

Exploring small airways using breath analyses

Emilia Viklund

Department of School of Public Health and Community Medicine
Institute of Medicine
Sahlgrenska Academy, University of Gothenburg



UNIVERSITY OF GOTHENBURG

Gothenburg 2022

Cover illustration: “Airway irritants visiting the alveolar region” by Tobias Pettersson Viklund

Exploring small airways using breath analyses

© Emilia Viklund 2022

emilia.viklund@amm.gu.se

ISBN 978-91-8009-781-9 (PRINT)

ISBN 978-91-8009-782-6 (PDF)

Printed in Borås, Sweden 2022

Printed by Stema Specialtryck AB



Thank you for reading!

Exploring small airways using breath analyses

Emilia Viklund

Department of School of Public Health and Community Medicine,

Institute of Medicine
Sahlgrenska Academy, University of Gothenburg
Gothenburg, Sweden

ABSTRACT

Background: Airway irritants such as allergens, tobacco smoke and viruses may cause damage in the small airways, usually undetected with routine diagnostic methods. Analyses of endogenously produced nitric oxide (NO) and endogenously produced small droplets (i.e. particles) in breath (PEx), have the potential to reflect exposure related changes in the small airways. Furthermore, in PEx, both exposure and effect markers can potentially be analyzed.

Aim: The overall aim of this thesis was to explore small airway effects of exposure to airway irritants in healthy subjects, in subjects with asthma, in cigarette smokers and in subjects with COVID-19 infection, by examining exhaled breath.

Methods: We explored methods to model alveolar NO (CANO), the number of PEx as well as content of major surfactant lipids and proteins in PEx. In addition, we explored if COVID-19 infection could be detected in PEx.

Results: Pollen season was not associated with an increase in CANO, although it was markedly increased in some subjects with asthma. Small airways ventilation inhomogeneity in subjects with asthma was associated with slightly increased CANO on group level, and markedly increased in some subjects, with no difference on levels of surface active lipids in PEx but markedly decreased number of PEx. Current compared to never smokers were associated with higher levels of lipids in PEx in subjects with normal lung function. Current smokers were associated with higher levels of surfactant protein A in PEx and number PEx in subjects with impaired lung function. Detection of SARS-CoV-2 RNA was possible in PEx, in some but not all subjects early in disease course of COVID-19. RT-PCR analyses were performed on PEx-samples generated from as little as 20 relaxed breaths, 10 deep breaths and 3 coughs.

Conclusions: These results of breath analyses including endogenously produced particles and alveolar NO highlight the potential of these methods to reveal effects of airway irritants in small airways prior to lung function decline. Furthermore, PEx are shown to contain SARS-CoV-2 RNA in subjects with confirmed COVID-19, highlighting the potential of detecting respiratory virus infection in exhaled breath.

Keywords: Exhaled particles, small airways, alveolar nitric oxide, SARS-CoV-2, birch pollen, smoking, lining fluid

ISBN 978-91-8009-781-9 (PRINT)

ISBN 978-91-8009-782-6 (PDF)

SAMMANFATTNING PÅ SVENSKA

I din utandningsluft finns, förutom olika gaser, mikroskopiskt små vätskedroppar. Dessa vätskedroppar, kallade partiklar i en aerosol, härrör från det skyddande vätskeskikt som bekläder luftvägsträdet. Vätskeskiktet i små luftvägar är rikt på ämnen med uppgift att bibehålla lungfunktion och skydda kroppen mot luftvägsirriterande ämnen. Ämnen som vi andas in, såsom tobaksrök, allergener och virus, kan trigga igång immunförsvaret i vätskeskiktet. Möjligheten att påvisa skada i de små luftvägarna orsakad av inandade ämnen är dock ytterst begränsad då de små luftvägarna just är så små ($<2\text{mm}$) och därför svåråtkomliga. Genom att andas med olika andningstekniker och med hjälp av speciella analysmetoder kan de små luftvägarna däremot indirekt studeras med hjälp av utandningsluft.

Denna avhandling syftade till att undersöka möjligheterna att identifiera påverkan på små luftvägar, genom att analysera utandningsluft med olika metoder från individer med sannolik påverkan på små luftvägar. De metoder vi har använt är analys av den kroppseget producerade gasen kvävemonoxid (NO), vars produktion ökar vid inflammation i luftvägarna, samt analys av utandningsluftens små vätskedroppar.

Avhandlingens huvudresultat visar att covidsmitta kan påvisas i utandade aerosolpartiklar förknippade med luftburen smitta (partiklar $<5\mu\text{m}$). Covidsmitta påvisades i utandningsprov hos några men inte alla provtagna individer med pågående COVID-19 infektion, insamlat vid så lite som 20 vanliga andetag, 10 djupandningar samt 3 hostningar. Vidare hade några, men inte alla, pollenallergiska individer med astma i pollensäsong markant förhöjda nivåer av NO-bidraget från små luftvägar. I en jämförande studie av vuxna med astma med normal respektive nedsatt lungfunktion fann vi ingen signifikant koncentrationsskillnad av de vanligast förekommande ytaktiva lipiderna i vätskeskiktet i de små luftvägarna, mätt i utandade partiklar. Förträngda små luftvägar var däremot associerat med mindre mängd utandade partiklar samt högre nivå av NO från små luftvägar, dock med stor spridning. I en explorativ studie hade nuvarande rökare jämfört aldrig rökare däremot högre nivåer av dessa lipider, trots annars normal lungfunktion. Vid påverkade luftflöden var nuvarande rökning associerat med högre nivåer av surfaktantprotein A i utandade partiklar, samt markant större mängd utandade partiklar.

