Analysis of Endogenous Particles in Exhaled Air

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ABSTRACT

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Exhaled air contains non-volatile particulate material from the respiratory tract. The precise location in which exhaled particles are formed is unknown, and details on their chemical content are scarce. The aim of this work was to chemically characterize and to study the mechanisms of formation of endogenous particles in exhaled air.

A new instrument for counting and sampling particles in exhaled air by impaction was developed, as a part of this thesis, at the Department of Public Health and Community Medicine, Occupational and Environmental Medicine in collaboration with the Department of Chemistry, Atmospheric Science at the University of Gothenburg. In the first instance, exhaled particles were analyzed using time-of-flight secondary ion mass spectrometry (TOF-SIMS), which is a very sensitive technique for surface analysis. This method was also used to compare the composition of particles in exhaled air from subjects with asthma to that in healthy controls. Second, a method for the quantitative determination of glutathione was developed and applied in the analysis of exhaled particles and exhaled breath condensate. In parallel to chemical analysis, the hypothesis that particles are formed during the reopening of closed airways was tested by measuring particle number concentrations in the air exhaled by healthy volunteers performing different breathing maneuvers.

This is the first study involving chemical analysis of particles in exhaled air. TOF-SIMS analysis revealed that exhaled particles contain several phospholipids (phosphatidylcholine, phosphatidylglycerol and phosphatidylinositol). These lipids are characteristic of the pulmonary surfactant which is present in the respiratory tract lining fluid (RTLF) that covers the epithelium in the alveoli and the airways. Using this method, it was found that the TOF-SIMS spectra of the particles exhaled by healthy subjects differed from those of the particles exhaled by subjects with asthma. These differences were attributed to differences in the abundance of phosphatidylcholine and phosphatidylglycerol between the two groups. By using the newly-developed method for glutathione analysis, it was possible to demonstrate the presence of

glutathione in exhaled particles for the first time. The method was used to compare glutathione levels in exhaled particles to those in exhaled breath condensate; it was found that analysis of particles was more revealing in terms of the levels of glutathione in exhaled air. Studies of particle formation showed that deep exhalations to residual volume (RV) caused significantly higher concentrations of particles in the subsequent exhalation than did exhalations to functional residual capacity (FRC).This supports the theory that film rupture during airway reopening after airway closure is an important mechanism of particle formation.

The results of these studies show that particles in exhaled air can be sampled by impaction, that surfactant phospholipids and glutathione are part of their chemical composition, and that they are largely formed in the peripheral airways, where airway closure takes place.

List of abbreviations

BAL	bronchoalveolar lavage
CF	cystic fibrosis
COPD	chronic obstructive pulmonary disease
CP	closing point
CV	closing volume
EBC	exhaled breath condensate
ERV	expiratory reserve volume
FENO	fraction of exhaled nitric oxide
FEV_1	forced expiratory volume in 1 second
FRC	functional residual capacity
FVC	forced vital capacity
GSH	glutathione
GSHt	total glutathione
GSSG	glutathione disulfide
HPLC	high performance liquid chromatography
m/z	mass-to-charge ratio
NPM	N-(1-pyrene)-maleimide
OPC	optical particle counter
PA	phosphatidic acid
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PEx	particles in exhaled air
PG	phosphatidylglycerol
PI	phosphatidylinositol
RTLF	respiratory tract lining fluid
RV	residual volume
TCEP	*tris*(2-carboxyethyl)phosphine
TLC	total lung capacity
TOF-SIMS	time-of-flight secondary ion mass spectrometry

LIST OF PUBLICATIONS

The thesis is based on the following papers, which are referred to in the text by their Roman numerals (I-IV).

I. **Almstrand AC**, Ljungström E, Lausmaa J, Bake B, Sjövall P, Olin AC.
Airway monitoring by collection and mass spectrometric analysis of exhaled particles.
Anal Chem, 2009, 81 (2), 662-668.

II. **Almstrand AC**, Josefson M, Bredberg A, Lausmaa J, Sjövall P, Larsson P, Olin AC.
Phospholipids in exhaled particles from subjects with asthma and healthy controls.
Revised version submitted to Eur Respir J

III. **Almstrand AC**, Bake B, Ljungström E, Larsson P, Bredberg A, Mirgorodskaya E, Olin AC.
Effect of airway opening on production of exhaled particles.
J Appl Physiol, 2010, 108:584-588.

IV. **Almstrand AC**, Evert L, Olin AC, Bredberg A, Larsson P, Mirgorodskaya E.
Collection and determination of glutathione in exhaled air.
Manuscript.

Papers not included in the thesis:

Bredberg A, Gobom J, **Almstrand AC**, Larsson P, Blennow K, Olin AC, Mirgorodskaya E.
Exhaled endogenous particles contain lung proteins.
Submitted to Eur Respir J.

Larsson P, Mirgorodskaya E, Andersson L, Bake B, **Almstrand AC**, Bredberg A, Olin AC.
Surfactant protein A and albumin in particles in exhaled air.
Submitted to Eur Respir J.

Holmgren H, Ljungström E, **Almstrand AC**, Bake B, Olin AC.
Size distribution of exhaled particles in the range from 0.01 to 2.0 μm.0
J Aerosol Sci, 2010, 41 (5): 439-446.

Romieu I, Barraza-Villarreal A, Escamilla-Nuñez C, **Almstrand AC**, Diaz-Sanches D, Sly PD, Olin AC.
Exhaled breath malondialdehyde as a marker of effect of exposure to air pollution in children with asthma.
J Allergy Clin Immunol, 2008, 121 (4): 903-909.

Barregård L, Sällsten G, Andersson L, **Almstrand AC**, Gustafson P, Andersson M, Olin AC.
Experimental exposure to wood smoke: effects on airway inflammation and oxidative stress.
Occup Environ Med, 2008, 65 (5): 319-324.

Svensson S, **Isacsson AC**, Ljungkvist G, Torén K, Olin AC.
Optimization and validation of an ion chromatographic method for the simultaneous determination of sodium, ammonium and potassium in exhaled breath condensate.
J Chromatogr B Analyt Technol Biomed Life Sci, 2005, 814 (1): 173-177.

Contents

INTRODUCTION

Lung diseases including respiratory infections, chronic obstructive pulmonary disease (COPD), tuberculosis, and lung cancer are among the leading causes of death in the world[1]. Asthma and COPD, the two major airway diseases, are chronic and cause individual suffering as well as high costs for the society.

In both asthma and COPD there is a need for better methods to assess airway inflammation so that exacerbations and progress of disease can be prevented. In occupational settings it is of interest to screen large populations in order to find risk factors for occupational lung disease and susceptible individuals. In lung cancer and acute lung diseases, such as pneumonia and influenza, early detection can be crucial. Methods that enable longitudinal and repeated assessment of airway inflammation and biological monitoring are therefore highly needed. To be clinically useful, these methods should also not be inconvenient to the patient. As a non-invasive technique, breath analysis satisfies all of these criteria. However it needs further thorough validation in terms of sampling and analysis of potential biomarkers.

Asthma and COPD are diseases that are characterized by limited airflow and airway inflammation. Their diagnosis is primarily based on typical symptoms and the use of spirometry to measure lung function and bronchial hyperresponsiveness which are indirect measures of airway inflammation. Direct methods for the assessment of airway inflammation are based on analysis of bronchial biopsies, bronchoalveolar lavage (BAL) or sputum. These methods are invasive or semi-invasive, may induce inflammation and are consequently not readily used in clinical practice[2, 3].

Breath contains thousands of volatile and non-volatile compounds that derive from the respiratory tract[4, 5]. Some reflect what we ate and drank for dinner (such as methyl mercaptan, which can be derived from the garlic and ethanol from the alcoholic beverages). Others can provide information on the individual's general health – for example, the breath of individuals with diabetes contains unusually high levels of acetone, while the breath of patients with kidney failure is unusually rich in ammonia. Many of the volatile organic compounds (VOCs) present in breath are blood-borne and are transported to exhaled air via the blood-gas interface in the alveoli[6]. Changes in the levels of VOCs can therefore reflect systemic disorders associated with oxidative or metabolic stress and are not necessarily indicative of respiratory disease. Non-

volatile compounds, such as lipids and proteins, are also present in breath and are more likely to derive directly from the respiratory tract lining fluid (RTLF) that covers the epithelium of the alveoli and the airways. The analysis of non-volatile compounds in exhaled air may therefore provide clearer insights into the biochemical processes occurring in the airways.

Research on proteins, lipids and their metabolites in exhaled air as well as their potential application as biomarkers of airway inflammation is still in the discovery phase, mainly because of a lack of useful sampling methods. The method of choice for the quantification of non-volatiles involves the analysis of exhaled breath condensate (EBC), which is based on the condensation of exhaled air. The American Thoracic Society/European Respiratory Society task force compiled a report on EBC standardization in 2005 with recommendations and considerations for future research[7]. The major identified issues concerned elucidating mechanisms and sites of formation of particles, determination of dilution markers and the improvement of reproducibility.

Non-volatile substances, such as proteins and lipids, are transported to the exhaled air via different mechanisms than do volatile substances, and are most likely to reach the gas phase as components of particles that are formed in the airways from the RTLF. To understand the mechanisms of formation and reproducibility of particle formation it will be necessary to devote greater attention to these exhaled particles. The exact content of the particles is unknown and their matrix can be considered to be new, with the potential to provide numerous useful new biomarkers. This thesis describes applications of a new method for simultaneously counting and collecting exhaled particles originating from the airways.

BACKGROUND

THE AIRWAYS

The primary function of the airways is to facilitate gas-exchange, i.e. the process whereby oxygen is transported into the bloodstream and carbon dioxide is transported from the bloodstream into the surrounding air. The lungs can be described as consisting of a "conducting zone", that conducts gas to and from the gas exchange regions, and a "respiratory zone" in which gas exchange takes place. The conducting zone, which is important also in the filtering and conditioning of the gas, consists of the nose, mouth and trachea; the trachea divides into the right and left main bronchi. The bronchi divide into smaller bronchi and then into terminal bronchioles which branch into the respiratory zone. The respiratory zone consists of respiratory bronchioles, which become alveolar ducts, then alveolar sacs, and finally the alveoli[8]. A human lung has are around 500 million alveoli which cover an area of about 80 m^2 and represent more than 95% of the lung's total surface area[8, 9]. Weibel's generally accepted model consists of a symmetrical branching structure, from trachea to the alveoli, in all 24 generations[10], see Figure 1. Airways with an internal diameter of 2 mm or less are usually called "small airways" or "peripheral airways"; this term denotes airways of the 8th generation and above, and can be used to describe both terminal and respiratory bronchioles. In reality, the airway tree branches rather asymmetrically[11]. For example, bronchioles with an internal diameter of 2 mm are found in generation 4 to 13[10].

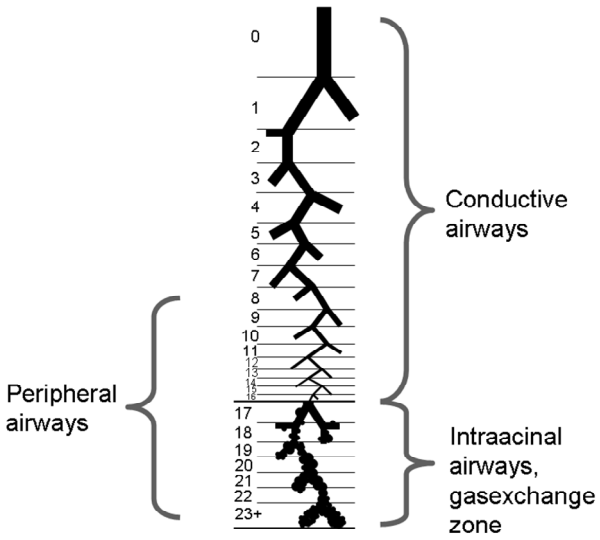

Figure 1 Schematic view of the branching structure of the airway according to Weibel's regular dichotomy model of the airways. Adapted with permission from Per Gustafsson.

EXHALED AIR, AEROSOLS AND PARTICLES – SOME DEFINITIONS

The exhaled air does not only consist of water vapor and carbon dioxide; thousands of gaseous substances and non-volatile compounds (i.e. compounds that do not readily evaporate) are also exhaled. It has been postulated that non-volatile substances are incorporated in aerosolized particles that derive from the respiratory tract[12]. The term *aerosol* is defined as a collection of solid or liquid particles suspended in a gas. There is no strict scientific classification for the physical form of the particles within the aerosol. The definitions worth mentioning in the context of this thesis include *bioaerosols* which are aerosols of biological origin (for example viruses, bacteria and fungi), *mist* and *fog* which are liquid-particle aerosols formed by condensation or atomization and *spray* - droplet aerosols formed by the mechanical breakup of a liquid (for example sea spray). The term *droplet* is used for liquid particles and *particulate matter* is used for either solid particles or liquid particles[13]. In the early literature on particulate matter in exhaled air, the terms droplet and particle both appear[14, 15]. The breath aerosol consists of gaseous substances (such as oxygen, carbon dioxide, nitric oxide and volatile organic carbons), water vapor and liquid particles/droplets/bioaerosols. In this thesis, the term *exhaled particles* and the abbreviation *PEx,* standing for *particles in exhaled air,* are used to denote exhaled liquid particles. In this context, the term *endogenous* is used to emphasize that the particles analyzed in this work derive from within the lungs and not from ambient air.

BREATH ANALYSIS

Previous studies on breath analysis have primarily focused on the measurement of exhaled gases. Usually, the compounds monitored in these studies have been simple inorganic species such as nitric oxide (NO), ammonia (NH_3) and carbon monoxide (CO), or volatile organic compounds (VOCs). Hundreds of VOCs have been identified in exhaled air[16]. Research targeting method development for analysis of gases in exhaled air has in many ways been successful. Several assays regarding volatiles in exhaled air have been adopted in the clinic, for example monitoring exhaled nitric oxide in patients with allergic asthma (FDA* approval in 2008) and the heartsbreath test used to monitor patients after heart surgery (FDA approval in 2003). Research into the analysis of exhaled volatile organic compounds is ongoing, with particular emphasis on the use of these compounds as markers for the diagnosis of lung cancer[17, 18] and breast cancer;[19, 20] additionally, considerable effort has been devoted to the development and optimization of sampling methodology. There is no standardized method for the analysis of VOCs; numerous different techniques for sample collection and preconcentration and quantification of analytes have been

* FDA=U.S. Food and Drug Administration

proposed, including real-time analysis[21]. Multivariate techniques are often employed to discriminate between patient groups[18, 22].

To date, exhaled NO is the best validated biomarker for airway inflammation in exhaled air. The fraction of exhaled nitric oxide (FENO) can be measured in real-time by chemiluminescence or with an electrochemical sensor using a single-breath approach. Levels of NO have been shown to be increased in asthma[23] and to be reduced in cystic fibrosis[24].

