when twenty-four lipid species were differentially abundant between the cases and controls. Analysis of repeated measures showed that the levels of twenty lipid species changed between baseline and follow-up in COVID-19 cases. Results are shown in Table 4. This shows that even a mild SARS-CoV-2 infection exerts significant effects on the pulmonary surfactant and small airway lipid profile. However, it is interesting that these changes were found to be most prominent months after the acute infection. These suggests that even a mild infection can lead to long-term changes in the composition of the pulmonary surfactant, which may be one of the reasons for the persistency of symptoms seen in some patients. Proportions of oxidized lipids were increased both during an acute infection but also at follow-up. They are produced during oxidative stress and can accumulate in inflamed tissues, where they have a pro-inflammatory role[186]. They also have the ability to induce coagulation as they can modulate the expression of coagulation factors[186]. Increased lipid oxidation during COVID-19 may therefore also contribute to the COVID-19-related thromboinflammation.

Additionally, a decrease in lipid species with an 18:2 component, i.e. linoleic acid, which is a polyunsaturated fatty acid and is considered pro-inflammatory, was seen in COVID-19. Linoleic acid is a precursor to arachidonic acid, which is a precursor to inflammatory mediators, such as prostaglandins and leukotrienes[187]. A decrease in lipid species with the 18:2 component may indicate consumption due to production of inflammatory mediators.

Due to difference in the disease duration in the individuals with COVID-19 at the time of sampling, the correlation between the days since symptom onset and the levels of lipids was tested. Twenty-three lipid species were correlated with the time since symptom onset. Those with the correlation coefficient (R) >0.60 are shown in Figure 19. As a similar pattern was observed for proteins, this shows that the composition of the respiratory tract lining fluid changes significantly during the course of an acute disease.

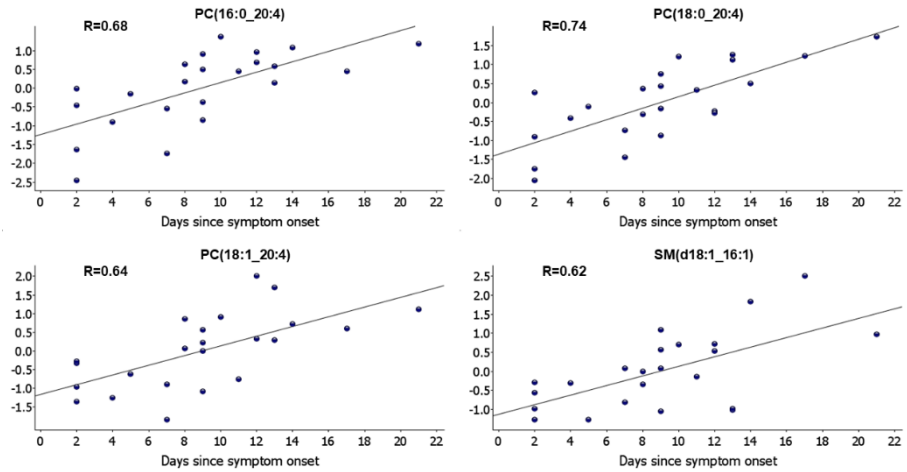

Figure 19. Correlation between the relative amounts of lipid species and the number of days between the symptom onset and sampling in cases with COVID-19.

Effects of circadian rhythm

To study whether different sampling times during a day had effect on the relative levels of lipid species, sampling time was noted and separated into three different groups, morning (8:00-10:30), noon (11:00-14:00) and afternoon (14:30-18:00). Levels of lipid species were compared in the context of the three different times of the day with the adjustment for the COVID-19 status (controls, and cases: baseline/follow-up). There were no significant differences in lipid species with regard to the sampling time.

Table 4. Differentially abundant lipid species in COVID-19 cases at baseline and at follow-up as compared to healthy controls.