Resultaten belyser potentialen med analys av partiklar och kvävemonoxid i utandningsluft för att påvisa luftvägsirriterande ämnens eventuella påverkan på små luftvägar. Påvisandet av luftvägsvirus i utandningsluften öppnar upp för fortsatta studier av utandningsluft utifrån exempelvis utbredning av virusinfektion i luftvägarna men också utifrån luftburen smittspridning.

LIST OF PAPERS

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

- I. Bake, B, **Viklund, E**, Olin, A-C. Effects of pollen season on central and peripheral nitric oxide production in subjects with pollen asthma. *Respiratory Medicine* 2014; 108: 1277e1283.
- II. **Viklund, E**, Kjellberg, S, Schiöler, L, Almstrand, A-C, Gustafsson, P, Olin, A-C. Major surfactant phospholipids, exhaled particles and small airways ventilation inhomogeneity in adult asthma (in manuscript).
- III. **Viklund, E**, Bake, B, Hussain-Alkhateeb, L, Koca Akdeva, H, Larsson, P, Olin, A-C. Current smoking alters phospholipid- and surfactant protein A levels in small airway lining fluid: An explorative study on exhaled breath. *PLoS ONE* 2021; 16(6): e0253825.
- IV. **Viklund, E**, Kokelj, S, Larsson, P, Nordén, R, Andersson, M, Beck, O, Westin, J, Olin, A-C. Severe acute respiratory syndrome coronavirus 2 can be detected in exhaled aerosol sampled during a few minutes of breathing or coughing. *Influenza Other Respi Viruses* 2022; 1-9. doi:10.1111/irv.12964.

CONTENT

ABBREVIATIONS.....	IV
DEFINITIONS IN SHORT	VI
1 INTRODUCTION	1
1.1 Why explore small airways using breath analyses?	1
1.2 The respiratory tract lining fluid- first line of defense	2
1.3 Airway irritants.....	4
1.4 Physiological methods to assess small airways impairment.....	5
1.5 Exhaled breath	7
1.6 Methods to assess small airways in exhaled breath.....	7
1.7 Previous studies using alveolar NO and PEx to assess exposures to airway irritants	14
1.8 Knowledge gaps adressed in this thesis.....	15
2 AIM	16
3 STUDY PARTICIPANTS AND METHODS	17
3.1 Study design.....	18
3.2 Study participants	18
3.3 Methods	21
3.3.1 Particles in Exhaled air (PExA) (Paper II, III and IV).....	22
3.3.2 Breath Explor (BE) (Paper IV)	26
3.3.3 Alveolar NO (Paper I and II)	28
3.3.4 Spirometry (Paper I, II and III)	29
3.3.5 Multiple breath washout (MBW) (Paper II).....	29
3.3.6 Single breath washout (SBW) (Paper III)	31
3.3.7 Asthma Control Test (ACT) (Paper II)	32
3.3.8 Systemic inflammatory variables (Paper I, II and III)	32
3.3.9 Stationary birch pollen measurement (Paper I).....	32
3.4 Outcomes	33
3.5 Statistical methods.....	33
4 RESULTS.....	35

4.1	Alveolar NO and birch pollen exposure (Paper I).....	35
4.2	Alveolar NO and small airways ventilation inhomogeneity (Paper II) ...	39
4.3	Amount of PEx and small airways ventilation inhomogeneity (Paper II)	41
4.4	Amount of PEx and active smoking (Paper III).....	43
4.5	Amount of PEx and SARS-CoV-2 infection (Paper IV)	44
4.6	Major surface active lipids in PEx and small airways ventilation inhomogeneity (Paper II)	46
4.7	Major surface active lipids in PEx and current exposure to tobacco smoke (Paper III).....	47
4.8	Surfactant protein A in PEx (III).....	50
4.9	Viral RNA in PEx (Paper IV)	51
5	DISCUSSION	54
5.1	Validity and reliability aspects of alveolar NO (Paper I, II)	55
5.2	Validity and reliability aspects of amount PEx (Paper II, III and IV)	59
5.3	Validity aspects of content in PEx (Paper II, III and IV)	63
6	CONCLUSION	67
7	FUTURE PERSPECTIVES	69
	ACKNOWLEDGEMENT	70
8	PAPERS ASSOCIATED TO THIS WORK NOT INCLUDED IN THE THESIS	71
	REFERENCES.....	72

ABBREVIATIONS

ACT	Asthma Control Test
BAL	Broncho alveolar lavage
BE	Breath explor
BMI	Body mass index
BW	Bronchial wash
CANO	Alveolar NO concentration
Ct	Cycle thresh-hold
DPPC	Dipalmitoyl-phosphatidylcoline
EBC	Exhaled breath condensate
ELISA	Enzyme linked immunosorbent assay
ERS	European Respiratory Society
FeNO	Fraction of exhaled nitric oxide
FENO50	Fraction of exhaled nitric oxide at 50 ml/s
FEV₁	Forced expiratory volume in first second of exhalation
FRC	Functional residual capacity
FVC	Forced Vital Capacity
ICS	Inhaled corticosteroid
JawNO	Bronchial NO flux
LCI	Lung clearance index
LLN	lower limit of normal
MBW	Multiple breath washout
NO	Nitric oxide