Exhaled breath condensate (EBC)

The presence of non-volatile compounds, like proteins, lipids and ions, in exhaled air has been demonstrated by analysis of EBC. Sampling of EBC is performed by cooling the exhaled air and collecting the resulting condensate. The patient breathes tidally into the device for a fixed time, normally 10 minutes. Sampling EBC is easy and inexpensive; moreover, because the condensate consists primarily of water, very little sample preparation is required. Aerosolized particles are believed to be captured in the EBC during sampling, probably due to gravitational settling when particles grow during condensation. The EBC contains numerous non-volatile and semi-volatile substances, including cytokines, peptides, lipid metabolites and hydrogen peroxide (H_2O_2). The EBC method has been in use since the 1980s. Early reports discussing EBC focused on its surface-active properties[25] and measurements of H_2O_2[26]. Several commercial instruments are available for EBC sampling; the most common according to the literature are the Ecoscreen (Jaeger) which is a stationary system and the RTube (Respiratory Research), which is a portable device. In addition, various different home-made devices have been described in the literature[27-29]. Methods for analysis of breath condensate are usually based on spectrophotometric or fluorometric assays or high pressure liquid chromatography (HPLC) in combination with different detection techniques such as fluorescence detection, UV detection or mass spectrometry, gas chromatography in combination with mass spectrometry and antibody-based assays such as enzyme immunoassay (EIA)/enzyme-linked immunosorbent assay (ELISA) and multiplex assays[30-33]. EBC pH is also measured[34, 35]. Nuclear magnetic resonance (NMR) has also been used, although it is less common in this context[36]. Many potential biomarkers, linked to airway inflammation and oxidative stress, have been detected in EBC and the method has been employed in the study of several different respiratory diseases, exposure studies and animal studies.

The main advantage of EBC collection is that samples can easily be obtained even from children and patients with severe airway disease. The major problems associated with EBC analysis, which are particularly severe for non-volatile compounds[37], are the low levels of certain analytes in the

condensate and the unknown degree of dilution. It has been shown that the coating material of the condenser affects recovery and concentrations of analytes in EBC[29, 38]. Losses of material from the sample can also be expected due to inefficient condensation of the exhaled air[29]. Different analytes will also have different condensation rates, depending on their physical and chemical properties, which in turn affect collection yield. No standards have been established for important parameters relating to sampling equipment such as the cooling temperature, material, and design; this strongly affects the reproducibility of the results obtained using this method[39]. Recent work on sampling instruments has therefore focused on preventing loss of sample by optimizing the material used in the construction of the condenser and enhancing condensation efficiency[29]. It has also been pointed out that collection conditions should be optimized for each marker studied[37, 40].

As is the case with BAL fluid[41], EBC lacks an internal standard determining its dilution, which makes it difficult to compare samples from different patients. A common finding in studies of particles in exhaled air is that there is high variability in the number concentration of particles;[14, 42] this will inevitably affect variability in the levels of particle-born substances in EBC. For example, the observed correlation of 8-isoprostane in EBC with the degree of airway closure in mild asthma could be a result of an increased particle production with increasing airway closure[43]. Various methods to correct for the variable dilution of EBC samples have been proposed, using parameters such as the exhaled volume[44], the concentrations of specific exhaled ions[45] or urea,[46] or the conductance of lyophilized samples[47]. These methods generally need further validation and have not found widespread use.

Avoiding contamination from the oral cavity is an important issue in breath analysis. Contamination is typically due to saliva leaking into the sample. One common way of determining the extent of saliva contamination in EBC samples is to measure their amylase content[48].

Sampling and analysis of particles and bioaerosols

Using X-ray dispersive analysis, Papineni et al collected exhaled particles on copper grids using a round nozzle impactor and found that exhaled particles contained potassium, calcium, and chloride.[14] Other approaches for sampling particles in exhaled air have been reported in the literature and deal mostly with detection of bacteria and viruses or narcotics. Sampling of particles in exhaled air for virus detection has been performed using a "cough aerosol particle collection system". The subjects coughed into the instrument and particles in the breath sample were collected in a centrifuge tube with a filter or a SKC BioSampler, which collects the aerosol

into a transport media[49]. Fabian et al used the commercial Exhalair (Pulmatrix, Lexington, MA) instrument, which integrates particle counting and sampling on Teflon filters to collect breath samples during tidal breathing and used quantitative polymerase chain reaction (qPCR) to detect influenza virus[50]. A similar approach was adopted by Hersen et al who collected particles, during coughing, on membrane filters for subsequent PCR analysis of viral material and recorded the particle concentrations using a real-time particle-size spectrometer (electrical low pressure impactor)[51].

Beck et al detected methadone, a small lipophilic substance, in exhaled air using a sampling method based on deep exhalations and suction of the exhaled air through a C18 filter[52]. Studies using the quartz crystal microbalance technique and a specially-designed interface for vapor to liquid sample transfer and capture are being conducted for detection of airborne virus[53, 54].

Real-time measurements of particles have been performed using Extractive Electrospray Ionization Mass Spectrometry (EESI)[55-57] or Bioaerosol Mass Spectrometry (BAMS)[58, 59]. Using EESI, urea, glucose[55], fatty acids[60] and narcotics[61] have been detected in exhaled air. In breath analysis, BAMS is primarily used for detecting respiratory infections such as tuberculosis[62].

PARTICLE FORMATION

It is commonly assumed that during tidal breathing, exhaled particles form in the airways due to shear forces[63, 64] or due to the reopening of airways following airway closure[42], the latter hypothesis is less-frequently invoked. In reality, studies of particle concentrations in exhaled air during normal breathing, and particularly studies of the mechanisms by which these particles are formed, are still lacking. Most of the published studies on particle concentrations in exhaled air were conducted in the context of airborne transmission of pathogens and hence focus primarily on particle production during talking, coughing and sneezing[65-67]. Early studies of endogenous particle concentrations in exhaled air during normal breathing were performed by Fairchild and Stampfer in 1987[15]. These authors found that the number of particles exhaled is extremely low, ranging from 0.1-4 particles/cm^3, and that most of the particles were <0.3 μm in diameter. Normal breathing produced the lowest numbers of particles while breathing during exercise produced the highest. On the basis of the finding that mouth breathing generated higher particle concentrations than coughing in one particular subject, it was concluded that particle generation may proceed via mechanisms that are not dependent on shear forces created by high-velocity air[14]. Edwards et al found that there was a huge variability in the concentrations of particles exhaled by the subjects, ranging from one particle per liter to >10 000[42].

Gebhart et al observed higher particle concentrations in the exhaled air after exhalations to closing or residual volume and that breath-holding after inspiration decreased particle concentrations[68] (unfortunately, it was not possible to obtain a full description of these authors' findings; this statement is based on the abstract of a conference presentation).

A recent study, analyzing exhaled radioaerosols deposited in the lung by different airway deposition patterns, found that particles collected with the EBC method originate mostly from the central airways[69]. Using this method, because tidal breathing is applied, airway closure should normally not be attained.

Airway closure

Airway closure is a phenomenon that involves blockage of the peripheral airways by the RTLF. Normally, it occurs in the terminal bronchioles of healthy humans at the end of expiration when the lung's RV is approached and the airway diameters are small[70, 71]. Instabilities of the liquid lining fluid and the tendency of the fluid to strive for minimal surface energy lead to airway closure by the formation of a liquid bridge. Closure through this mechanism is facilitated by a "compliant collapse" of the airway walls due to wall elasticity. The development of instabilities in the fluid is affected by the surface tension of the liquid lining fluid, wall stiffness and the initial film thickness[72, 73]. The liquid bridge normally ruptures during early stage of inspiration. This was observed by Macklem et al in cat bronchioles using a dissecting microscope. At a certain transmural pressure the airway diameter increases abruptly and closed airways "pop" open[74, 75]. This can also be detected as crackling sounds[76]. Under pathological conditions, airways do not always reopen after closure and this could lead to atelectasis (lack of gas-exchange in the alveoli) or local hypoventilation (excess of carbon dioxide/decrease of oxygen in the blood).

The closing volume (CV) denotes the volume of gas that remains in the lungs (RV not included) when airway closure begins, and is also known as the closing point (CP). The CV can be estimated by e.g. the nitrogen technique[77], further described in the Methods section of this thesis. Increases in the CV and increased tidal airway closure have been observed in patients with severe asthma[78] and is a useful indicator of small airway disease[79]. Tidal airway closure occurs when the CV is greater than the expiratory reserve volume (ERV) and is also common in e.g. obesity[80].

THE RESPIRATORY TRACT LINING FLUID AND SURFACTANT

The particles are believed to derive from the respiratory tract lining fluid (RTLF) which is a thin and heterogeneous lining layer that covers the respiratory epithelium. A wide range of chemical and immunological components of the RTLF protect the epithelial cells and help to minimize inflammatory reactions induced by inhaled toxic compounds, viruses and bacteria that are present in ambient air. Like the epithelium itself, the characteristics of the RTLF vary along the respiratory tree. In the upper airways, the epithelium is pseudostratified and the RTLF contains high levels of mucins, secreted by goblet cells and (to a lesser extent) mucous cells. Lipids, lipid metabolites, proteoglycans, proteases and antimicrobial proteins and peptides are also important constituents of the RTLF in the upper airways[81-83]. The epithelium in the distal airways is cuboidal and the RTLF is arranged as a two-phase layer with an aqueous phase containing lamellar bodies and tubular myelin[84] and a superficial layer composed of pulmonary surfactant[85]. The alveolar epithelium is very thin, consisting of two main cell types - alveolar type I cells, which mediate gas-exchange, and alveolar type II cells, which are important in innate immunity and repair processes[86, 87], see Figure 2. The major function of type II cells is to synthesize, secrete and recycle surfactant, a complex mixture consisting of about 80% glycerophospholipids, 10% neutral lipids and 10% proteins (comprising the surfactant proteins A, B, C and D). Secretion of surfactant by exocytosis is regulated by various stimuli; the most important physiological stimulus in this context is ventilation of the alveoli[88, 89]. The pulmonary surfactant is crucial in pulmonary physiology because it prevents the collapse of the alveoli during expiration by reducing the surface tension. It also facilitates airway reopening after airway closure[75, 90]. Furthermore, both the proteins and the phospholipids of the surfactant have immunological functions[91, 92]. Surfactant protein A and surfactant protein D are important for the innate immune system in the clearance of inhaled particles and pathogens[93].

The surfactant system is dynamic; its composition has been observed to change during for example exercise[94]. Several lung and airway diseases, such as asthma[95], interstitial lung disease[96] and cystic fibrosis[97] are associated with defects of the surfactant. Suggested mechanisms leading to impaired function of the surfactant are related to leakage of plasma proteins into the airway lumen[95], altered proportions of surfactant components or oxidative degradation of surfactant components[91].

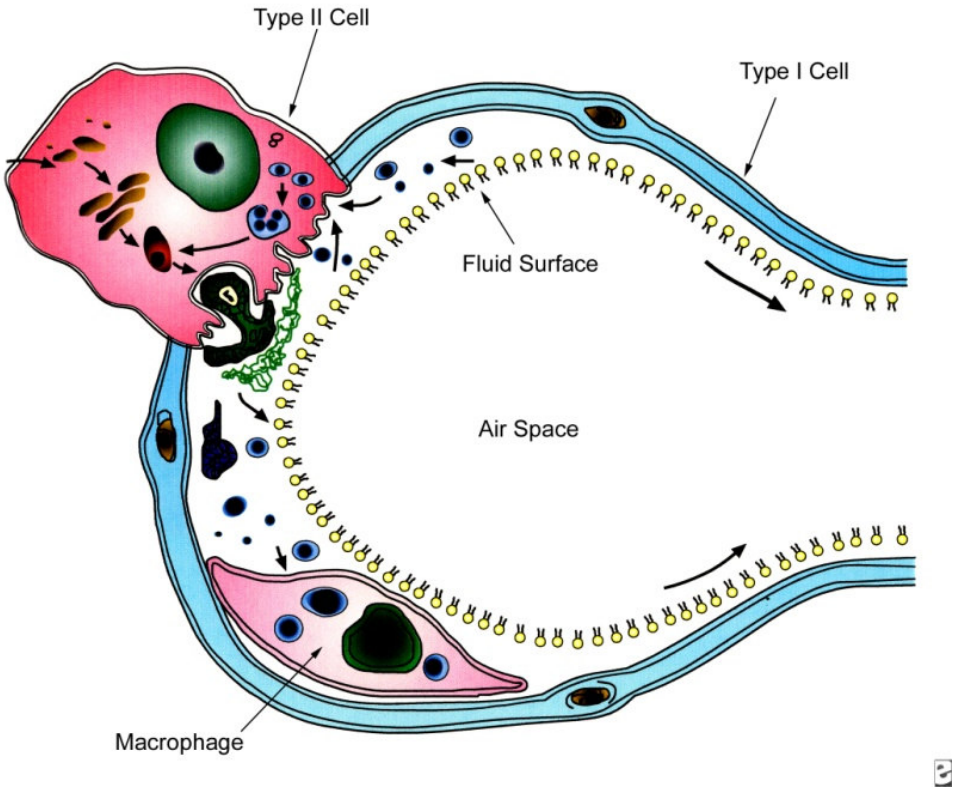

Figure 2 Schematic view of an alveolus and the surfactant metabolism. Surfactant components, synthesized in the alveolar type II cell, are stored intracellularly in lamellar bodies. After secretion, surfactant phospholipids are organized into tubular myelin which subsequently forms a monolayer of phospholipids at the air-liquid interface in the alveolus. The macrophage is a free-moving cell that has defensive properties, removing particles and bacteria. Image reprinted with permission from Medscape.com, 2011. Available at: http://emedicine.medscape.com/article/976034-overview

Surfactant composition can be analyzed in the cell-free BAL fluid. Surfactant is also often classified according to the way it separates in the fluid during centrifugation. Depending on the method used, two (large and small aggregates) or three fractions (ultraheavy, heavy and light) may be recovered. These fractions can then be analyzed to determine the surfactant's composition and surface tension. When comparing and interpreting the results of different studies, it is important to be aware of which approach was used, as different approaches will not generate directly comparable results[98].

Surfactant lipids

It is believed that the surfactant's spreading properties and ability to reduce surface tension, both of which are necessary in the maintenance of alveolar stability, are due to its various contents, including saturated and unsaturated lipids and proteins[99, 100].

Glycerophospholipids, which are also important membrane constituents, consist of a three-carbon glycerol backbone, a phosphorylated headgroup (attached to carbon-3 of the glycerol backbone) and two hydrocarbon fatty acid chains (attached to the other two carbon atoms of the glycerol backbone). Glycerophospholipids are amphiphilic molecules; the phosphate and glycerol form the hydrophilic end and the fatty acid chains form the hydrophobic end of the molecule. The chains differ in length and number of unsaturated carbon bonds†. The major glycerophospholipids present in surfactant are *phosphatidylcholine* (PC) and *phosphatidylglycerol* (PG). PG has been suggested as a marker of lung surfactant because of its unusual abundance in RTLF compared to other parts of the body. The lipid composition of surfactant is shown in Table 1. *Phosphatidylinositol* (PI), *phosphatidylethanolamine* (PE), and *phosphatidylserine* (PS) are present in minor amounts. *Phosphatidic acid* (PA), an important intermediate for the biosynthesis of phospholipids, is also present in surfactant[101].

Dipalmitoylphosphatidylcholine (PC32:0), see Figure 3, with palmitate (C16:0) at both the 1- and 2-positions, is the dominant form making up about 70% of the PC pool and is believed to be the most important component for reducing airway tension[102].

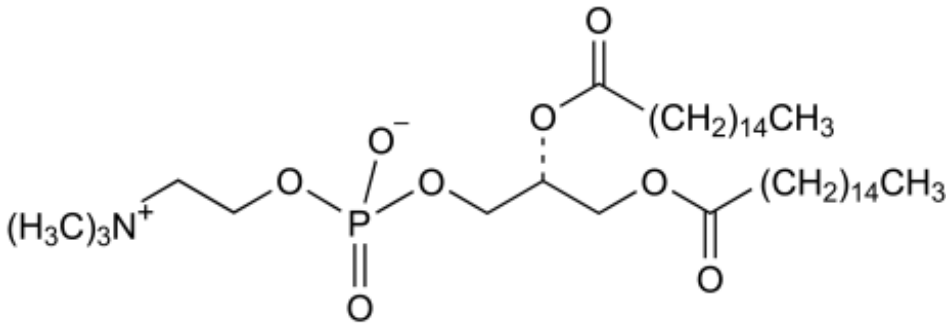

Figure 3 Molecular structure of dipalmitoylphosphatidylcholine (PC32:0).