	COVID-19 at baseline vs Healthy controls			COVID-19 at follow-up vs Healthy controls			COVID-19 at baseline vs at follow-up		
Lipid species	**p-value**	**q-value**	**Difference**	**p-value**	**q-value**	**Difference**	**p-value**	**q-value**	**Difference**
PC(14:0_18:1)	0.319	0.705	-0.04	0.033	0.155	-0.08	0.016	0.207	-0.04
PC(14:0_18:2)	0.035	0.266	-0.02	0.048	0.190	-0.02	0.969	0.986	0.00
PC(15:0_16:0)	0.186	0.618	0.09	0.022	0.126	0.15	0.369	0.558	0.03
PC(16:0_8:0;O)	0.003	0.192	0.01	0.060	0.205	0.01	0.600	0.740	0.00
PC(16:0/16:0)	0.158	0.601	0.06	0.023	0.126	0.09	0.030	0.207	0.05
PC(16:0_17:0)	0.512	0.771	0.03	0.025	0.131	0.13	0.046	0.221	0.05
PC(16:0_18:1)	0.943	0.977	0.00	0.290	0.552	-0.07	0.036	0.213	-0.09
PC(16:0_18:2)	0.036	0.266	-0.12	0.003	0.051	-0.21	0.096	0.318	-0.08
PC(16:0_18:3)_2*	0.687	0.907	-0.01	0.043	0.181	-0.03	0.015	0.207	-0.03
PC(16:0_20:4)	0.428	0.767	-0.02	0.059	0.205	-0.04	0.029	0.207	-0.03
PC(16:0_20:5)	0.015	0.192	-0.01	0.406	0.653	-0.01	0.220	0.499	0.01
PC(16:0_22:6)	0.016	0.192	-0.01	0.310	0.577	0.00	0.129	0.365	0.00
PC(O-16:0/16:0)	0.018	0.192	0.03	0.087	0.253	0.02	0.142	0.375	-0.02
PC(P-16:0/16:0)	0.007	0.192	-0.04	0.091	0.253	-0.03	0.624	0.759	0.01
PC(16:1_18:0)	0.946	0.977	0.00	0.214	0.452	-0.02	0.029	0.207	-0.03
PC(16:1_18:2)	0.095	0.476	-0.02	0.015	0.121	-0.03	0.100	0.318	-0.01
PC(16:0_7:0;O)	0.027	0.229	0.04	0.004	0.054	0.06	0.112	0.336	0.02
PC(16:0_9:0;O)	0.009	0.192	0.22	0.002	0.051	0.26	0.271	0.514	0.07
PC(18:0_18:1)	0.477	0.771	-0.03	0.187	0.412	-0.07	0.041	0.217	-0.06
PC(18:0_18:2)	0.009	0.192	-0.11	0.003	0.051	-0.14	0.096	0.318	-0.04
PC(18:0_20:4)	0.150	0.594	-0.01	0.041	0.181	-0.02	0.205	0.485	-0.01

PC(18:1_18:2)	0.109	0.517	-0.11	0.015	0.121	-0.17	0.250	0.505	-0.06
PC(18:1_20:4)	0.145	0.594	-0.01	0.006	0.054	-0.01	0.098	0.318	-0.01
PC(18:2/18:2)	0.025	0.229	-0.09	0.018	0.126	-0.10	0.795	0.853	0.01
PC(18:0_9:0;O)	0.052	0.355	0.04	0.020	0.126	0.04	0.732	0.814	0.01
PE(16:0_16:1)	0.404	0.767	0.00	0.438	0.667	0.00	0.026	0.207	-0.01
PE(16:0_18:1)	0.675	0.903	-0.01	0.044	0.181	-0.03	0.008	0.207	-0.03
PE(16:0_18:2)	0.961	0.977	0.00	0.000	0.016	-0.03	0.015	0.207	-0.02
PE(16:1_18:1)	0.737	0.922	0.00	0.021	0.126	-0.01	0.010	0.207	-0.01
PE(18:0_18:1)	0.652	0.900	-0.01	0.286	0.552	-0.02	0.038	0.213	-0.02
PE(18:0_18:2)	0.016	0.192	-0.02	0.003	0.051	-0.03	0.067	0.279	-0.01
PE(18:0_20:4)	0.638	0.900	0.00	0.269	0.532	0.00	0.005	0.207	-0.01
PE(18:1/18:1)	0.241	0.651	-0.03	0.005	0.054	-0.09	0.016	0.207	-0.07
PE(18:1_18:2)	0.016	0.192	-0.04	0.001	0.028	-0.07	0.087	0.318	-0.03
PE(18:1_20:0)	0.413	0.767	0.00	0.005	0.054	-0.02	0.017	0.207	-0.01
PG(18:0_20:4)	0.180	0.618	-0.01	0.029	0.143	-0.01	0.022	0.207	-0.01
PI(16:0_18:1)	0.360	0.760	0.05	0.668	0.824	-0.02	0.028	0.207	-0.05
PI(18:0_20:4)	0.964	0.977	0.00	0.137	0.318	-0.02	0.037	0.213	-0.02
SM(32:2;O2)	0.083	0.456	0.00	0.664	0.824	0.00	0.047	0.221	0.00