NOS	Nitric oxide synthase
PE_x	Particles in exhaled air
POPC	Palmitoyl-oleoylphosphatidylcholine
RT-PCR	Real time Polymerase chain reaction
RV	Residual volume
S_{acin}	MBW derived index of ventilation inhomogeneity at the entrance to the acinar airway zone
SBW	Single breath washout
S_{cond}	MBW derived index of ventilation inhomogeneity in the small conductive airways
SF₆	Sulfur hexafluoride
Sn_{III}	Concentration normalized phase III slope
SP-A	Surfactant Protein A
TLC	Total lung capacity
ULN	upper limit of normal
V_t	Tidal volume

DEFINITIONS IN SHORT

PE _x	Exhaled particles with a size range between 0.41-4.55 µm in diameter
PE _x A	The instrument used for sampling of PE _x
Small airway	Airways with an inner diameter of <2mm
Small airway dysfunction (SAD)	Quantifiable impaired function in the small airways, assessed physiologically
Small airway disease	Specific pathology in small airways
Early disease	Pathological changes early in the disease course

1 INTRODUCTION

1.1 WHY EXPLORE SMALL AIRWAYS USING BREATH ANALYSES?

Exposure to airway irritants, such as allergens and tobacco smoke but also viruses are known to affect the small airways (Bosken, Wiggs, Pare, & Hogg, 1990; Hogg, Pare, & Hackett, 2017; Sungnak, Huang, Bécavin, & Berg, 2020). One important step towards detection of exposure related impairment is the sampling of biological material where inflammation takes place and to sample at the right time and in a correct way. Due to the inaccessible nature of the small airways (defined as airways with an inner diameter of $<2\text{mm}$), alterations in this region are difficult to detect, and are usually not discovered until a larger and/or irreversible damage is present (Hogg et al., 2017). The large variety in how/when we respond to inhalable airway irritants highlights the need for exploring patient friendly methods with potential to detect alterations in the small airways in a repeatable and reproducible way.

Methods used today to obtain a sample from the small airways, such as Broncho alveolar lavage (BAL) and biopsy sampling are invasive, complicated and difficult to repeat and reproduce (Balbi et al., 2007; Balzar, Wenzel, & Chu, 2002). Fortunately, exhaled breath consists of gases and small droplets (i.e. particles in an aerosol) originating in the small airways, and therefore allows for non-invasive sampling with the potential to reflect exposure related effects on the small airways. Aerosol particles, originating in the small airways lining fluid protecting the airways from inhalable airway irritants, have the potential to reveal exposure effects of airway irritants on the small airways lining fluid and to reveal the inhaled substance itself if deposited in the fluid. In addition to particles from the lining fluid, the airways also produces nitric oxide (NO), increased in type-II driven inflammatory conditions, which can be monitored in exhaled breath. As the diffusion of NO into the exhaled air is dependent on the concentration gradient and exhalation flow, the contribution of NO produced in the alveolar region may be separated from that in the more central by the use of mathematical models.

There is a long tradition in the research group *Airways and Health* at Occupational and Environmental Medicine, University of Gothenburg, of working with methods to assess exposure related effects within the airways. The research group works in the intersection of physics, medicine, chemistry and physiology. The method to sample particles in exhaled air (PExA) was developed by the research group. Prior to the development of PExA, the research group had also had a history of analyzing exhaled NO in different exposure settings.

This thesis takes off in the further development of the PExA method and the use of exhaled NO to explore its potential to address small airways involvement in different exposure settings.

1.2 THE RESPIRATORY TRACT LINING FLUID- FIRST LINE OF DEFENSE

The respiratory tract lining fluid (RTLFL) covers the airways and constitutes the first barrier of defense against inhalable irritants (Griese, 1999; Macklem, 1998). In the alveolar region, the lining fluid also functions as a preventative force that lowers the surface tension, preventing airways and alveoli to collapse (Goerke, 1998).

The composition of RTLFL is different along the respiratory tract, rich in mucins in the larger airways (Thornton, Rousseau, & McGuckin, 2008) and constituting of mainly surfactant in the small airways (Chronos, Sever-Chronos, & Shepherd, 2010). The surfactant is a complex mixture of lipids and proteins, with phospholipids representing the larger part of the surfactant mass. Surfactant phospholipids form the surface-active film that reduces the surface tension, by placing the hydrophobic part of the lipid structure in the airspace of the alveoli and the hydrophilic part in the aqueous phase (Goerke, 1998), as illustrated in Figure 1. Around 40 % of the surfactant mass consists of dipalmitoylphosphatidylcholine (DPPC), produced and recycled by the alveolar type II cells (Griese, 1999; Parra & Pérez-Gil, 2015). The surfactant function is improved by other phospholipids such as the most abundant of the unsaturated phosphatidylcholines, palmitoyloleoylphosphatidylcholine (POPC) (Bernhard et al., 2001; Griese, 1999). The levels of DPPC and POPC have been found to be reduced in BAL fluid from former smoking subjects with COPD (Agudelo et al., 2020).

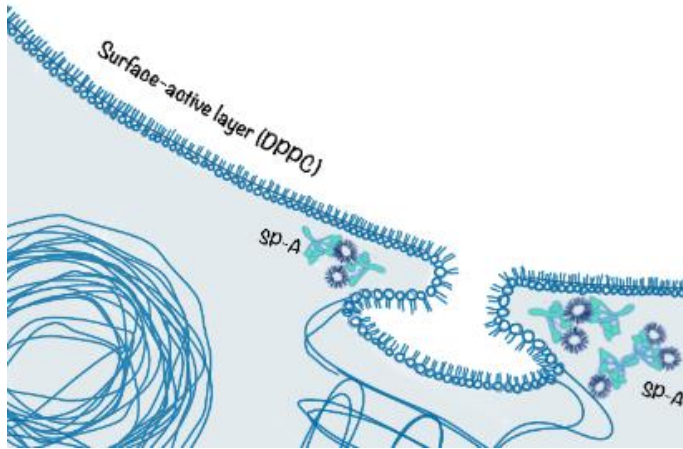


Figure 1. Illustration of the surface active film in the alveolar region, with the “head-legs” shape of DPPC oriented with legs in the airspace of the alveoli. SP-A with its characteristic look of a flower bouquet, binding to a foreign substance.