† In this thesis, phospholipids are referred to as *a*:*x*, where *a* is the total number of carbon atoms in the fatty acid chains and *x* is the total number of unsaturated bonds in the chains.

The anionic phospholipids PG and PI have been suggested to be important for the stabilization of the surfactant film[100, 103]. The additional roles of PG and PI are less well-understood. Recently, the Voelker group presented results showing that these phospholipids play important roles in suppressing inflammatory responses[104, 105]. PI has been shown to interact with SP-D but the importance of this interaction is not well understood[106]. It has been suggested that PI is involved in the development of surfactant dysfunction in adult respiratory distress syndrome (ARDS)[107].

Cholesterol is a sterol that is also present in surfactant. Increased levels in surfactant were observed in ARDS[108] and are believed to contribute to surfactant dysfunction[109, 110].

Table 1 The lipid composition of human alveolar surfactant. The neutral lipid and phospholipid (divided in two classes) values are expressed as % of total lipid (in their respective classes). Adopted from Harwood et al[111].

Chemical substance		**%**
Phospholipids		90
	Dipalmitoylphosphatidylcholine	73
	Phosphatidylglycerol (PG)	12
	Phosphatidylinositol (PI)	6
	Phosphatidylethanolamine (PE)	3
	Other	6
Neutral lipids		10
	Cholesterol	71
	Triacylglycerol	26
	Others	3

ASSESSMENT OF AIRWAY DISEASE AND AIRWAY INFLAMMATION

The measurement of lung volumes and exhalation flow by spirometry is important for understanding lung function in health and disease. Airway narrowing, a common feature in asthma and COPD, is measured by performing forced exhalation or by determining airway resistance. Spirometry can be supplemented by bronchial challenge tests to examine bronchial hyperresponsiveness, which can be used to confirm the asthma diagnosis, and the reversibility test, which is used to differentiate between COPD and asthma[112]. Table 2 lists the definitions of various measures of lung volume that are used in this thesis.

Table 2 Definitions of different measures of lung volume.

Lung volume	Definition
Total lung capacity (TLC)	Volume of gas in lungs at the end of maximal inspiration
Functional residual capacity (FRC)	The volume of air in the lung at the end of a normal exhalation
Residual volume (RV)	The volume of air in the lungs after a maximum exhalation
Expiratory reserve volume (ERV)	The volume of air that can be expired from the resting expiratory level, at FRC
Forced expiratory volume in 1 s (FEV_1)	The maximum volume of air that can be expired during 1 s after a maximum inspiration
Forced vital capacity (FVC)	The maximum volume of air that can be expired forcefully after a maximum inspiration

FEV_1 and the slow vital capacity (VC) or FVC are measures of airflow limitation. A low FEV_1/FVC indicates airway obstruction. FEV_1 values are corrected for age, height and gender and compared to a reference value; the values are then presented as the predicted FEV_1% (FEV_1%pred).

Airway inflammation plays an important role in airway diseases such as asthma[113]. Inflammation is a protective mechanism involved in healing injury and defending the body against foreign substances and pathogens. Several inflammatory cells and different mediators are involved in the inflammatory process[114].

In general, the measurement of biomarkers‡ can be helpful in diagnosing disease, disease phenotyping, and disease monitoring, and can be used to identify optimal treatments and to predict the onset and severity of disease. Discovery of potential molecular or cellular biomarkers in airway inflammation is generally based on samples from sputum induction or bronchoscopy.

Bronchoscopy is performed with a flexible fiber-optic instrument that allows visual inspection and also sampling of the airways. There are several approaches for sampling, including *bronchial washings*, *bronchial brushings*, *bronchial biopsy*, *BAL* and *transbronchial lung biopsy*. Samples are analyzed in terms of their number of inflammatory cells and mediator levels. Bronchoscopy is a valuable research tool and also an important supplement for the diagnosis of e.g. suspected lung cancer and diffuse lung disease[115]. The BAL procedure is used to collect material from the lower respiratory tract, while bronchial sampling using techniques such as the bronchial wash collects material from the larger airways[2].

Induced sputum is important for the study of airway inflammation in asthma, COPD and chronic cough. Sampling is performed by inhalation of a nebulised saline solution, which induces expectoration of secretions into a Petri dish[116]. Cells and solutes are analyzed in the sputum sample, which is considered to reflect the larger airways. A drawback of this method is that it induces inflammation and is therefore not suitable for repeated sampling. Another limitation is the success rate of the procedure. It has been reported that the success rate is only 80% in healthy subjects[117]. In children and subjects with severe asthma it can be even lower[118, 119].

Sampling of *blood* and *urine* can be used for the measurement of inflammatory markers but there are difficulties with low levels of analyte and determining the location from which it originates[120].

Breath analysis is attractive because it is completely non-invasive, and can thus be used for repeated measurements. However, unlike invasive methods such as sputum induction or BAL, breath samples do not contain any human cells.

‡ A biological marker (biomarker) is defined as "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention" [116]

Glutathione (GSH) as a biomarker of oxidative stress in RTLF

Glutathione (GSH) is the most abundant thiol-containing peptide in mammalian cells, being found in millimolar concentrations. GSH is also highly abundant in the RTLF, occurring at concentrations of 200-300 μM - 10 to 100 times higher than its concentration in plasma[121]. It primarily acts as an antioxidant protecting the airways against oxidants in inhaled air or from inflammatory cells. The major redox forms of GSH are GSH and glutathione disulfide (GSSG), see Figure 4.

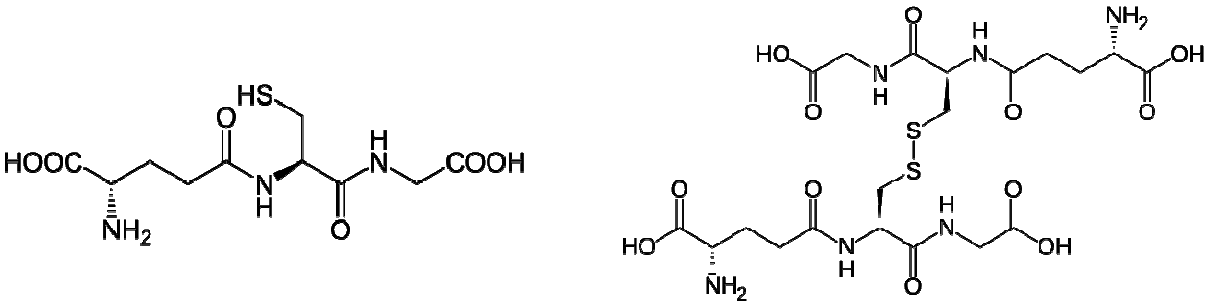

Figure 4 Molecular structure of glutathione (left) and glutathione disulfide (right).

GSH is a tripeptide composed of glutamic acid, cysteine and glycine. The presence of cysteine provides GSH with a nucleophilic sulfhydryl group that gives it the ability to detoxify substances by conjugation, which is catalyzed by glutathione transferase, by chemical reaction with a reactive metabolite to form a conjugate, or by donation of a proton or hydrogen atom to reactive metabolites or free radicals[122]. The latter reaction involves superoxide dismutase (SOD).

$$R^{\bullet} + GSH \rightarrow RH + GS^{\bullet}$$

$$GS^{\bullet} + GS^{-} \rightarrow GSSG + O_2^{\bullet -}$$

$$2\ O_2^{\bullet -} + 2\ H+ \rightarrow H_2O_2 + O_2 \text{ (catalyzed by SOD)}$$

If the reactive metabolite is a peroxide (e.g. hydrogen peroxide) GSH is oxidized and converted to GSSG. This reaction is catalyzed by the glutathione peroxidase enzymes, also highly abundant in the RTLF[121]. The reduction of GSSG is then catalyzed by glutathione reductase.

$$H_2O_2 + 2\ GSH \rightarrow GSSG + H_2O$$

$$GSSG + NADPH + H^{+} \rightarrow 2\ GSH + NADP^{+}$$

Other end products of GSH oxidation by oxygen radicals include the sulphenic acid (GSOH) (unstable), sulphonic acid (GSO_3H)[123] and S-nitrosoglutathione (GSNO) derivatives of GSH[124]. These GSH derivatives are likely to be involved in cell signaling[124]. GSH also helps in the regeneration of other antioxidants.

In airway diseases such as asthma and COPD, inflammatory cells generate excessive amounts of $O_2^{\bullet-}$ and $^{\bullet}OH$[125] which are referred to as reactive oxygen species. These species are extremely reactive free radicals that cause oxidative stress. Oxidative stress, characterized by an imbalance between reactive oxygen species and antioxidant defenses, can lead to lipid peroxidation and damage to proteins and DNA[126]. Under conditions of oxidative stress, GSH synthesis is up-regulated and the relative amount of GSSG increases[127]. An elevated level of GSH or total GSH is an indication of an adaptation to oxidative stress, and elevated levels of GSSG are a marker of oxidative stress[128]. Levels of GSH are decreased in the lung lining fluid in several airway diseases such as cystic fibrosis and acute respiratory distress syndrome[129]. Elevated levels of both total GSH as well as GSSG have been observed in BAL and/or sputum in COPD and asthma[130-135].

EXAMPLES OF RESPIRATORY DISEASES INVOLVING SMALL AIRWAYS

Asthma

Asthma is one of the major respiratory diseases, affecting around 300 million individuals world-wide[136]. It is a complex disease characterized by chronic inflammation and airway narrowing with airway hyperresponsiveness. Diagnosis is based on clinical history and lung function testing[137]. Exacerbations of the disease involve episodic wheezing, chest tightness, coughing and shortness of breath.

The causes of asthma are poorly understood, but it has been concluded that genetic and environmental factors are important in its development. Because of its heterogeneous nature, attempts have been made to divide asthma into many subtypes, such as allergic asthma, non-allergic asthma, occupational asthma and obesity-related asthma[137]. In some cases, asthma has overlapping features with COPD and the two diseases may thus be difficult to distinguish from one another[138].

The prevention of exacerbations and the restoration and maintenance of normal lung function are important goals in asthma treatment; ideally they would be achieved with a minimum of medication [23, 137]. Analysis of bronchial biopsies, BAL fluid and sputum from patients with asthma have given more detailed information on the inflammation process, inflammatory cells and mediators[139-141]. Asthma exacerbations have been shown to be related to airway

inflammation, which is not assessed by current diagnostic tests. The use of inflammatory markers in monitoring the disease might therefore provide important information that could improve treatment. Inflammatory markers such as FENO[23] or the eosinophilic cell count in sputum[142, 143] may be useful in this context.

Studies of surfactant function and composition in asthma have been performed on sputum and BAL fluid but knowledge is very limited. Surfactant dysfunction, which is probably attributable to the infiltration of plasma proteins into the airway lumen, may contribute to airway obstruction in asthma. Evidence for this has been presented primarily for acute asthma. Surfactant tension, phospholipids and the ratio of phospholipids-to-total proteins in sputum were reported to increase during an acute asthma attack[144]. The PC-to-PG ratio was significantly increased in BAL fluid and correlated with surfactant dysfunction and increased protein concentration after a local allergen challenge in subjects with asthma, due to infiltration of plasma lipoproteins and not catabolism of phospholipids[145]. These results are consistent with a recent study of lung injury in children where an increase in plasma PC and a decrease in PC32:0 and PC30:0 in the BAL fluid were observed[146]. Segmental allergen challenge was shown to cause inhibition of surfactant function in patients with mild asthma[147, 148]. Wright et al observed a decrease in the proportion of PC32:0 (PC16:0/16:0) in sputum but not in the BAL fluid in patients with stable asthma, suggesting that phospholipid composition is altered in the airway and not in the alveolar surfactant[95]. In children with asthma, it has been reported that levels of PC32:0 are elevated in sputum samples compared to controls and that there is a positive correlation with sputum inflammatory cells and sputum eosinophil cationic protein[149, 150].

Cystic fibrosis

Like asthma, cystic fibrosis (CF) is a disease characterized by a chronic airway inflammation and airway obstruction. It is a genetic disease caused by a mutation in the CF transmembrane conductance regulator (CFTR) gene which leads to dysfunction of the CFTR protein, expressed in epithelial cells and blood cells. The entire body is affected, but the pulmonary dysfunction is the most important factor for morbidity and mortality in CF[151]. The dysfunction of CFTR leads to defects in the innate immune system so that airways in CF rapidly become colonized by bacteria. The severity of the infection is associated with the progress of the disease. Monitoring of CF is therefore primarily focused on early detection of respiratory infection[152].

AIMS

The general aims of the work were to study the chemical composition of particles in exhaled air and their mechanisms of formation.

In **Paper I,** the specific aim was to study if particles in exhaled air can be collected by impaction. We hypothesize that exhaled particles originate from the respiratory tract lining fluid and not from saliva.

In **Paper II,** the specific aim was to compare the chemical composition of particles from healthy subjects and subjects with asthma. We hypothesize that it is possible to discriminate between groups using multivariate analysis of spectra obtained by Time-of-Flight Secondary Ion Mass Spectrometry (TOF-SIMS) analysis and that the composition of phospholipids differs between the groups.

In **Paper III,** the specific aim was to understand the impact of airway closure and airway reopening on number concentrations of particles in exhaled air. We hypothesize that film rupture during airway opening following airway closure is an important formation mechanism for particles in exhaled air.

In **Paper IV,** the specific aim was to develop a sensitive method for the determination of total glutathione (GSHt), glutathione (GSH) and glutathione disulfide (GSSG) in EBC. We hypothesize that GSH and GSSG can be quantified in EBC and that GSHt can be determined in particles in exhaled air.

MATERIALS AND METHODS

STUDY DESIGN AND SUBJECTS (PAPERS I-IV)

Pilot study: Healthy non-smoking volunteers (n=10, age 22-56 years, 6 females) breathed either tidally or forcefully into the instrument for three minutes. Each measurement period was performed twice in a random order. Particle number concentrations were recorded during the whole sampling period.

Paper I: In an introductory study, sampling of exhaled particles from four healthy volunteers was performed. Subjects breathed forcefully into the instruments for 15 minutes. Saliva samples were also collected, for comparative purposes. Four healthy volunteers, four subjects with asthma and four subjects with cystic fibrosis participated in a subsequent patient study. Participants breathed particle-free air for 3 minutes and then performed 10 forced exhalations in succession into the instrument. All samples were analyzed by TOF-SIMS.

Paper II: Fifteen non-smoking subjects with physician-diagnosed asthma and eleven non-smoking healthy controls were included. Subjects were free from symptoms of respiratory infection for at least three weeks prior to sampling. Prior to sampling of particles in exhaled air, FENO was measured and participants filled out a questionnaire regarding age, allergies and medication and performed spirometry and skin prick tests for common inhalant allergens. Participants breathed particle-free air for 3 minutes and then performed 10 forced exhalations in succession into the instrument. Samples were analyzed by TOF-SIMS and spectra were analyzed and compared using multivariate analysis (PCA and OPLS).

Paper III: Particle number concentration in exhaled air after exhalations to RV, CV or FRC were measured and compared. Ten healthy volunteers participated in the study. Spirometry and the single breath nitrogen test were performed in order to determine FEV_1 (% pred), FVC (% pred), ERV and CV. Participants breathed according to a designed protocol described in detail in the paper.