***When there are isomers with results in two peaks, the second peak is annotated with an ending of _2.**

Difference is calculated based on the $\log_2$ data.

5 STRENGTHS AND LIMITATIONS

Paper I

The major strength of paper I is the high number of repeated measurements for each study participant, as exhaled particles were collected from each individual on eleven different occasions. Another important strength is that all the collections were performed by the same person, which eliminates the potential variation that would occur between different investigators. However, an important limitation of the study is the small number of subjects, which is probably one reason for the high coefficient of repeatability reported. Moreover, the time interval between the measurements was not the same for all the individuals, which could also be considered a limitation.

Papers II-IV

This thesis shows that the collection and analysis of exhaled particles allow for studying the proteomic and lipidomic changes in the composition of the RTLF, enabling the characterization of the inflammatory profiles in the small airways and giving us new insights about the underlying pathogenesis of different pulmonary diseases. Hence, these findings offer a step closer to achieving precision medicine in lung diseases.

The non-invasive nature of the method for the collection of small airway proteome and lipidome is considered a major strength of this thesis, as previously this has mainly been done by the use of invasive BAL fluid. The non-invasive nature of the method also allowed us to study individuals with a mild COVID-19 infection on two occasions, something that would have been much more difficult if an invasive method would have been used. By using exhaled particles, where the number and mass of the sampled material is known, we are able to avoid the problem of unknown dilution, which is an important drawback in BAL, induced sputum and EBC.

However, in all three studies the sample size was small, and an independent validation cohort was lacking. As many variables are being tested in omics studies, and if the sample size is small, there is a higher likelihood that some results are significant due to chance. In order to account for multiple testing, q-values were reported and used to interpret the results.

As reported in paper I, SP-A and albumin levels are subjected to diurnal variation, but not the amount of exhaled particles. Sampling time was not accounted for in papers II-IV as individuals were examined at different times of the day. The samples in papers II and III were collected before the study in paper I was even started, and in paper IV, due to a global pandemic and examining highly infectious individuals, it was not possible to examine all the study participants at the same time of the day. However, differences between different times of the day were tested for in all three papers and no significant effect of diurnal rhythm was observed on the protein and lipid profiles.

Furthermore, in papers II and IV, proteins with RFU values >50% in more than half of all samples were included, which could be considered another possible limitation. This was done due to the explorative nature of the study and choosing a lower detection limit enables us to study proteins that would have otherwise been missed. In paper IV, dilution samples were also analyzed, and the coefficient of determination (R^2) calculated for each protein based on their dilution and only proteins with R^2 >80% were included in further analysis which could be considered a strength. SOMAscan analysis is not optimized for exhaled particles with very little biological material collected (in the range of nanograms). It is mainly used for the analyses of blood samples. Therefore, there is a much smaller number of proteins that were detected in our samples as compared to all the proteins analyzed (approximately 1300 in paper II and 7000 in paper IV). SOMAscan data has also been normalized to the total amount of exhaled particles as all samples were diluted to contain the same particle number concentration. Other possible ways to normalize the data were considered, e.g. normalization based on the total protein mass or to a housekeeping protein. However, due to a small amount of biological material in our samples, measuring the total protein mass would be difficult, and knowledge about housekeeping proteins is still limited due to PExA being a rather novel method. There is also difficult to find a protein with stable levels that are not affected by a pathological condition.