The hydrophilic surfactant protein A (SP-A), also illustrated in Figure 1, is the most abundant of the surfactant proteins and is, together with the hydrophilic SP-D, mainly involved in host-defense mechanisms. The hydrophobic membrane proteins SP-B and SP-C are important for the stability and the function of the surfactant (Griese, 1999). SP-A, expressed mainly in the alveolar type II cells, have several important immunological functions such as promoting the re-uptake of oxidized surfactant lipids (Cañadas, Olmeda, Alonso, & Pérez-Gil, 2020). It also binds to inhaled material, which increases the phagocytosis by alveolar macrophages (Pastva, Wright, & Williams, 2007). Surfactant protein/lipid interactions are essential for maintaining the homeostasis of surfactant (Castillo-Sánchez, Cruz, & Pérez-Gil, 2021).

The RTLF is usually sampled by bronchial wash (BW), broncho alveolar lavage (BAL) or by induced sputum (Balbi et al., 2007; Pouwels, Burgess, Verschuuren, & Slebos, 2021). All these methods are more or less invasive and unpleasant for the subject undergoing the examination. In addition, the fluid collected comes in an unknown dilution, complicating the interpretation of the sampled fluid (Baughman, 1997). Therefore, other ways of retrieving a sample from the RTLF are warranted.

1.3 AIRWAY IRRITANTS

Birch Pollen allergens

In Europe, the most allergenic tree pollen is produced by birch, with the major birch allergen Bet v 1, and is the major cause of allergic rhinitis and, possibly, asthma symptoms (Caillaud et al., 2015; Canova et al., 2013; Smith et al., 2014). Birch pollen, due to its size of roughly 20 μm in diameter, has a low probability to enter the small airways (Wilson, Novey, Berke, & Surprenant, 1973), but when the pollen grains come into contact with humidity, they burst and a huge amount of allergens are released. Birch pollen allergens as Bet v 1 can be found both in association with fine particles and as free allergen molecules as small as 30 nm (Taylor, Flagan, Miguel, Valenta, & Glovsky, 2004), with potential to deposit in the small airways.

Cigarette smoke

One of the main pollutants that we inhale is particulate matter (PM). The particle size determines where deposition occurs in the lung, where smaller particles deposit to a greater extent in the peripheral airway regions (Usmani, Biddiscombe, & Barnes, 2005). Cigarette smoke contains of PM of a mean diameter of $< 1\mu\text{m}$, from which a high rate deposits in the small airways. By smoking one cigarette, the respiratory tract is estimated to be exposed to 10 000-40 000 μg of PM (Ghio et al., 2008).

Respiratory viruses

Most respiratory viruses are built up of RNA, packed in a protein structure, which is the case with the Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). As a respiratory virus, the SARS-CoV-2 needs to enter the cells lining the respiratory tract. The virus is known to infect Angiotensin-converting enzyme 2 (ACE2) and TMPRSS2 expressing cells, which for the most part consists of the epithelial cells in the upper respiratory tract and to a lesser extent the ciliated bronchial cells and the alveolar type II cells (Hou et al., 2020). Chest computed tomography (CT), however, within 3 to 5 days from symptom debut on subjects diagnosed with COVID-19, have shown presence of viral pneumonitis in $>90\%$ of the cases (Bernheim et al., 2020), indicating viral infection also in the lower respiratory tract.

1.4 PHYSIOLOGICAL METHODS TO ASSESS SMALL AIRWAYS IMPAIRMENT

1.4.1 SPIROMETRY TO ASSESS FLOW AND VOLUME IMPAIRMENT

The word *spirometry* is a composition of the latin words *spiro*, “to breathe” and *meter*, “to measure”, that is, spirometry refers to “to measure the breathing”.

Dynamic spirometry is the widely used lung function method in the clinical setting, where maximal airflow at a forced and complete exhalation and inspiration are being measured (M. R. Miller et al., 2005). From the airflow and time registered, the volume of air exhaled and inhaled is being calculated by the relationship between flow signal F , volume V and time t , so that

$$V = F \times dt$$

Due to the forceful and maximal effort needed to achieve a correct measurement, the method primarily reflects how fast you can empty and fill your lungs. This means that neither the diffusive part of gas transport nor the ventilation with exchange between carbon dioxide and oxygen is being captured with spirometry. That is, spirometry is not very sensitive to impairment in the small airways due to the low flow resistance in this region.

1.4.2 INERT GAS WASHOUT TO ASSESS IMPAIRED GAS DISTRIBUTION

The ventilation distribution, which is the efficiency in which the mixture of inhaled gas with resident gas occurs within the airways, can be studied with the method inert gas washout (IGW), presented already during the mid 20th century (Fowler, 1949; Robertson, Siri, & Jones, 1950). In present time, the procedure of IGW has been presented in an ERS/ATS consensus statement document (Robinson et al., 2013). The ventilation distribution, assessed with IGW, can be measured by following the concentration of an inert gas in the exhalation if the previous inhalation is without the inert gas.