Paper IV: An analytical method based on HPLC and fluorescent detection was developed for the determination of GSHt, GSH and GSSG in EBC. The method was applied to determination of GSHt, GSH and GSSG in EBC from four healthy volunteers. In addition, particles in exhaled air were collected (in total 80 liters) and analyzed with respect to GSHt. Sampling of particles in

exhaled air was achieved using a modified breathing maneuver developed on the basis of the results of the study described in **Paper III**.

OPTICAL PARTICLE COUNTING (PAPERS I-IV)

In all of the different studies, aerosol particles in the exhaled air were counted using an optical particle counter (OPC) (Grimm Model 1.108, Grimm Aerosol Technik, Ainring, Germany). The instrument sizes particles according to the amount of light scattered by each particle when counted. The instrument's size calibration is based on the light scattering caused by monodisperse latex spheres (certified by National Institute of Standards and Technology); analyzed particles are assumed to have the same diameter as latex spheres that scatter the same amount of light. This was accounted for in **Paper IV** by recalculating particle sizes on the basis of the scattering observed with water droplets, whose properties more closely resemble those of the exhaled particles [153]. The particle counter draws in air at 20 ml/s and counts and sizes particles over 15 size intervals with a time resolution of 6 s (0.3-20 μm; this mode was used in **Paper I**) or over 8 size intervals with a time resolution of 1 s (0.3-2.0 μm; this mode was used in **Papers II-IV**).

In the early pilot studies only particle number concentrations in exhaled air were studied. In these experiments, the particle counter was housed in a box with a temperature of 36°C as above but no sampling of particles or exhaled flow monitoring was performed.

PARTICLE SAMPLING (PAPERS I-IV)

Sampling of particles was assessed using a commercial three-stage cascade impactor (PM10 Impaktor, Dekati Ltd, Tampere, Finland); see Figure 5. The principle of sampling by impaction is to direct the aerosol through a nozzle and to direct the output stream towards a flat surface known as the impaction plate. The impaction plate causes the flow to bend 90° and particles that cannot follow the airstream will, due to their high inertia, continue in a straight line and strike the impaction plate[13]. In a cascade impactor, a number of impactor plates are connected in series. The impactor used in this work had three stages, but only the last stage, collecting particle sizes in the range 0.5-2.0 μm, was used for sampling. In order to increase the flow through the impactor and decrease the cutoff for impacting particles, half of the nozzles were covered with tape, leaving ten open nozzles above the impaction plate.

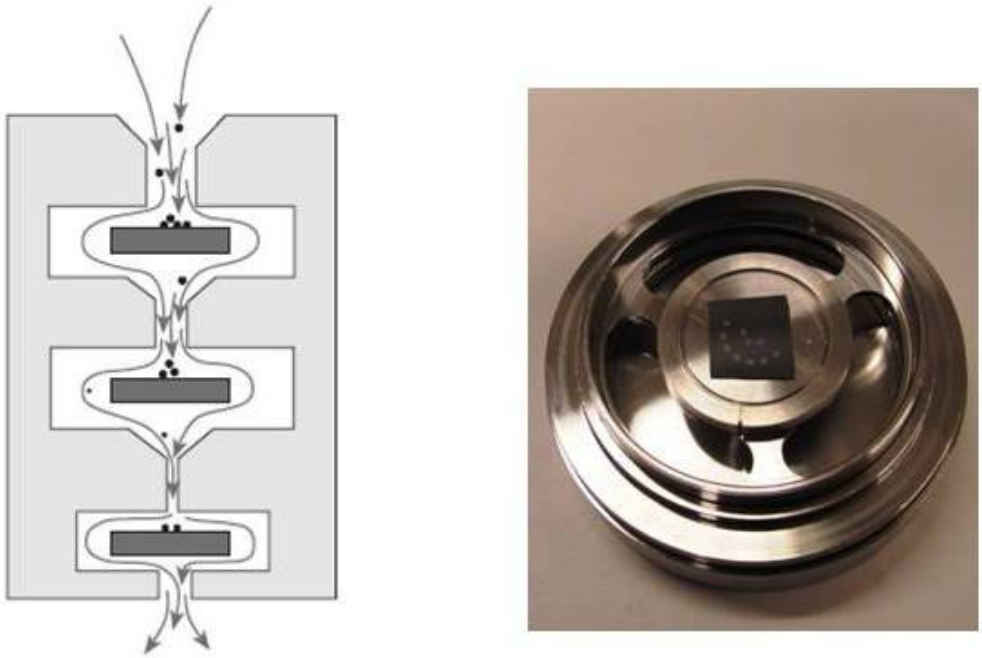

Figure 5 Particle sampling by impaction on silicon wafers. A) Cross-sectional schematic illustration of the impactor. Reprinted with permission from Dekati (www.dekati.com). B) Picture of the silicon wafer (size about 1.5 cm^2) that was used as the impaction plate. An authentic sample of exhaled particles collected in 10 spots is visible on the wafer.

The cascade impactor and related parts are housed in a thermally insulated box with a temperature of 36°C to avoid condensation, see Figure 6. The appurtenant components are described in more detail in **Paper I**. Outside the box, an insulated silicon tube and a mouthpiece is connected to the inlet of the instrument**.** The mouthpiece consists of a two-way valve that allows inhalation of particle-free air and exhalation into the instrument. In the studies described in **Papers III-IV**, the instrument was modified by attaching an additional two-way valve between the mouthpiece and the silicone tube. The purpose of this valve was to make it possible to direct the exhaled air either into the instrument or into the room.

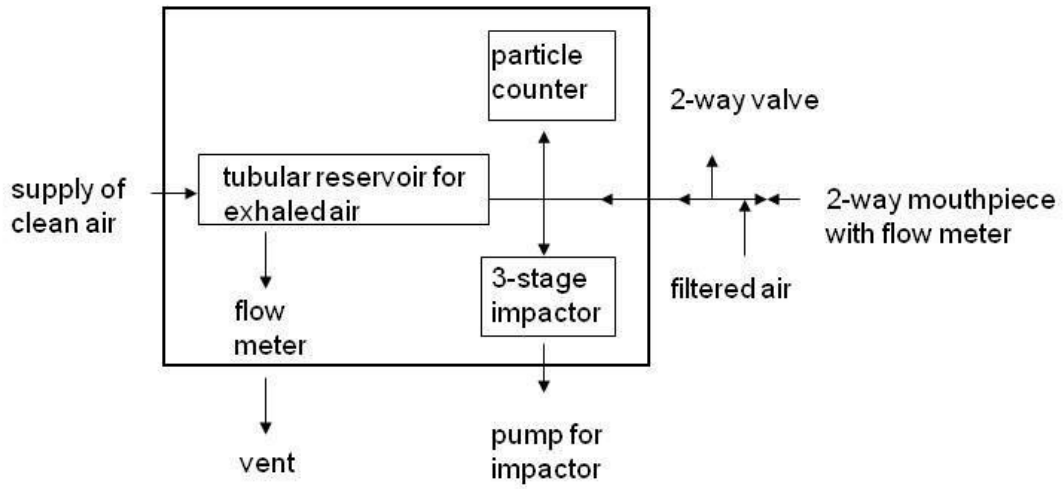

Figure 6 A schematic illustration of the instrument for collection and counting of particles in exhaled air.

The insulated silicon tube outside the box is coupled to a copper tube housed inside the box. In addition to providing a connection to the impactor used for particle sampling, the copper tube also serves as a reservoir for the exhaled air. The purpose of the reservoir is to prevent the loss of exhaled particles and to give the system flexibility to cope with different exhalation flows and volumes. Clean, particle-free air, saturated with water vapor at 34°C is added at the end of the reservoir at 18.5 L/min to serve as a buffer when no exhaled air is entering the system and also to prevent room air from entering the instrument through the vent. The air is saturated with water vapor in order to resemble exhaled air as closely as possible.

Air was drawn through the impactor continuously at a rate of 16 L air/min using a vacuum pump. Silicon wafers were used as impaction plates and placed on the last stage with cutoff size of 0.5 μm. The impaction plates were selected on the basis of their compatibility with subsequent TOF-SIMS analysis. Initially, aluminum filters were assessed, but were discarded because their use introduced contaminants complicating the interpretation of the TOF-SIMS analysis results.

The expiratory flow rate was measured using an ultrasonic flow meter (OEM Flow Sensor Spiroson-AS, ndd Medical Technologies, Zürich, Zwitzerland). The flow rate was displayed on a computer screen so that patients could observe and control their breathing. In **Paper III**, an

additional ultrasonic flow meter was connected to the mouth-piece enabling control of the inhalation flow.

Patients rinsed their mouths with water and breathed particle-free air for at least 3 minutes before sampling. They were asked to breathe particle-free air for 3 minutes prior to sampling in order to ensure that only endogenous particles from the lung were studied and not particles from the ambient air. The washout time was chosen on the basis of a study on washout time for particles in exhaled air after smoking[154]. Prior to patient particle counting and sampling, the instrument and optical particle counter were tested to make sure the detected background particle concentration was zero.

SAMPLING OF EBC

EBC was collected for determination of GSH and GSSG using the EcoScreen breath condenser (Jaeger, Wurtsberg, Germany). Before sampling, subjects washed their mouth with purified water. Subjects wore a nose clip during sampling and exhaled for ten minutes with tidal breathing. Exhaled volume was recorded with an Ecovent spirometer (Viasys Healthcare GmbH, Hoechberg).

After centrifugation for 5 minutes, EBC was immediately transferred to Eppendorf tubes (Protein LoBind, Eppendorf AG, Hamburg, Germany).

ANALYTICAL METHODS

Time-of-Flight Secondary Ion Mass Spectrometry (TOF-SIMS) analysis (Papers I and II)

In **Papers I and II**, static TOF-SIMS was used for the chemical analysis of silicon wafers. No sample preparation was required for the collected particles, and the analysis protocol exploited the fact that the particles were delivered in the form of small concentrated spots.

TOF-SIMS is a technique that provides detailed molecular information about the sample surface. It can also be used an imaging technique. It is a well established surface analysis technique in materials science and semiconductor research, and has also been extensively used for surface studies of polymers and, more recently, biomolecules[155]. The main principle of TOF-SIMS is mass spectrometric analysis of ionized atoms, molecules and molecular fragments (secondary ions) that are emitted from the sample surface upon irradiation with a focused beam of energetic (primary) ions[156], see Figure 7. The emitted secondary ions, which can be either positively or negatively charged, are analyzed in a time-of-flight mass spectrometer according to their mass-to-

charge ratio, m/z. Both positive and negative secondary ion mass spectra can be measured, although not simultaneously. TOF-SIMS can detect ions with masses up to 10 000 Dalton.

In the studies described in this thesis, the TOF-SIMS measurements were carried out in the so-called static regime, which means that a very low dose of primary ions was used ($<10^{13}$ ions cm^{-2}). Using this mode, less than 1% of the top surface layer of atoms or molecules is hit by ions resulting in minimal damage and alterations to the surface, thus providing a mass spectrum that is representative of an essentially undamaged surface.

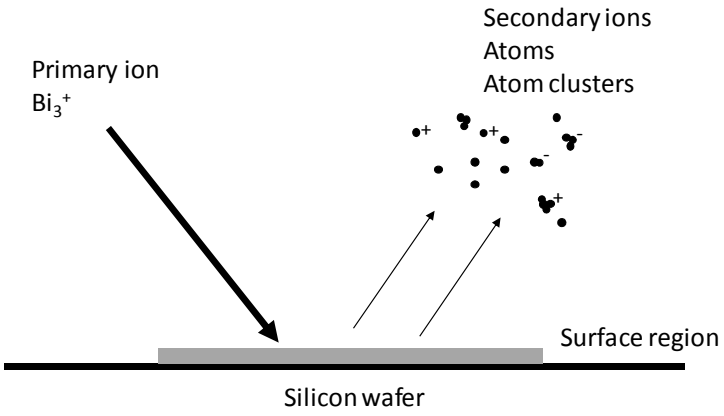

Figure 7 Picture of the TOF-SIMS process.

The degree of fragmentation of molecules being analyzed is determined by the type of primary ion (mass and number of atoms) used. In this thesis, Bi_3^+ cluster ions were used as the primary ions. Cluster ions, such as Bi_3^+, are more efficient at inducing the emission of intact molecular ions and larger fragment ions than monoatomic species, which is an advantage because detection of both intact molecular ions and characteristic fragments facilitate secondary ion identification.

TOF-SIMS is a semi-quantitative technique. The yield of secondary ions is dependent on a number of different factors, including not only the concentration of a given molecule but also the surrounding environment, the matrix. The latter factor, which means that the measured secondary ion signal intensities for a given analyte concentration may vary considerably due to differences in the surface composition and chemical binding state is often referred to as "matrix effect" [157]. Matrix effects make quantification of and comparisons between samples difficult. In the case of biological samples, this problem is exacerbated by the fact that TOF-SIMS generates information-rich spectra containing many peaks corresponding to fragments derived from larger

molecules within the sample. Efforts to overcome these problems have focused on the use of internal standards[158, 159] and multivariate statistical techniques[160, 161].

In this work, mass spectra of positive and negative ions were recorded with the instrument optimized for mass resolution (typically $m/\Delta m > 5000$ at $m/z=28$). In most cases, a surface area of 500x500 μm^2 was analyzed in each of the measurements. Positive and negative secondary ion mass spectra and ion images were recorded from two randomly selected particle spots on each collection plate. Data were stored in raw data files in which the analyzed area is represented by 128x128 pixels, and a full mass spectrum is associated with each pixel. From the raw data file, total spectra representing the entire analyzed area, or spectra from regions of interest representing selected parts of the analyzed area, or ion images showing the intensity distribution for single selected ion peaks within the analyzed area, can be retrieved. In **Paper II**, an additional approach was used for the purpose of multivariate analysis; to this end, all of the spectral data were processed using software developed in-house and described in detail in the paper.

HPLC and fluorescence detection for determination of GSH and GSSG (Paper IV)

Paper IV describes the development of an analytical method for the determination of GSHt (GSH+GSSG), GSH and GSSG in EBC.

The most sensitive assays for GSH analysis are either based on fluorescence or mass spectrometric detection. For fluorescent detection, derivatization with a fluorophore is required since GSH and GSSG do not have any fluorophores in their molecular structure. High-performance liquid chromatography (HPLC) is used for separation of the fluorescent derivatives before detection and is necessary for improving sensitivity and selectivity.

Ideally, the derivatization agent should be non-fluorescent before derivatization and should form a stable product with the analyte of interest. Derivatization of GSH can be performed at the carboxylic acid, amino, or thiol group; GSSG can be derivatized at either the amino group or the carboxylic acid. Derivatization of the thiol group is preferred because of its high specificity and because it prevents the autooxidation of GSH. There is a wide range of fluorophores available for the derivatization of thiols including *o*-phthalaldehyde[162], naphthalene-2,3-dicarboxaldehyde[163], monobromobimane[164] and different maleimide derivatives[165]. The reaction pH, selectivity, incubation temperature, and time period vary for the different assays.

Analysis of GSH is difficult because it undergoes facile auto-oxidation at pH>7 and is enzymatically converted to cysteinyl-glycine at neutral pH by γ-glutamyl transpeptidases[166]. It is

therefore important to keep sample pH in the acidic range during sample preparation. Refrigeration is also important to minimize oxidation and proteolysis. Adding EDTA to the samples is believed to prevent oxidation of the –SH group. Protein removal is encouraged because, if abundant in the sample, they may affect the analytical procedure and shorten lifetime of the HPLC column and instrument.