Furthermore, it would have been preferable to use a more specific method for the analysis of complement proteins in our studies, due to low affinity of SOMAmers for certain very important complement factor, such as C3a and C5a. A new method of analyzing the complement system in exhaled particles is now being tested, which could offer better understanding of the role the complement system plays in the airways.

Lastly, one of the major limitations with the SOMAscan analysis is that in paper IV, almost one third of our samples were destroyed due to a lab error at SomaLogic, which reduced the already small sample size even further. In paper

IV, major limitations were also the small amount of material collected from some individuals, as well as the varying time period from symptom onset to sampling and to follow-up between the individuals with COVID-19. Although all subjects were planned to be examined as soon as possible after symptom onset and a positive PCR test, and then again at follow-up after three months, that was difficult to achieve due to practical issues.

6 CONCLUSION

This thesis shows that non-invasive breath analysis, by the sampling the respiratory tract lining fluid from small airway in the form of exhaled particles, allows us to explore its composition and study the proteomic and lipidomic changes over time, related to different exposures and diseases. Exhaled particles together with high sensitivity protein and lipid analyses offer a promising platform for identification of biomarkers and novel drug targets for COPD, asthma, and COVID-19.

The intra-individual variability and repeatability of the composition of exhaled particles is comparable with other methods used to collect the biological material from small airways, such as BAL and induced sputum. Due to non-invasiveness of exhaled particle collection, they present an attractive alternative to other more invasive methods for studying the pathogenesis of small airway inflammation. The ability to use a safe and simple non-invasive method to study the changes in the small airway proteome and lipidome will facilitate achieving precision medicine.

The recognition of the importance of the complement system in small airway inflammation, both in asthma and smoking, contributes a piece of knowledge to the understanding of the mechanisms underlying the development of airway diseases and highlights its potential as a possible therapeutic target in small airway inflammation.

Additionally, the alterations in the small airway lipid profile demonstrate the significance of the surfactant homeostasis in respiratory infections. Although the role of the surfactant phospholipids in the pathogenesis of airway inflammation is not clear, they appear to be of importance and should be studied further.

Finally, due to the heterogeneity of lung diseases between women and men, it is imperative to consider sex differences when searching for biomarkers and studying biological processes that drive the inflammation associated with these diseases.

7 FUTURE PERSPECTIVES

Despite advances in the understanding of different endotypes in lung diseases in the recent years, there is still a large knowledge gap regarding the underlying inflammatory mechanisms driving these diseases.

The respiratory tract lining fluid, rich in proteins and lipids representing the first line host defense against inhaled materials, provides a valuable source for studying biomarkers, mechanisms and identification of novel therapeutic targets and offering a step closer to precision medicine.

When searching for biomarkers for airway diseases and the underlying biological mechanisms, the heterogeneity of these diseases needs to be considered. The importance of biological sex when studying the effects of smoking on the composition of the small airway lining fluid was observed. There are many other factors that should be considered as well, such as gender (the socio-cultural determinant), comorbidities and early-life exposure (the prenatal period and the growth phase), as all these factors have been shown to contribute to the development of asthma and COPD.

Cross-sectional studies are the most common studies used when studying the mechanisms for the development of COPD and asthma. However, a longitudinal approach that follows individuals through a longer period of time and can account for how exposures during different life stages contribute to the development of these diseases is needed in order to understand the phenotypes and endotypes of these diseases better.

This thesis is based on the first studies using exhaled particles and an omics approach to study the inflammation in small airways related to different disease. However, the nature of these studies was exploratory and therefore validation studies are necessary to confirm the findings and explore the role of the specific alterations further. Additionally, broadening the cohorts by including patients with different severities of COPD, asthma and COVID-19 would expand the understanding of the changes observed in this thesis.

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