By following the inert gas concentration during either a normal or a full exhalation, three or four distinct phases of the exhalation can be found (Robinson et al., 2013), as illustrated in Figure 2. The first phase of the expirogram (phase I)

consist of zero inert gas concentration and represents the part of the conducting airways from where the last inhalation of inert gas free air did not mix with the resident gas volume. The second distinct phase of the expirogram (phase II) is where the inert gas concentration rapidly increases and represents the volume of resident a mixture of gas from the conducting and the alveolar region. The third phase of the expirogram (phase III) is where the inert gas starts to reach a plateau representing the alveolar concentration, and, the fourth phase (phase IV), present mainly at full exhalation, is referred to as the closing volume. Analyses of the plateau of gas concentration at phase III, referred to as S_{nIII} analysis, can give information on gas mixing due to convection and/or diffusion within the lung (Crawford, Makowska, Paiva, & Engel, 1985). Ventilation inhomogeneity at more specific locations such as in the conductive and in the acinar airways can be addressed with the S_{nIII} analysis (Verbanck & Paiva, 1990).

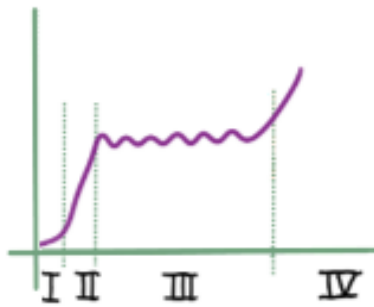


Figure 2. Schematic illustration of an expirogram. Marker gas concentration on the y-axis, volume exhaled on the x-axis. The typical four phases explained in the main-text.

Inflammation, mucus plugging, epithelial disruption and remodeling of airways causes narrowing of the airways. Obstruction in the small airways may also be due to parenchymal destruction leading to loss of lung elastic recoil. Obstructed small airways have little effect on lung mechanics, due to its low contribution to the total airway resistance. At the same time, small airways obstruction has a major effect on ventilation distribution (Robinson, Goldman, & Gustafsson, 2009), wherefore small airways ventilation inhomogeneity may be considered as an indirect measure of small airways obstruction.

To understand the tissue response due to airway irritants within the lungs, the very complicated dynamics of the airway tree needs to be addressed but also the tissue itself.

1.5 EXHALED BREATH

When you exhale, your lungs push air out to reduce the concentration of carbon dioxide to make room for more oxygen-rich air to be inhaled. The vital transport of air in and out of our lungs normally occurs without our attention is vital and important to understand to enable the understanding and potentially also the prevention of an impaired transport. Monitoring exposure effects of inhaled airway irritants is warranted but unfortunately difficult to perform in the inaccessible small airways, the site where airway irritants particularly may cause huge damage. Exhaled breath, however, carries signals from the entire respiratory tract and may, indirectly, assess small airway impairment.

1.6 METHODS TO ASSESS SMALL AIRWAYS IN EXHALED BREATH

Analyses of breath usually focus on either volatiles, i.e. gases, or non-volatiles, such as droplets, i.e. particles. Listed below (Table 1) are some currently available methods that sample and analyze exhaled breath. As of today, none of them have been implemented into clinical practice due to lack of either analytical or clinical validation, or both. The criteria to fulfill an analytical validation are clear, whereas the clinical validation to verify the clinical usability of a method are far more vague (Fijten et al., 2017).

Table 1. Different methods to, in-direct, assess small airways by analyses of exhaled breath

	Method based on	Breathing maneuver	Content in sample	Clinical validation
Exhaled breath Condensate (EBC) (Hoffmeyer, Raulf-Heimsoth, & Brüning, 2009)	Cooling exhaled breath and collecting the resulting condensate	Tidal breathing	Volatiles, Non-volatiles, Semi-volatiles	Not efficient sampling of non-volatiles, unknown dilution and origin, low concentration

Electronic nose (Queralto et al., 2014)	“Breathprints”/ machine learning model	Tidal breathing	Volatiles	Insufficiently validated
Alveolar NO	Mathematical calculations	A steady flow- rate exhalation	Volatile	Need further validation
Particles in exhaled air (PEx) (Almstrand et al., 2009)	Impaction	Airway opening maneuver	Non-volatiles	Not validated, low conc.
Breath explor (BE) (Seferaj et al., 2018)	Impaction	Tidal breathing/airw ay opening maneuver	Non-volatiles	Not validated

In this thesis, the main focus is on the PExA method and, the FENO method at multiple flows, to explore their potential to reveal alterations in small airways induced by inhalable airway irritants. Other methods targeting small airways non-invasively may be of outmost interest to explore in different exposure settings but are beyond the scope of this thesis.

1.6.1 PARTICLES IN EXHALED AIR (PEX)

Background

At relaxed breathing, the smallest sizes of particles exhaled are thought to originate from the RTLTF in the small airways (Almstrand et al., 2010). Formation is mainly thought to occur at reopening of collapsed small airways due to film bursting of the lining fluid covering the airways (Figure 3) (Almstrand et al., 2010; Dollfuss, 1967; Haslbeck, Schwarz, Hohlfeld, Seume, & Koch, 2010). This mechanism of producing particles is affected by the breathing pattern, as shown in studies where deep breathing to residual volume substantially increases the number of exhaled particles (Almstrand et al., 2010; Holmgren, 2010; Johnson & Morawska, 2009).