Reduction of disulfide bonds is performed to determine bound GSH, such as GSSG. The selection of reducing agent depends on the identity of derivatization agent used for GSH labeling; the reducing agent must not interfere with the labeling agent. Other issues that must be considered and optimized include the reaction pH, stability, buffer selection, and incubation time.

In this work, N-(1-pyrene)-maleimide (NPM) was used for the derivatization of GSH. NPM reacts readily with free sulfhydryl groups to form fluorescent products. Winters et al developed the original method and used it to detect GSH in different cell lines[167].

The concentration of GSHt in exhaled particles at the point of formation was calculated on the basis of values measured in the NPM assay and estimated particle volumes, as described in the paper.

STATISTICAL ANALYSIS

Multivariate analysis (PCA and OPLS) (Paper II)

TOF-SIMS analysis of biological samples, which often contain a multitude of different substances, generates complex spectra that are difficult to interpret visually. In order to reduce data complexity and extract differentiating traits in the data, multivariate analysis techniques are often used[161]. In **Paper II**, a multivariate analysis was used to differentiate patterns in the TOF-SIMS spectra from subjects with asthma and healthy controls by principal component analysis (PCA) and orthogonal projections to latent structures (OPLS) using SIMCA-P+ 12.0 (Umetrics AB, Umeå, Sweden). A hypothesis-generating approach was employed by considering all of the ion signals in the relevant spectra.

Multivariate projection methods such as PCA and OPLS provide visual overviews of complex data matrices. PCA is an unsupervised method that reduces a large data matrix to few principal components that account for as much of the variation in the data set as is possible[168]. Each principal component is an independent latent variable (including all original variables and their angular impact on the principal component). A s*core plot* is constructed based on these principal

components and a corresponding *loading plot* summarizes the influence of the individual variables. PCA is descriptive and is usually the first step in an analysis, providing a means of detecting things such as systematic variation and outliers in the data matrix.

OPLS is a supervised regression method to find and explain relations between X variables and Y variables (response variables). OPLS separates the systematic variation in X into two parts, one part that is correlated to Y and one part that is orthogonal to Y (not correlated). According to the same principles as for PCA, OPLS produces scores that describe relations between measured spectra from test persons and loadings that describe the relative importance of the variables, in this case the mass spectral m/z values. In contrast to PCA, the scores and loadings in OPLS are rotated to show the variation that is connected to the variation in Y. OPLS is a modification of PLS for enhanced interpretability. Compared to PLS, OPLS will however yield models with the same prediction capability[169].

The purpose of discriminant analysis is to separate two or more groups based on differences in X variables. A simple form of discriminant analysis was introduced by Fisher in 1936[170]. A linear combination of variables was determined to separate two or more known groups by maximizing the ratio of the between-groups variance to the within-groups variance using a regression function. Class membership is expressed by a categorical value in the response variable, for example Y=1 for one group and Y=0 for the other group. This approach can be applied to multivariate regression models such as PLS and OPLS where one or more principal components will comprise the linearly correlated variables that best separate known groups. In OPLS-discriminant analysis (OPLS-DA), the discriminatory information for class separation is contained in the first, predictive, component. Orthogonal components comprise variation in X that is not related to Y.

Pre-processing of data was performed by normalizing the peak intensities of individual ions signals against the total peak intensity in the corresponding spectrum. This is done to minimize systematic variation, such as time-related variations in the instrument[155]. This was followed by scaling of data, mean-centering and Pareto scaling, which is done to balance the influence from peaks with large and small magnitudes, still keeping the influence from the base-line low.

Cross-validation is an important tool to test the validity of the obtained model. Cross-validation is a procedure in which fractions of data are systematically excluded. Q2 is the measure of the quality of multivariate models, based on cross-validation. Generally, a Q2 value larger than 0.5 is considered good, indicating that the model provides useful systematic information. For OPLS-

DA, overlapping groups yield lower Q2 while groups that are well separated in the present spectral ~5000 dimensional space yield higher Q2. The cross-validation may be performed by generating a model using 6/7 of observations (here mass spectra) at a time. A sum of squares is then calculated for the deviations between the modeled responses (group ratings) and the assigned group (Y). These sums of squares, called Prediction Error Sum of Squares (PRESS), are summed for all excluded fractions and compared to the total sum of squares for the variation in Y (SS(Y)) by the formula: Q2=1-PRESS/SS(Y).

R2 is the fraction of the sum of squares (SS) of all the X's explained by the current component.

Univariate analysis (Papers II and III)

In **Paper II**, the Mann-Whitney test was applied to assess differences between groups regarding the ratios of ions in TOF-SIMS spectra. In **Paper III**, the Wilcoxon signed rank test was used to assess differences in particle numbers between the different respiratory maneuvers. Differences were considered significant when $p<0.05$. Pearson correlation was used to correlate particle number concentrations between maneuvers.

CLINICAL TESTS

Exhaled NO (Paper II)

In **Paper II**, fraction of exhaled NO (FENO) was measured using a NIOX mini handheld device (Aerocrine, Sweden). A single breath test at a flow rate of 50 ml/s was performed according to American Thoracic Society/European Respiratory Society recommendations[171].

Single breath nitrogen test (Paper III)

Measurements of closing volume were performed by the single breath nitrogen method with custom-made equipment and following the procedures described by Oxhoj and Bake[172]. The flow rate was about 0.2 l/s during inspiration of the initial 0.5 liters from residual volume (RV), i.e., during distribution of the nitrogen in the anatomic and apparatus dead space. The flow rate during expiration from TLC back to RV was about 0.3 l/s. The closing point, i.e. the transition between the alveolar plateau (phase III) and phase IV, was defined as the volume point corresponding to the first permanent, convincing, upsloping departure from a straight line through the last part of the plateau (Figure 8).

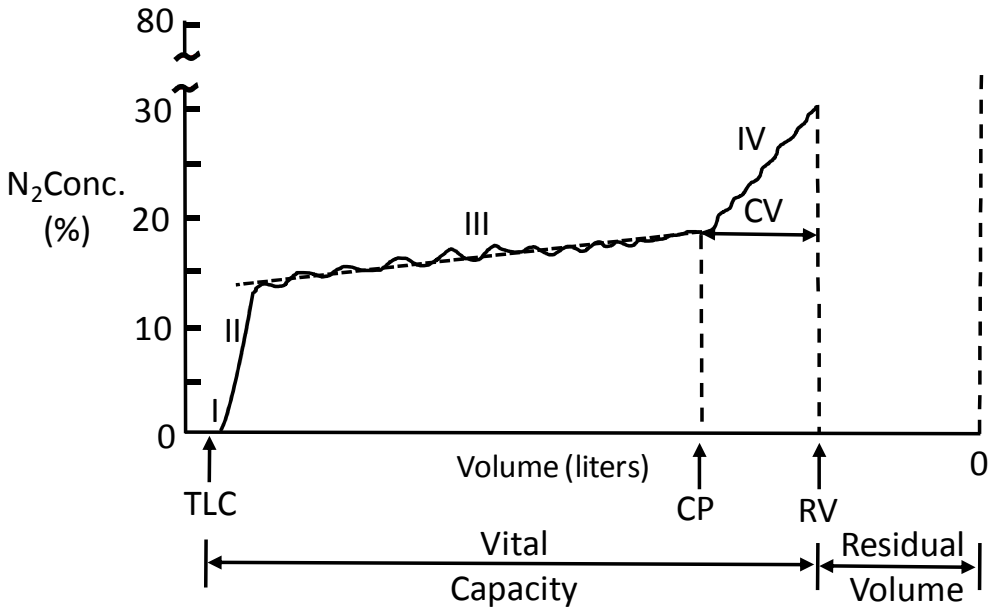

Figure 8 Diagram from a single breath nitrogen test. The subject expires to residual volume (RV), inspires oxygen to total lung capacity (TLC) and expires into the equipment. During the subsequent exhalation the nitrogen concentration is continuously recorded together with expired volume. CP=closing point, CV=closing volume.

Spirometry (Papers I-III)

FVC and FEV_1 were determined in accordance with European Respiratory Society guidelines[173]. Lung function values were expressed as percentage of predicted values[174].

ERV values presented in **Paper III**, are based on measurements performed in parallel with the other experiments and using the same spirometer as was used during particle measurements.

SUMMARY OF RESULTS

PILOT STUDY

The effects of forced and tidal breathing on particle concentrations were studied. Mean number concentrations over the studied time period were calculated and compared. Particle number concentrations for size intervals 0.30-2.0 μm were increased between 6 and 80-fold when breathing forcefully compared to breathing tidally. In the group of 10 subjects, particle number concentrations ranged from 22 to 98 particles/liter during tidal breathing and 320 to 5100 particles/liter during forced breathing.

PAPER I

Introductory study

Inter- and intra- individual variability in particle number concentrations using forced exhalations was high and not related to FEV_1. Most particles were <2.0 μm. The size distribution was similar in all subjects, with the highest numbers in the smallest size interval (0.30-0.40 μm) and then decreasing gradually for each size interval.

TOF-SIMS analysis of particles from healthy subjects revealed the presence of several different phospholipids, see Table 3. In the positive ion spectra, both fragments and molecular ions ($[M+H]^+$, $[M+Na]^+$ and $[M+K]^+$) of phosphatidylcholines were observed. A strong fragment signal was detected at m/z 184 corresponding to the phosphocholine ion. A low intensity signal corresponding to cholesterol was observed in the positive ion spectra. Of the phosphatidylcholines species detected in the positive spectra, the strongest signals were from PC 32:0. In negative ion spectra, deprotonated molecular ions, $[M-H]^-$ corresponding to phosphatidylglycerols, phosphatidylinositols and phosphatidic acid were detected. The strongest signals were assigned to phosphatidylglycerols with PG 34:1 being the strongest. The acquired spectra suggest that similar mixtures of phospholipids were present in different spots on the same silicon wafer and in samples collected from the same individual on both sampling days. Spectra were rich in fragments derived from phospholipids and other fragment ions such as CN^- and CNO^- that presumably originated from proteins.

TOF-SIMS images of a particle spot collected from a healthy control subject are shown in Figure 9. Spectra from saliva samples and particle samples did not show any similarities in the high m/z range; see Figure 10.

Table 3 Phospholipids detected in the TOF-SIMS spectra. Positive ions were detected as molecular ions $[M+H]^+$, $[M+Na]^+$ and $[M+K]^+$ while negative ions were detected as $[M-H]^-$.

	Assignment	*m/z*
Positive ions	PC 28:0	678.5
	PC 30:0	706.6, 728.5, 744.5
	PC 32:1	732.6, 754.5, 770.6
	PC 32:0	734.5, 756.5, 772.5
	PC 34:1	760.6, 782.5, 798.6
	PC 34:0	762.6, 784.5, 800.6
Negative ions	PA 32:1	645.5
	PA 32:0	647.4
	PA 34:2	671.5
	PA 34:1	673.5
	PG 28:1	663.4
	PG 28:0	665.4
	PG 32:0	721.5
	PG 34:1	747.5
	PG 36:1	775.5
	PI 34:2	833.6
	PI 34:1	835.6
	PI 36:2	861.6
	PI 36:1	863.6

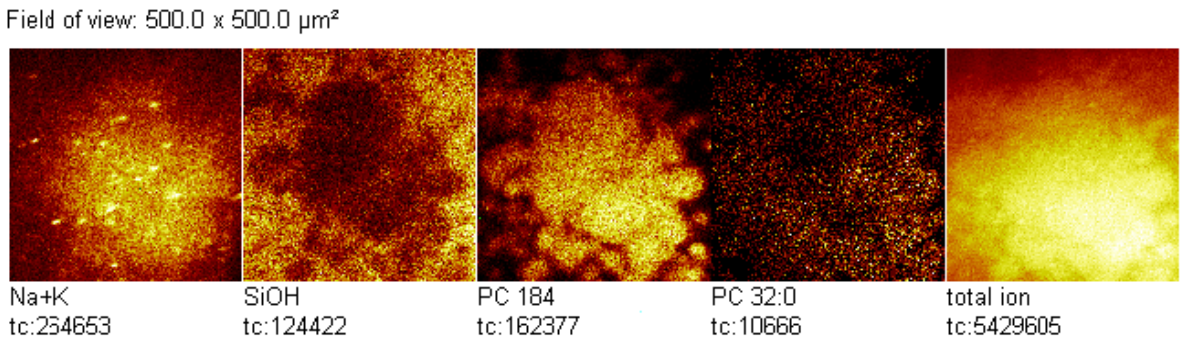

Figure 9 TOF-SIMS images from a single spot of particles sampled from a healthy control subject. A selection of positive ions is shown. The PC32:0 signal corresponds to the sum of protonated and alkali metal cationized molecular ion species. tc denotes the total count for the respective ion species in the analyzed area. The intense signal surrounding the particle spot is attributed to SiOH, originating from the silicon surface oxide covering the silicon wafer.

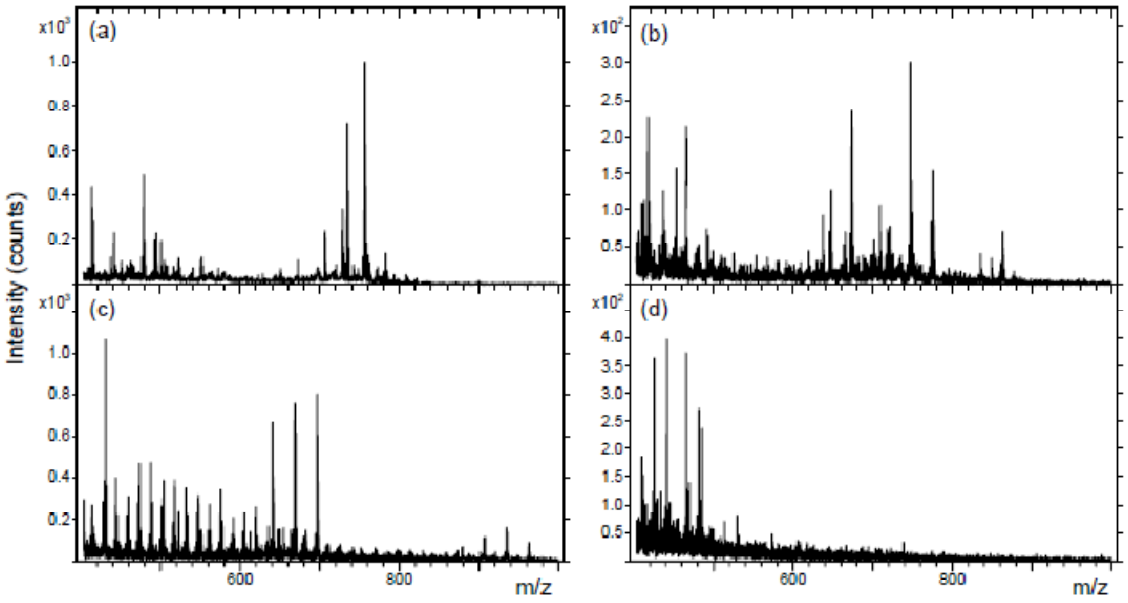

Figure 10 Positive (a and c) and negative (b and d) TOF-SIMS spectra (*m/z* 400-1000) of a particle spot sample (a and b) and a saliva sample (c and d). Exhaled particles were collected from a non-smoking healthy control subject performing forced exhalations for a period of 15 min.

In Table 4, a comparison between exhaled particles, BAL fluid (BALF) and sputum regarding the relative amount of each phospholipid in each class (PC and PG) is shown.