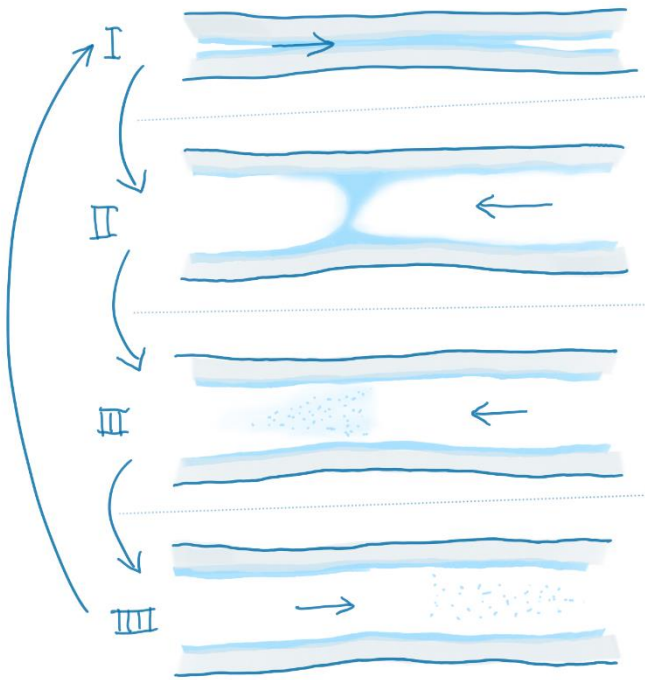


Figure 3. Schematic illustration of film bursting and formation of particles at airway closure followed by airway-reopening. I) small airways close during exhalation and meniscus of RTL formed between airway walls, II-III) reopening of small airways with rupture of meniscus and particle formation during inhalation, and IIII) exhalation of particles.

Particles in exhaled breath can be sampled in a diluted form, through cooled sampling systems as with exhaled breath condensate (EBC) (Bondesson, Jansson, Bengtsson, & Wollmer, 2009) or in an un-diluted form by impaction, as with PExA (Almstrand et al., 2009), with instrument version 1.0 and 2.0 presented in Figure 4, and Breath explor (BE) (Seferaj et al., 2018).

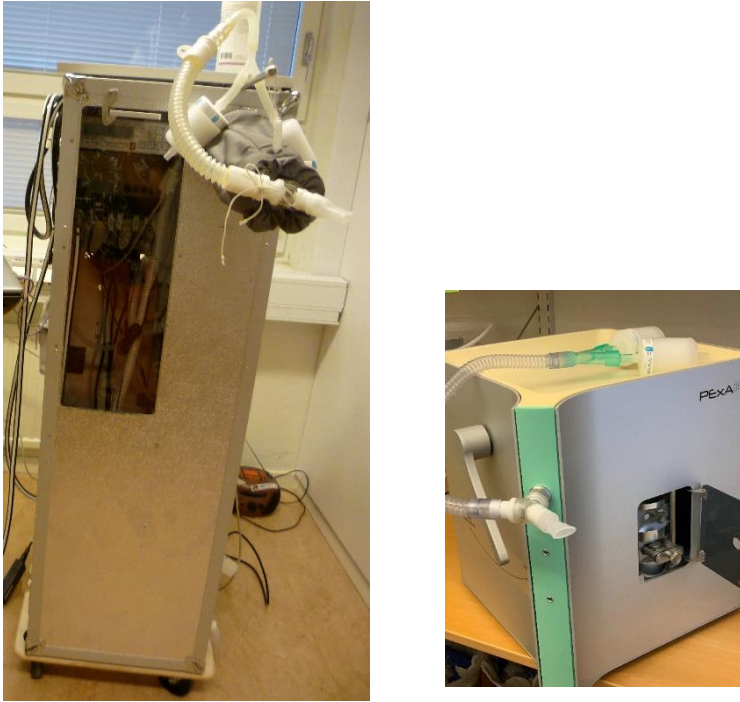


Figure 4. The PEXA 1.0 (left) and 2.0 (right) instruments.

While breathing in a relaxed manner, a portion of the smallest airways are thought to close upon expiration. The volume of the lung at which a substantial number of airways are closed is called the closing volume. The aging of the lung, likewise lung diseases such as COPD, have been shown to increase the amount of airway closure (Milic-Emili, Torchio, & D'Angelo, 2007). Studies conducted during the seventies showed that smokers have increased closing volume (Buist & Ross, 1973; McCarthy, Spencer, Greene, & Milic-Emili, 1972).

The method

Aerosol particle sampling with the method PEXA is based on impaction but also simultaneous counting and size fractioning by an optical particle counter (OPC)(Almstrand et al., 2009). Sampling by impaction means that particles of a certain size in an airstream will impact on a surface. Due to the inertness of the particles, they are separated in the airstream at a given threshold resulting in a selection of particles that will impact on the sampling surface. A pump draws the aerosol through the impactor in the PEXA instrument, with a certain velocity, through small holes. This pre-determined velocity, together with the known size of the small holes, results in a well-defined size range of the particles that impact.

Calculations on the mass of the sampled particles can therefore be performed based on the information on particle number and size from the OPC. Particles with a diameter size range of 0.41–4.55 μm is sampled and is referred to as PEx.

The breathing maneuver

Over the years, the breathing maneuver to sample aerosol particles has been slightly modified. To maximize the sampled amount of particles, the breathing maneuver today includes a deep exhalation to residual volume followed by 5 seconds of breath-hold before maximal inspiration and the final relaxed exhalation where the sample is retrieved. Despite the use of a standardized breathing maneuver, there are large inter-individual and intra-individual differences in particle emissions found in subjects (Bake, Ljungstrom, et al., 2017; Kokelj et al., 2020; Schwarz, Biller, Windt, Koch, & Hohlfeld, 2015).

The sample

The exhaled particles impact on a Teflon membrane, and this membrane is later picked out from the impactor and stored in $-80\text{ }^{\circ}\text{C}$ prior to chemical analyses. The mass of the analyte in the particles on the sampling surface can later on be analyzed by, for instance, enzyme-linked immunosorbent assay (ELISA) or liquid chromatography mass spectrometry (LC-MS). The concentration of the analyte in the particles can be determined and is expressed as weight percent (wt%) of the total mass of particles sampled, enabling comparison of analyte-concentration between subjects with different particle emissions.

1.6.2 FRACTION OF EXHALED NITRIC OXIDE (FENO)

Background

The very small molecule nitric oxide (NO) is involved in a wide range of biological functions within our body, such as vasodilatation but acts also as signaling molecule in inflammation (J. W. Coleman, 2001). Specific enzymes named nitric oxide synthases (NOS) are expressed in the airways and induces the NO production by different stimuli (Ricciardolo, 2003). NO has many roles in the airways, for instance, it helps clearing the airways from inhaled particles by stimulation of the ciliary frequency and of the secretion of the submucosal glands (Barnes & Belvisi, 1993; Ricciardolo, 2003). It also acts as a scavenger for more harmful free radicals (J. W. Coleman, 2001). In addition, it is a bronchodilator, enhancing oxygenation (Ricciardolo, 2003).

From first being found in exhaled air (Gustafsson, Leone, Persson, Wiklund, & Moncada, 1991), studies that followed showed a large variation in the concentrations of exhaled NO. This was later explained by a high dependence on exhalation flowrate of the exhaled concentrations (Silkoff et al., 1997). Nowadays,

the measurement of NO during a steady flowrate (Fractional exhaled nitric oxide; i.e. FENO) has become a standard procedure for indirect assessment of airway inflammation in asthma (Dweik et al., 2011). Normal values from healthy subjects, for the standard procedure of sampling NO at exhalation of 50 mL/s (FENO50) have been published (Högman et al., 2017; Olin, Bake, & Toren, 2007). FENO has recently been recommended by the American Thoracic Society (ATS) to be used in patients with asthma in whom treatment is being considered (Khatri et al., 2021). In the latest guidelines for asthma diagnosis by the European Respiratory Society, it is recommended when spirometry is inclusive for diagnosis (Louis et al., 2022).

The NO registered at the ordinary sampling consists of a mixture of NO produced in different parts of the airways, wherefore FENO50 alone cannot distinguish between NO produced in the central airways from that produced in the peripheral airways. Different mathematical models are currently being used to distinguish NO production in small airways to that of larger airways (Horváth et al., 2017; Lehtimäki, Karvonen, & Högman, 2020).

Based on the notion that NO is flow dependent (Tsoukias, Tannous, Wilson, & George, 1998), a simple two compartment model (2CM) of pulmonary NO dynamics was introduced (Tsoukias & George, 1998), dividing the lung into a bronchiolar and an alveolar compartment, as illustrated in Figure 5. The bronchiolar, representing the conducting airways to the transitional bronchioles, and the alveolar representing the gas exchanging zone from the respiratory bronchioles to the alveoli.

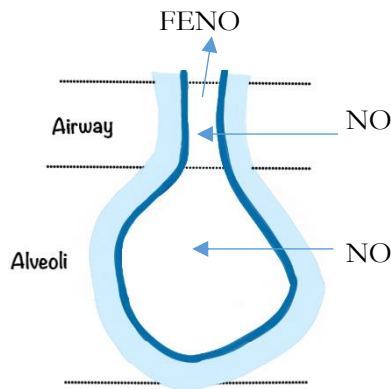


Figure 5. A schematic drawing of the two-compartment model of pulmonary NO dynamics.

The transport of NO from the airways and the alveoli occurs via convection and axial diffusion, where the NO from the airways also “back-diffuses” towards the alveoli, as shown by experimental studies using heliox (Kerckx, Michils, & Van Muylem, 2008).

The extent of small airway inflammation due to pollen exposure in birch pollen sensitized subjects with asthma is unclear (Lopez, Prieto, Perez-Frances, Barato, & Marin, 2012; Tufvesson, Aronsson, Ankerst, George, & Bjermer, 2007). In addition, neither the NO site production nor the peripherally produced NO that may diffuse towards the alveoli have a clear interpretation (Kerckx et al., 2008; Shin, Condorelli, Rose-Gottron, Cooper, & George, 2004). There is yet no generally agreed upon model to assess the alveolar NO production (Horváth et al., 2017; Lehtimäki et al., 2020).

The method

The method used in this thesis is the chemiluminescence method, based on a reaction between NO and ozone (O_3), the gold standard in how to detect NO in exhaled air (Hetrick & Schoenfisch, 2009). A light is emitted in the reaction, proportional to the amount of NO in the exhaled air, which is being quantified by a photomultiplier.

The sampling procedure

NO-free air is being inhaled to total lung capacity (TLC) via a mouthpiece, followed by a full and steady-state exhalation. By exhaling against a positive pressure, which is the recommended method today, the soft palate closes and prevents NO from the nose to contaminate exhaled NO (Kharitonov & Barnes, 1997).

The standard procedure of today includes an exhalation of 50 mL/s, referred to as the measurement FENO50 and is a sample of NO from the entire lung. However, an increased FENO50 cannot separate an inflammation in the central airways from that in the peripheral airways.