Table 4 Relative amounts of PG and PC in exhaled particles, BAL fluid (BALF) and sputum from healthy subjects.

	Exhaled particles*	BALF[95]**	Sputum[95]**
PC30:0	17	9	8
PC32:1	10	10	9
PC32:0	60	50	53
PC34:1	8	3	3
PC34:0	6	13	15
PC36:2	nd	2	3
Other	1	7	6
PG32:0	14	7	7
PG34:2	nd	8	8
PG34:1	29	33	28
PG36:3	nd	5	7
PG36:2	11	22	21
PG36:1	18	21	26
Other	28	2	3

*% Intensities of the signals corresponding to the indicated group of ions. For PC, ($[M+H]^+$, $[M+Na]^+$ and $[M+K]^+$) for each ion was summed first. **(mol% of total PL in the indicated group of ions (PC, PG or PI). Analysis was performed using electrospray mass spectrometry[95].

Patient study

The mean particle number concentrations generated during forced exhalations did not differ between the groups. No differences in phospholipid composition were observed compared to the introductory study or between patient groups and healthy controls. It was however found that the ratio of CN^- and CNO^- to PO_3^- was higher in spectra acquired from samples collected from patients with asthma and patients with cystic fibrosis. The ratio of unsaturated to saturated phospholipids (PC) did not differ between groups.

PAPER II

Subjects with asthma exhaled significantly lower number of particles compared to healthy controls ($p=0.03$).

In keeping with the results described in **Paper I**, the dominant peaks in the TOF-SIMS spectra (in the high m/z range) were attributed to phospholipids molecular ions. There was a high variability in signal intensities between spectra from different individuals. The ratio of CN^- and CNO^- to PO_3^- did not differ between groups while the ratio of unsaturated to saturated phospholipids (PC) was significantly lower in samples from subjects with asthma.

An initial PCA analysis revealed a grouping due to a change of sample containers in the beginning of the study. The original sample containers were discarded due to problems with condensation on the wafers during sample storage, and so were replaced with containers more suitable for use with silicon wafers. Condensation of water on the sample surfaces may lead to reorganization of the collected sample, thereby influencing the results of the TOF-SIMS analysis which only probes the surface. The first PCA analysis thus led to the exclusion of 9 samples (5 subjects with asthma and 4 controls). The following PCA analyses on positive and negative ion spectra, respectively, showed that the final data set does not have any outliers or groupings attributable to factors such as sample handling.

OPLS-DA analysis showed good separation between subjects with asthma and controls (Model 1 in Table 5). Score plots and loading plots are included in the paper. Most of the separation was attributable to fragment ion data. According to the OPLS loading plot, the most important phospholipid group in terms of differences between the groups in the positive spectra was PC 32:0; several different PG species were responsible for the between-group difference in the negative ion spectra. The impact of the total amount of particles on the TOF-SIMS spectra was evaluated using an OPLS model for particle number concentrations in healthy subjects (Model 2 in Table 5). Low particle amounts were shown to correlate with increased PC 32:0 (in positive

spectra) and PG 34:1 and PG 36:1 (in negative spectra) and vice versa. In the data set, there were two control subjects with very high particle number concentrations. In the original OPLS model, the two orthogonal components were plotted against each other in order to evaluate other factors (aside from asthma) that may affect spectra. It was observed that the two subjects with very high particle number concentrations group together; this shows that the covariance of spectra and particles does not obscure the discriminative information in the predictive component, see Figure 11. A separate OPLS model was performed without the two control subjects with very high particle number concentrations resulting in an improved model compared to the original one (Model 3 in Table 5). The most important molecular ions for the separation remained the same (PC 32:0 and PG 28:0).

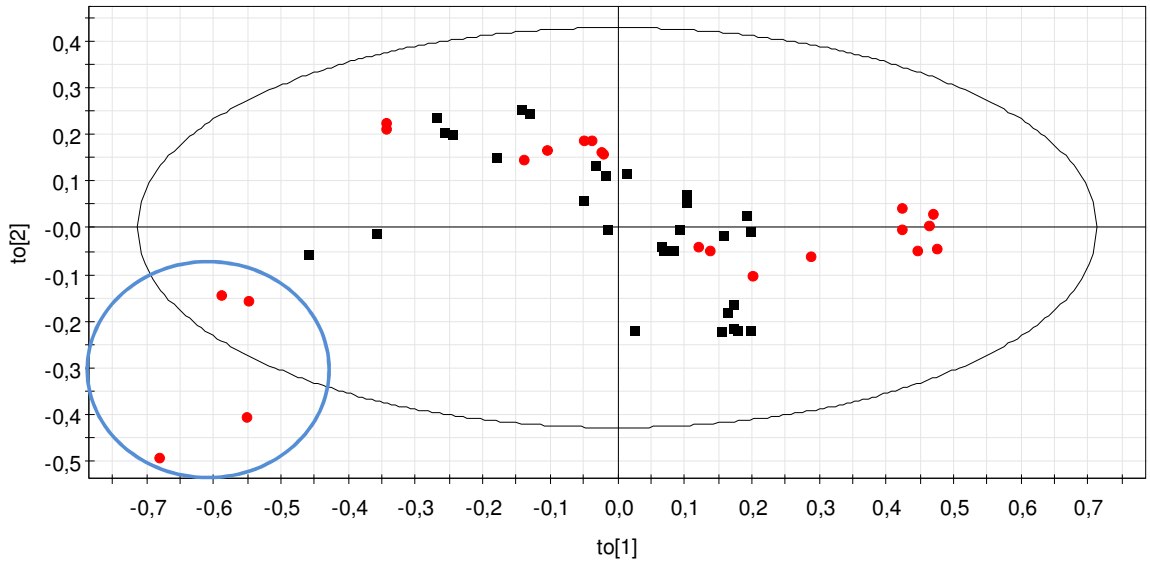

Figure 11 The diagram with the two orthogonal components in Model 1 plotted against each other shows that the two subjects with large particle amounts group together (blue circle).

An OPLS-DA analysis comparing the positive ion spectra from subjects with asthma who were receiving inhaled glucocorticosteroid treatment with subjects with asthma not receiving such treatment showed that PC 32:0 was stronger in samples from patients taking glucocorticosteroids (Model 4 in Table 5). For the negative ion spectra, Q2 was 0.286, indicating the model to be weaker than the model for positive ions.

Table 5 A presentation of R2X, R2Y and Q2 for each OPLS model that was performed. Shown are also the 2-3 signals (m/z) in the high mass range that were most important for the separation in the score plot. The m/z in parenthesis (negative spectra) is likely to correspond to a phospholipid but is unidentified.

Model		n	Positive spectra			m/z	Negative spectra			m/z
			R2X	**R2Y**	**Q2**		**R2X**	**R2Y**	**Q2**	
1	**Asthma group vs. control group**	15/11*	0.606	0.655	**0.517**	734.6 756.6	0.694	0.602	**0.568**	(637.4) 665.4
2	**Control group and particle numbers**	11	0.96	0.999	**0.99**	734.6 756.6 772.6	0.904	0.997	**0.982**	747.6 775.6
3	**Asthma group vs. control group (high-producers excluded)**	15/9*	0.918	0.983	**0.914**	734.6 756.6	0.838	0.894	**0.636**	(637.4) 665.4
4	**Asthma group and glucocorticosteroid intake**	4/11**	0.862	0.972	**0.934**	734.6 772.6	0.641	0.62	**0.286**	747.6 775.6

R2X and R2Y are the fraction of the sum of squares (SS) of all the X's explained by the current component.Q2 is the measure of the quality in multivariate models, based on cross-validation.

**Subjects with asthma/control subjects

***Subjects not taking glucocorticosteroids/subjects taking glucocorticosteroids

Low Q2 values were obtained for OPLS models of TOF-SIMS spectra and FEV_1 (Q2=0.0362) and FENO (Q2=0.0418), respectively.

PAPER III

Three different breathing maneuvers, described in detail in the paper, were performed. Briefly, subjects were asked to exhale to one of the following depths before inhaling to TLC: (1) zero, corresponding to inhalation from FRC and referred to as the FRC maneuver; (2) CP, referred to as the CP maneuver; and (3) RV, referred to as the RV maneuver. Particle number concentrations were recorded in the exhalation following the inhalation to TLC. All inhalation flows and exhalation flows were carefully chosen and monitored so as to prevent dynamic compression. The RV maneuver caused significantly higher number concentrations of particles in the following exhalation than did the CP maneuver (p=0.012) and the FRC maneuver (p=0.012 compared to the CP maneuver). This was true also for each individual size interval

except for the size interval 1.6-2.0 μm where the difference between the CP maneuver was not significantly different from the FRC maneuver.

The differences in number of exhaled particles during the three maneuvers were more evident after correction for the difference in inspired volume (and particles produced in the different volume intervals, i.e. particles produced in the FRC maneuver subtracted from the particles produced in the CP maneuver for CP-FRC volume interval) between the three maneuvers. The mean particle number concentrations produced during inspiration of the three volume intervals, "RV to CP", "CP to FRC" and "FRC to TLC" are presented in Figure 12. For each unit of volume inspired from "RV to CP", the particle number concentration is considerably higher than for each unit of volume in the "CP to FRC" interval and in the "FRC to TLC" interval.

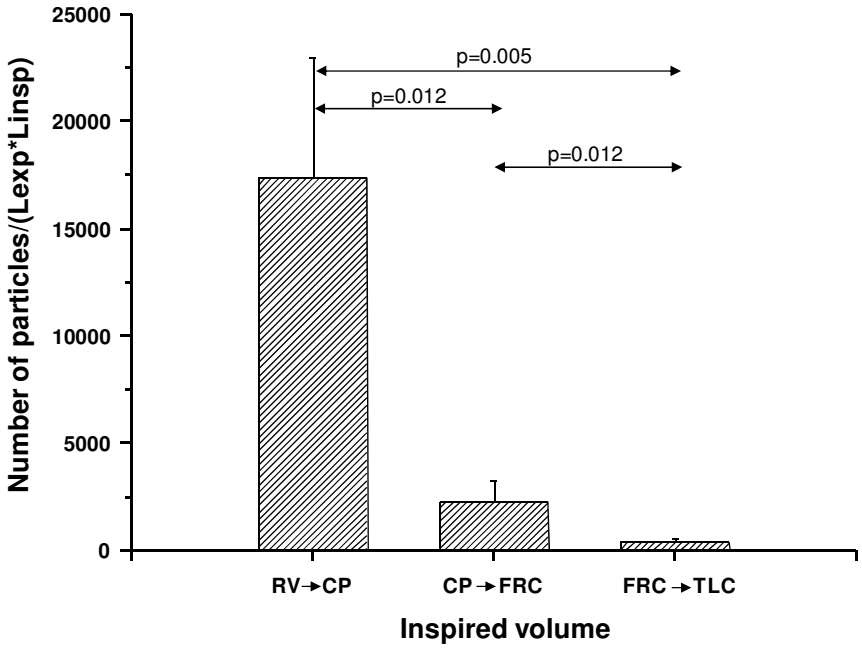

Figure 12 Particle production during inspiration of the three different volume intervals (RV to CP, CP to FRC and FRC to TLC). The particle number concentrations are expressed per unit volume of the interval concerned.

The particle size distributions were similar to those observed in previous studies, with the highest number concentrations being observed in the smallest interval and gradually decreasing as the particles size increased.

It was observed that the number of particles/liter expired*liter inspired for the CP maneuver correlated strongly to the number of particles/liter expired*liter inspired for the FRC maneuver, although this observation was not discussed in the paper.

PAPER IV

Method development

Derivatization conditions (pH and incubation time) were chosen on the basis of the work of Winters et al[167]. The concentration of NPM was decreased to 0.1 mM and instead of serine borate buffer, phosphate buffer (pH 7.3) was used. For determination of GSHt, disulfide groups were reduced using immobilized tris(2-carboxyethyl)phosphine (TCEP); this was done prior to the NPM derivatization step. Reduction was achieved by incubating the samples at 80°C for 30 minutes at pH 8.0. The GSH derivative was separated isocratically by hydrophilic interaction chromatography on a ZIC-HILIC column with a mobile phase of 22% 30 mM ammonium acetate and 78% acetonitrile (v/v) at a flow rate of 0.5 ml/min. The composition of the mobile phase was selected on the basis of the observed retention time and peak shape. The retention time for the NPM-GSH adduct was 6 minutes. A typical chromatogram of a derivatized sample is shown in Figure 13. Results from the validation are presented in the paper.

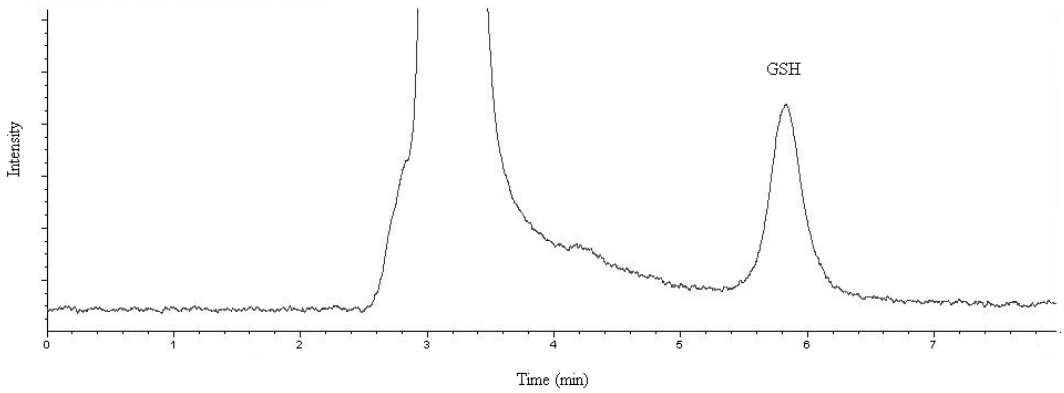

Figure 13 Chromatogram showing the separation of the NPM reagent and the NPM-GSH derivative (GSH), 20 nM GSH standard.

Application to EBC and particle samples

The method was used to determine GSHt, GSH and GSSG in EBC and GSHt in exhaled particles from four healthy volunteers; see Table 6. The concentrations of GSHt in particles were approximated using the obtained substance amount of GSH and estimations of the initial volume of particles at the point of formation, based on particle number concentrations and recent work on the shrinkage of particles during sampling[175]. Particle sizes were adjusted according to Holmgren et al[153].

Table 6 Concentrations (nM) of GSH, GSSG and GSHt in EBC and exhaled particles.

Subject	**EBC**		**Exhaled particles**	
	GSH (nM)	GSSG (nM)	GSHt (nM)*	GSHt (mM)**
1	2.7	nd	28	1.3
2	nd	nd	18	5.3
3	nd	7	15	2.4
4	nd	nd	18	2.1

*Concentration of GSH after reduction with TCEP in a particle extract with a volume of 250 µl

**Estimated concentration in particles at the point of formation in the airways

nd=not detected

DISCUSSION

Non-invasive methods that can be used to collect and analyze material from the lower respiratory tract are very desirable in the study and treatment of respiratory diseases. The availability of sampling methods that are convenient for the patient and can be performed on a regular basis would greatly facilitate the early detection of airway disease and the monitoring of disease progression and the patient's response to therapy. Furthermore, non-invasive methods are unlikely to harm the airways during sampling and the procedure does not imply any risk for the patient. Consequently, there is considerable interest in breath analysis, which has resulted in numerous publications on potential biomarkers. To date, EBC has been the method of choice for sampling exhaled non-volatile compounds such as proteins and lipids. The utility of the EBC method is however restricted by the large inter- and intravariability in the (low) concentrations of these analytes in the condensate and the uncertain origin of potential biomarkers. Given the fact that exhaled non-volatile compounds are likely to be incorporated in particles, there should be other, better, approaches for studying them.

Our pilot studies on particle counting showed that particle number concentrations in breath during tidal breathing are extremely low. On the basis of the assumption that particles are created by the action of shear forces on the respiratory tract lining fluid during breathing, we chose to perform a comparative study of particle formation during forced and tidal breathing. During pilot studies, it became obvious that particle number concentrations were dramatically affected by breathing technique and that as such, any method for collecting exhaled particles should be optimized in terms of the breathing technique used as well as the sampling method adopted. Therefore, a new method was developed that is based on particle impaction and which can be used to count and collect particles at different exhalation flows.

PARTICLE NUMBER CONCENTRATION AND MECHANISMS OF FORMATION

We found that forced breathing produced far more particles than tidal breathing. The mechanism of particle production during forced exhalations is uncertain; it was suggested that it might be similar to that which is believed to occur during coughing, in which particle formation is effected by waves that shear the mucus layer in the large airways[176]. Our observations contradict the findings of Johnson and Morawska, who concluded that "rapid exhalation had little effect on the measured concentration"[177]. However, these authors did not record the

exhalation flow during "rapid exhalation". In **Papers I** and **II**, the inhalation flow and depth of both inhalation and exhalation were different during tidal and forced breathing; this is likely to affect particle formation because of the influence of airway closure and reopening. It is probable that airway reopening occurred during the forced exhalations and that this was partially responsible for the increased number of exhaled particles. Further studies are needed to understand the impact of depth of exhalation and exhalation flow on the origin and number concentration of exhaled particles.

We found that deep exhalations to CP caused significant increases in the number concentrations of exhaled particles in the following exhalation and that the particle concentration increased further when exhaling to RV. The theory behind particle formation during this maneuver is that deep exhalations cause airway closure in the distal airways by the formation of a liquid bridge. During inhalation when the airways expand, the liquid bridge ruptures[70]. This rupture is likely to produce a cascade of particles originating from the RTLF[178]. Particles will be exhaled in the following exhalation. Our results are consistent with those of other recent studies which measured particle number concentrations in exhaled air during deep exhalations [177-179]. However, in these studies CP was not determined and exhalation flow rates were not controlled. Further support for the theory that particles forming before exhalation derives from the fact that breath-holding after inspiration causes a decrease in particle number concentrations in the following exhalation (particles are subject to gravitational settling)[68, 177]. In normal lungs, airway closure occurs in the terminal bronchiole zone. Consequently, most of the exhaled particles originate from the small airways after deep exhalations to RV. This is an exciting finding because it implies that analysis of exhaled particles provides a way to monitor processes occurring in the small airways on the molecular level; the inaccessibility of these airways has made such studies difficult in the past. Because of this difficulty, little is known about the pathology of the small airways in diseases such as asthma and COPD, especially in the early stage of the disease[180]. It should however be noted that airway closure in patients with respiratory diseases may occur further up the airway tree than is the case in healthy subjects, and that this would probably change the location from which the exhaled particles originate. For example, the site of airway closure in asthma is not well known[181].

On the basis of another recent study, which focused on the analysis of exhaled radioaerosols that had been deposited in the lung by different airway deposition patterns, it was concluded that particles collected by the EBC method originated mainly from the central airways[69]. This indicates that there is at least one additional and as-yet unidentified mechanism of particle

formation that does not involve airway closure. In a recent study by our group, we observed that the size distribution of exhaled particles generated by tidal breathing differed from that resulting from deep exhalations, also indicating two different mechanisms of formation. In addition, there was no correlation between the total number of particles generated by tidal breathing and that generated by breathing to RV[153]. The observations made in the study of **Paper III** provide some support for the existence of a second mechanism of formation. After correcting for the differences in inspired volume between the maneuvers, we found an unexpected and strong correlation between the numbers of particles produced after inhalation from FRC (i.e. for a total inspired volume of FRC-TLC) and the numbers of particles produced after inhalation from CP (i.e. for an inspired volume of CP-FRC), see Figure 14. However, there was no correlation between any of these measures and the number of particles produced after inhalation from RV (i.e. for an inspired volume of RV-CP). This indicates that some mechanism other than airway closure and reopening must influence particle production for the inspired volume CP-TLC. If no attempt is made to correct for the differences in inspired volumes, the numbers of particles exhaled per unit volume expired correlate well between the different breathing maneuvers.

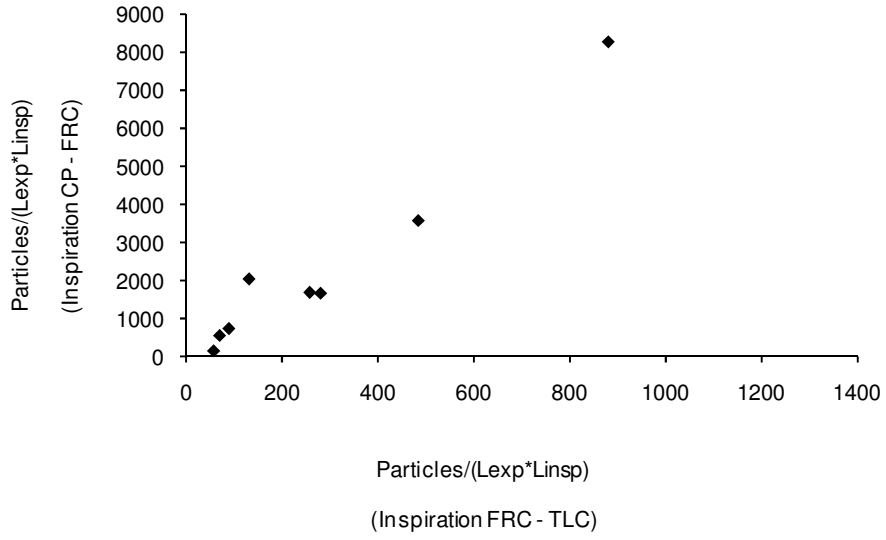

Figure 14 Particle number concentrations (particles/(Lexp*Linsp) for the CP-FRC and FRC-TLC volume intervals. Two individuals that did not perform the CP maneuver (exhalation to CP) are excluded.

There are also studies that have analyzed different analytes in EBC during different breathing maneuvers (without recording particle concentrations) [182, 183]. Changes in the tidal volumes were found to affect the collected volume of EBC but not the protein concentrations within the

EBC[183]. However, the relevance of any results based on the total protein content of EBC is questionable because EBC samples are typically contaminated with human skin keratins derived from the ambient air; these keratins have in some cases been mistakenly assigned as the major EBC proteins[184].

During tidal breathing, inter-individual variation in particle concentrations among healthy subjects can differ immensely. Edwards et al were able to distinguish two groups of particle-producers, and defined individuals exhaling <500 particles/liter as "low-producers" and individuals exhaling >500 particles/liter as "high-producers" (particle size >0.15 μm). In the studies presented here (specifically, the pilot study and that discussed in **Paper III**), this phenomenon was not observed (particle size >0.3 μm). In **Paper II**, where forced exhalations were performed, two subjects had extremely high particle numbers compared to the rest of the subjects. Fabian et al found that 50% of the subjects examined (5 from a group of 10) exhaled >500 particles/liter[50]. Inter-individual differences in exhaled particle concentrations may be due to differences in the physical properties of the RTLF[42]. The variability in the number of particles produced by different individuals may also be attributable to differences in lung architecture. For example, airway closure may occur at different airway dimensions even in healthy individuals.

The size distribution of the exhaled particles was similar in all of the studies presented in this thesis. The highest particle numbers were always observed in the smallest size interval (0.30-0.40 μm); the numbers then became gradually smaller for increasing particle sizes. Particles >2.0 μm were rarely observed, most probably because such large particles are deposited in the lung due to gravitational settling[185]. Because the OPC was initially calibrated using latex spheres, the particle sizes reported in **Papers I** and **II** are not entirely correct. This issue was addressed by our group in a recent paper, where interval limits were recalculated on the assumption that exhaled particles have the same physical dimensions as aqueous droplets[153]. We were able to show that the majority of particles exhaled during airway opening after airway closure were between 0.2 μm and 0.5 μm in diameter.

Does airway disease affect the concentrations of exhaled particles?

It is not well known whether particle concentrations or particle size distributions differ between patients with respiratory disease and healthy subjects[186]. One study that used PCA to compare particle sizes between "symptomatic" patients (cough, cold etc.) and healthy individuals found that during coughing, patients emitted more particles of certain sizes but this did not give rise to a specific size distribution characteristic for symptomatic patients[51]. In **Paper I**, particles were sampled from healthy subjects, subjects with asthma and subjects with cystic fibrosis. The

groups were however too small to detect any significant differences between them. In **Paper II**, particles in exhaled air from somewhat larger groups of healthy subjects and subjects with asthma were compared. Particle concentrations were shown to be significantly lower among subjects with asthma compared to healthy controls. This may be due to differences in the physical properties of the lung lining fluid between subjects with asthma and the healthy controls, which would in turn influence particle formation. In asthmatic airways, airway closure may take place at earlier generations of the bronchial tree than in healthy lungs and this may also affect particle number, size and composition. The mass median aerodynamic diameter was the same in both groups (particles <2.0 μm), indicating that the size distribution of the exhaled particles is similar in both groups. It must be stressed that the groups in this study were small and when removing the two "high-producers" there was no longer any significant difference between the groups regarding particles numbers.

CONDITIONS FOR SAMPLING PARTICLES IN EXHALED AIR

High particle concentrations are desirable in order to minimize the sampling time required to collect sufficient particles for further analysis. Increasing the exhalation flow could potentially result in increased particle production, but may also cause changes in the site of particle formation. In view of this, an attempt to standardize sampling was performed in **Paper I** and **Paper II** by adjusting the forced flow to the individual FEV_1.

Forced breathing can be physically challenging, especially if the patient is in bad respiratory condition, and is therefore not optimal for routine sample collection. However, forced exhalations were initially used to increase the number of exhaled particles in comparison to tidal breathing.

In addition to generating an adequate number of particles, a good sampling technique should generate particles with a known origin. Further studies on particle production, described in **Paper III**, established that high concentrations of particles can be achieved at low exhalation flows. These breathing maneuvers with low exhalation flows were designed to restrict the probable origin of the particles to the distal airways and were adopted in **Paper IV**.

PARTICLE CHARACTERIZATION

Considering the size of exhaled particles (<5 μm) it can be assumed that exhaled particles contain no human cells (typically 10 μm in size). Chemical analyses of particles are discussed in **Papers I, II** and **IV**. In **Papers I and II**, a hypothesis-generating approach was adopted, using direct analysis by TOF-SIMS, without any sample preparation. In **Paper IV,** the particles were

extracted from the wafer to allow for the subsequent determination of GSH, a biomarker for oxidative stress that is known to be abundant in the RTLF.

TOF-SIMS analysis

The TOF-SIMS analyses of particle samples reported in **Papers I** and **II** showed the presence of several surfactant phospholipids. According to BAL and sputum studies, PC 32:0 is the most abundant phosphatidylcholine in the surfactant, followed by PC 32:1, PC 34:1 and PC 30:0 (mol% of total PC). Similar relationships were observed between the signals from peaks assigned to PC species in the TOF-SIMS spectra, providing further support for the hypothesis that the sampled particles were derived from the respiratory tract lining fluid. Judging from their lipid-rich content, it is reasonable to assume that they at least partly consist of micelles or liposomes.

In the studies described in **Papers I and II,** stronger signals from phospholipid molecular ions (for example PC molecular ions) were observed at the rim of the spots. This was most likely a matrix effect because PC 184, which is a fragment derived from phosphatidylcholine and less affected by matrix effects, did not show the same distribution but was more evenly distributed throughout in the spot. Even though only phospholipids were detected in the TOF-SIMS analysis, it is difficult to estimate the relative amount of phospholipids in the sample. Phospholipids are surface-active compounds which are expected to migrate to the sample surface as the particles dry on the silicon plate. However, material below the surface is not amenable to analysis by TOF-SIMS. TOF-SIMS has been useful for the detection and imaging of lipids in several applications involving biological samples[187, 188]. TOF-SIMS analysis of surfactant proteins gives rise to many unspecific fragments some of which will overlap with phospholipid fragments making it difficult to identify them. For instance, the signal at m/z 104 (which was an important differentiator in the OPLS model of TOF-SIMS spectra from subjects with asthma and healthy controls) could be assigned to a PC fragment but might also be assigned to a fragment derived from the amino acid methionine[189]. Numerous other assignments are also possible. The molecular ion of surfactant protein C was undetectable when mixed with PC and PG[190].

It is important to prevent the contamination of exhaled particles with saliva because many interesting biomarkers may be present in high concentrations in saliva and so such contamination would make it impossible to draw reliable conclusions regarding those markers. Normally, the absence of saliva contribution in breath samples is verified by the absence of amylase, an abundant protein in saliva. However, this approach was not adopted in the studies reported in this thesis. Instead, TOF-SIMS spectra of saliva samples and particle samples were

compared. Spectra from saliva had much lower signal intensities from phospholipids than did particle samples. Whereas the signal corresponding to PC32:0 was persistently the most abundant PC signal in all particle samples, there were no dominating signals among signals corresponding to PC species in spectra from saliva. The dominant signals in spectra from saliva were not the same as those in the spectra of particle samples. It is therefore unlikely that the particle samples were contaminated with saliva. Furthermore, recent studies by our group on proteins in exhaled particles did not identify any evidence for the presence of amylase and thereby confirmed the conclusion that particle samples are unlikely to be contaminated with saliva[191].

In **Paper I**, it was observed that different spots from the same sample gave rise to very similar TOF-SIMS spectra (as judged by visual inspection). This high degree of spectral reproducibility was also observed in **Paper II**, where duplicate samples from single individuals generally clustered together in the multivariate analysis score plots. This observation indicates that all of the exhaled particles collected on wafers are similar in terms of their surface lipid composition and that TOF-SIMS analysis has good within-sample reproducibility.

Determination of GSH and GSSG

The GSH content of particle samples was determined after reducing the disulfide bonds within the sample; the measured values therefore reflect the combined levels of GSH and GSSG in the original sample. After reduction, GSH was detected in all of the particle samples examined (n=4).

In EBC, the amount of collected particles is unknown, both because the exhaled concentration is not monitored and also because the method's ability to sample particles is unknown. It is therefore not possible to determine whether differences in GSH levels between individuals reflect actual differences in the concentration of GSH in the RTLF or just differences in the concentration of exhaled particles. This issue can be ignored by considering ratios of biomarkers instead of absolute values. The GSH/GSSG ratio has been suggested as a marker of oxidative stress on the basis of the observation that GSSG increases in relation to GSH during oxidative stress. The initial aim of **Paper IV** was therefore to develop a method for determining of this ratio.

NPM was chosen as the derivatization agent because it allows for a rapid, sensitive and specific assay. The derivatization reaction inhibits autooxidation, but does not allow detection of GSSG. Reduction of GSSG, which is a necessary prerequisite for derivatization, was accomplished with

immobilized TCEP. The method developed here exhibited better limit detection than has previously been reported for NPM. Separation of fluorescent compounds was performed on a ZIC-HILIC column with a hydrophilic stationary phase. In RP-HPLC, NPM and its hydrolysis products are detected at different retention times in the chromatogram. These NPM-related products complicate the interpretation of the data because they may interfere with the analyte of interest. Using the ZIC-HILIZ column, the NPM-related species eluted as one peak early in the chromatogram and were well separated from the NPM-GSH adduct.

The EBC approach is designed for sampling tidal breaths, which contain very low concentrations of exhaled particles. This may explain why GSH and GSSG were only detected in two of the EBC samples discussed in **Paper IV**. Even though low detection limits were obtained for GSH, both GSH and GSSG levels were generally below detection limits in most of the EBC samples. This was also observed in pilot studies, including both healthy control subjects and subjects with asthma, during the development of the method. In light of these results, it seemed sensible to examine the glutathione levels in exhaled particles. The estimated concentrations of GSH in particles at the point of formation are somewhat higher but in good agreement with previous estimations of GSH in RTLF (based on analysis of BAL fluid). Because the GSH/GSSG ratio cannot reliably be determined by analysis of EBC, methods for glutathione analysis should be optimized for use with particle samples and employed in studies looking at larger groups of subjects. A few attempts were made to detect GSH (without prior reduction of disulfides) in particle samples but they were unsuccessful. It will therefore be necessary to develop an optimized sample preparation procedure in order to properly evaluate the scope for detecting GSH in particles. Kleinman et al showed that in plasma, there was a 75% loss of GSH after 30 min because of disulfide (GSSG and cysteine-GSH) formation[192], implying that an accurate determination of the GSH levels in particles (at the point of formation) is not feasible. However, it is important to remember that GSH could potentially be oxidized to end products other than disulfides during particle sampling. Estimated concentrations of GSH at the point of formation were somewhat higher than the GSH concentrations reported for BAL fluid indicating that oxidation during sampling to other oxidation products than disulfides may not be a big problem. Future studies should focus on optimizing and validating the method for determining GSH and GSSG in particles.

The recorded particle number concentrations can be used to calculate the total number of exhaled particles. In **Paper IV**, we tried to calculate the volume of particles collected by the impactor. Attempts were made to estimate the particle size distribution and the fraction of

particles lost because the impactor cut-off did not coincide with the lower limit of the smallest size bin. In addition, the obtained volume was corrected with a factor taking the shrinkage of particles due to drying during sampling into account. Taking these corrections into account, the concentration of GSH in the particles as they are formed from the RTLF was estimated. This approach represents a first attempt to calculate the concentrations of analytes in the RTLF at the site of particle formation, and needs to be validated, possibly by comparing the estimated results to experimentally-determined values for BAL and other analytes in particle samples. This method was introduced in **Paper IV** primarily in order to confirm that the GSH concentrations measured in the particles were reasonable compared to those observed in BAL fluid.

Can the chemical information obtained in this work tell something about the origin of exhaled particles?

The results presented in **Paper III** suggest that the exhaled particles probably originate from the distal airways. In the TOF-SIMS studies (**Papers I** and **II**), forced exhalations were used and the origin of particles under these conditions is more uncertain. The TOF-SIMS analyses of particles revealed the presence of several phospholipids, which are known to be abundant in surfactant that is synthesized and secreted by alveolar type II cells. However, this finding does not constitute evidence that particles are formed in the distal airway because surfactant is also present in the conducting airways[193]. The same is true of glutathione, which is abundant in saliva, sputum, and BAL fluid[121, 132, 194].

PARTICLES AND AIRWAY DISEASE

In **Paper I**, the TOF-SIMS spectra of exhaled particles from small groups of patients with asthma, patients with cystic fibrosis, and healthy subjects were compared. No apparent differences were observed in the mass spectra measured from samples from the different groups. Although TOF-SIMS spectra may be difficult to interpret, especially quantitatively, the sensitivity of the technique and the fact that it requires no sample preparation are great advantages. To evaluate the potential of the method, a study using larger groups was therefore performed (**Paper II**). The aim was to compare the TOF-SIMS spectra of particles from subjects with asthma and healthy controls in order to determine whether the two groups could be distinguished by multivariate analysis based on mass spectra and if so, to identify ions that separate the groups. A similar approach using TOF-SIMS and discriminant analysis for separation and discrimination of bacterial samples has recently been described[195, 196].

According to the OPLS analysis, signals assigned to PC 32:0 and species of PG were more intense in the TOF-SIMS spectra from subjects with asthma than in those generated by the

controls. Few studies have examined the composition of the surfactant in subjects with asthma. It has been reported that sputum samples from subjects with asthma have low surface activity;[144, 148] it was suggested that this may be due to leakage of plasma proteins[145]. PG and PI have been reported to be decreased in BAL fluid from patients with asthma after antigen challenge,[197] whereas PC 32:0 has been reported to be elevated in children with asthma[149].

An additional OPLS model was created to compare subjects with asthma on the basis of their glucocorticosteroid intake. This model gave a high Q2 showing that PC 32:0 was higher among subjects taking glucocorticosteroids, which could be taken to indicate that the medication affects the spectra of the exhaled particles. It must however be stressed that there is a risk for over-fit because the group without steroid intake was very small.

The ratio of CNO^- and CN^- (fragments that are considered to be derived from proteins and peptides) to PO_3^- (fragment from phospholipids) illustrates the difficulty of discriminating between groups on the basis of differences in the relative intensities of specific peaks in TOF-SIMS spectra. The rationale for using this ratio is that the ratio of total phospholipids to total proteins may give an indication of plasma leakage. As described in **Paper I,** the ratios of CNO^- and CN^- to PO_3^- were higher among asthmatics, but the number of subjects was very small. In **Paper II,** no significant difference in this ratio between groups was observed. However, studies have also shown that the concentration of plasma PC 34:2 in BAL is increased in asthma[95, 145], implying that an elevated protein-to-phospholipid ratio might not be an appropriate indicator of plasma leakage. In particle samples, the molecular ion of PC 34:2 was not detectable using TOF-SIMS, but fragments of the species might be present, and contribute to the PO_3^- signal, in the spectra. In the loading plots, both CN^- and CNO^- were elevated in subjects with asthma, indicating differences between subjects with asthma and controls regarding the relative amounts of peptides/proteins in particles. Multivariate analyses do not focus on the ratios of two individual species; instead, they allow for the simultaneous analysis of all of the detected ions relative to one-another. However, their interpretation can still be complicated by matrix effects and the CN^- and CNO may not solely derive from proteins and peptides. The relationship between the topography of a sample and the amount of material it represents can have an influence on the results obtained, as reported in **Paper II**. Restricting the analyzed area used in the multivariate analysis, i.e. not including spectra from the silicon wafer outside the spot, would probably facilitate interpretation of the results because different samples may have different spot sizes. It is also crucial to handle all samples in the same way throughout the study; the importance of this point was emphasized by the observation that changing the type of sample

container used had a pronounced effect on the results obtained and necessitated the exclusion of the first samples collected.

The ratio of unsaturated to saturated phosphatidylcholines was significantly lower in samples from subjects with asthma than in samples from controls. This difference could possibly be due to a higher degree of oxidative stress in asthmatic airways.

A validation of the TOF-SIMS analysis and the sampling technique regarding storing, inter-individual and intra-individual variation prior to the patient study would have facilitated data interpretation. On the other hand, it was important to evaluate the potential of the method to detect differences in phospholipid composition before doing extensive validation. No specific ions, notably plasma phospholipids, were detected in samples from subjects with asthma. Future comparative studies of phospholipid composition should therefore first focus on developing quantitative methods for determining individual phospholipids using electrospray ionization mass spectrometry or internal standards in conjunction with TOF-SIMS. However, such methods are more time-consuming and may lead to loss of sample (and information) due to need for additional sample preparation steps.

Asthma is a heterogeneous disease and the subjects included had varying degree of severity and not all were taking regular medication. Furthermore, the groups in the study were small. The clinical relevance of the present results is hence limited but still suggests that phospholipid composition in particles is an interesting area of research in asthma.

CONCLUDING REMARKS

Like other breath tests, the procedure presented here is non-invasive making it possible to do repeated sampling. Because it is non-invasive, it can also be used as a screening tool, useful for early detection of disease.

The principal purpose of developing a method for sampling particles was to address the major problem of exhaled air analysis concerning non-volatiles, namely that the amounts of potentially interesting substances that are exhaled are extremely low. Optimizing the sampling efficiency of exhaled material is essential for the development of robust and reliable assays using instruments that are available in most labs in clinics and hospitals. The results from **Paper IV** indicate that particle impaction is a more efficient technique than EBC for sampling of non-volatiles, such as glutathione. The results reported in **Paper III** show that particle production can be enhanced using an appropriate breathing technique which is important because it allows for the efficient acquisition of large samples of exhaled material. A possible problem for some biomarkers is that the temperature and sampling time required may allow for the modification of some biomarkers during the process of sampling exhaled particles.

Another equally important issue for the development of the sampling technique was to address the fact that there is a high inter-individual (in some cases also high intra-individual) variation in particle concentrations that will give confusing results for quantitative assays unless they are corrected for. Studies examining the correlation between particle numbers and measurements of the substance amounts of specific analytes will be needed to confirm whether or not the current method for sampling particles in exhaled air constitutes a useful solution to this problem. There is strong indication that this will be possible as indicated by our recent data[198].

In this thesis, it is shown that breathing technique is very important. The breathing technique adopted probably affects the site of formation of particles. This observation illustrates the difficulty with exhaled breath sampling compared to urine and blood sampling. However, an individual who is cooperative and has experience with the technique will probably generate samples with low variability concerning breathing technique and particle numbers. It is very important to take this factor into consideration during method development. Differences in the origin of particles that arise from differences in the degree and location of airway closure may make it difficult or impossible to reliably compare samples from different individuals even if the sampling maneuvers are done correctly. This is especially important when comparing healthy subjects and subject with a respiratory condition affecting the site of airway closure. An optimal

way of sampling, according to observations made in this thesis, may be using a well controlled maneuver. However, such a maneuver may be difficult to perform in children and subjects with severe respiratory disorders.

The principal aim in this work was to chemically characterize exhaled particles and to thereby explore the scope for using such particles for biomarker detection and monitoring. The data presented in this thesis show that this new technique for sampling and counting exhaled particles has considerable potential for future use in biomarker detection and monitoring in airway disease.

SUMMARY OF CONCLUSIONS

The main findings of this work are listed below.

- Particles in exhaled air can be sampled by impaction in a thermostatted sampling environment.
- Exhaled particles contain phospholipids characteristic of the pulmonary surfactant and also glutathione, which is an important anti-oxidant in the airways. This indicates that particles in exhaled air originate from the RTLF.
- The phospholipid composition of particles differs between subjects with asthma and healthy controls, and this difference may be partly related to the intake of glucocorticosteroids.
- Particles are formed during airway reopening after airway closure.
- Glutathione is detectable in exhaled particles. Collection of exhaled particles seems to a more suitable method for determination of glutathione in exhaled air than is EBC.

FUTURE RESEARCH

In terms of *chemical characterization*, research into the protein content of exhaled particles is ongoing, using both global mass spectrometry and immunoassays targeting specific proteins like surfactant proteins[191, 198]. A global approach, similar to the one presented here, could be used for detailed lipid characterization using advanced mass spectrometric technique. In addition, a targeted approach should be used for interesting biomarkers such as cytokines, eicosanoids and oxidized phospholipids. Extraction procedures must be developed and optimized for the different chemical analyses.

In terms of the *mechanisms of particle formation*, it remains to be determined which other mechanisms are operative during tidal and forced breathing. It is still not clear why the number concentrations of particles in exhaled air differ so much among healthy individuals.

Suggestions for the improvement and development of matters studied in this thesis:

TOF-SIMS and OPLS analysis can be evaluated for group comparisons but particle sampling and data analysis should be further optimized. A quantitative method for analysis using liquid chromatography-mass spectrometry could be developed for detailed studies of the phospholipid composition in terms of PC and PG. An important factor to consider during method development is the stability of lipids during particle sampling.

An optimized sample preparation procedure for the determination of GSH in exhaled particles should be developed. The normal GSH levels in particles exhaled by healthy individuals should then be determined. Measured GSH levels in exhaled particles could be used to study oxidative stress in diseases such as asthma. The method presented here could also be expanded upon for the determination of other important thiols such as cysteine and homocysteine.

Some general comments for future research:

- The possible utility of particle number concentrations as a biomarker for airway disease should be investigated
- Validation of particle sampling and counting including the possible loss of particles during sampling, either to the walls of the instrument's tubes or because of impaction inefficiency, should be performed.
- The quantification of the volume of particles collected on the wafers should be validated.

- Breathing techniques for particle sampling must be carefully designed so that patients with severe respiratory diseases are able to perform the breathing correctly. This is also important in order to obtain good reproducibility for the marker of interest.

POPULÄRVETENSKAPLIG SAMMANFATTNING

En helt ny metod för insamling av partiklar i utandningsluft har utvecklats vid Göteborgs Universitet (Arbets- och Miljömedicin i samarbete med Atmosfärskemi). En unik egenskap, i detta sammanhang, är att metoden möjliggör kvantitativ mätning av de partiklar som andas ut och samlas in.

Luften vi andas ut innehåller tusentals olika ämnen som härrör från luftvägsslemhinnan. Dessa ämnen har potential att fungera som indikatorer för olika sjukdomar som angriper luftvägarna. En klass av ämnen som inte studerats i stor utsträckning i utandningsluft är proteiner och lipider. Proteiner och lipider är inte flyktiga men återfinns ändå i utandningsluft. Det har länge antagits att små partiklar som bildas från luftvägsslemhinnan för med sig dem i utandningsluften. Partiklar som andas ut under vanlig viloandning har emellertid aldrig samlats in och studerats specifikt. Det har heller inte utretts hur och var i lungorna partiklar bildas. Denna typ av kunskap är vital för att kunna studera potentiella sjukdomsmarkörer i utandningsluft. Koncentrationer av partiklar i utandningsluft under vanlig viloandning varierar stort mellan individer och beror också på andningsmönstret.

I det här arbetet kunde vi visa att en viktig mekanism för partikelbildning är när små luftvägar öppnar sig efter en djup utandning då så kallad luftvägsstängning förekommer. Detta innebär att partiklarna kommer från de små luftvägarna. Vi kunde också visa att partiklarna innehåller flera olika fosfolipider. Fosfolipider är ytaktiva ämnen som återfinns i surfaktanten i luftvägsslemhinnan. Surfaktanten sänker ytspänningen i lungblåsorna och bidrar därmed till att hålla dem och de små luftvägarna öppna. En jämförelse av partikelprover från patienter med astma och friska försökspersoner visade att sammansättningen av dessa fosfolipider skiljde mellan grupperna. Signalerna från de två viktigaste fosfolipiderna i surfaktant, fosfatidylkolin och fosfatidylglycerol, var relativt förhöjda i partiklar från patienter med astma jämfört med friska försökspersoner. I det här arbetet utvecklades även en metod för bestämning av glutation som är en viktig anti-oxidant i luftvägarna och som bland annat skyddar lungorna mot reaktiva ämnen som finns i omgivningsluften. Vi kunde visa att glutation är detekterbart i partikelprover från friska individer. Den kemiska analysen av ett partikelprov ger mängden glutation i provet. För att kunna jämföra prover mellan patienter behöver man emellertid koncentrationen av sjukdomsmarkören. Detta är möjligt tack vare registrerade partikelkoncentrationer under

partikelinsamlingen och en teoretisk uppskattning av partiklarnas storlek vid bildningsögonblicket.

Insamling och analys av partiklar i utandningsluft öppnar för nya möjligheter att utveckla icke-invasiva metoder för att undersöka luftvägssjukdomar. Framtida studier bör fokusera på att vidare studera innehållet i utandade partiklar och att validera insamlingsmetoden för olika biomarkörer i partiklar från friska och sjuka individer.

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