By the use of the two-compartment model and exhalations at multiple flow rates, the contribution of NO from central and peripheral airways can be calculated (Högmán, Drca, Ehrstedt, & Meriläinen, 2000; Pietropaoli et al., 1999; Silkoff, Sylvester, Zamel, & Permutt, 2000; Tsoukias & George, 1998).

1.7 PREVIOUS STUDIES USING ALVEOLAR NO AND PEX TO ASSESS EXPOSURES TO AIRWAY IRRITANTS

Allergen exposure and/or asthma

Exposure to birch pollen in birch pollen sensitized subjects with asthma have showed a reduction in the amount PEx during compared to outside pollen season, but no change in lung healthy controls (Larsson, Lärstad, et al., 2017). No statistically significant association was found between amount PEx and the inflammatory marker FENO50 for subjects with asthma. SP-A and albumin in PEx was not associated with exposure to birch pollen in this study population.

Subjects with asthma and with small airway dysfunction (impaired impulse oscillometry- and Inert gas washout-indices), have been found with decreased amount of PEx and lower levels of SP-A and albumin in PEx, in comparison to healthy controls (Soares et al., 2018). Soares et al. however, did not find any associations between SP-A and albumin in PEx across GINA step treatments.

CANO has been found increased in subjects with stable asthma, likely explained by small airway inflammation (Fujisawa et al., 2013; Högman et al., 2002; Kerckx et al., 2008). CANO has also been found increased in some but not all, children with asthma and acinar ventilation inhomogeneity (Keen, Olin, Wennergren, & Gustafsson, 2011).

Cigarette smoke

In a previous study comparing active smokers to non-smokers, no significant difference was found in the amount of PEx (Bredberg et al., 2013). Former smokers diagnosed with COPD have been found to exhale less amount of PEx in comparison to lung healthy controls (Larstad et al., 2015).

Subjects with COPD have also shown with lower levels of SP-A in PEx, with differences increasingly pronounced with worsening in lung function assessed with spirometry (Larstad et al., 2015).

Increased content of DPPC and POPC in PEx in current compared to never smokers have recently been found in the cohort study by Hussain-Alkhateeb et al (Hussain-Alkhateeb et al., 2021).

Respiratory Viruses

Only explorative experiments have been performed to identify virus RNA in samples retrieved by the PExA method.

1.8 KNOWLEDGE GAPS ADRESSED IN THIS THESIS

There are several gaps in knowledge that needs to be addressed with both alveolar NO and PEx-outcomes before these can be considered as valid effect markers of exposures in small airways. This thesis has focused on the explorative approach in different exposure settings to generate a ground for further research within this field. The following gaps will be addressed:

Studies on birch pollen exposure in subjects with and without birch pollen sensitization, and its effect on alveolar NO production, have previously been reported with some contradictory results. The interdependence of FENO50 and alveolar NO, as well as the influence of back diffusion of NO, will be addressed in **Paper I**, in an attempt to contribute to the understanding of alveolar inflammation in subjects with birch pollen allergy. Detection of small airways inflammation could implicate a need for treatment aiming specifically at the small airways.

The association between levels of surface-active lipids in the small airway lining fluid, and inhomogeneous gas mixing in the small airways, is currently unknown. Inhomogeneous gas mixture in the small airways is an indirect measure of inhomogeneous small airway narrowing. Furthermore, the association between small airways narrowing and small airways inflammation is unclear. This will be addressed in **Paper II**. Combining indirect measurements of small airways impairment might amend knowledge regarding subject characteristics.

Current compared to former and never smokers, and analyses of content of SP-A, albumin and major surface-active lipids in PEx as well as number of PEx, has not been addressed previously but will be addressed in **Paper III**. Altered composition of PEx in current smokers, in comparison to former smokers and never smokers, might reveal changes in the small airway lining fluid that may be reversible.

Detection of virus infection in samples retrieved with PExA has not been possible in previous experiments. The COVID-19 pandemic and collaboration with researchers at the Department of infectious diseases, Sahlgrenska University hospital, enabled the possibility of addressing detection of SARS-CoV-2 RNA in exhaled breath using the PExA, as presented in **Paper IV**.

2 AIM

The overall aim of this thesis was to explore the ability of different non-invasive methods to assess the effect of airway-irritant exposure on small airways. More specifically, we wanted to explore if alveolar nitric oxide, the number of endogenous exhaled particles as well as their main contents of lipids and proteins is affected in subjects exposed to birch pollen, or tobacco smoke. Moreover, we also wanted to examine if SARS-CoV-2 could be detected in exhaled particles from small airways.

Specific research questions in Paper I-IV

Paper I

Is birch pollen exposure associated with increased alveolar NO (CANO) in birch pollen sensitized asthmatic subjects compared to controls?

Is CANO corrected for back diffusion of NO (CANO_{corrected}) elevated at birch pollen exposure in birch pollen sensitized asthmatic subjects compared to in controls?

Paper II

Is small airways ventilation inhomogeneity, assessed with Sacin and Scand, associated with altered levels of the major surfactant lipids DPPC and POPC in exhaled endogenous particles, and/or with altered levels of the number exhaled particles?

Is small airways ventilation inhomogeneity associated with altered levels of alveolar NO production?

Paper III

Do current smokers have altered levels of the number exhaled particles and/or the content of DPPC, POPC, and surfactant protein A in exhaled particles, in comparison to former and never smokers?

Paper IV

Can the PExA method and sampling of exhaled particles from a few breathing maneuvers and coughs detect viral load in subjects newly diagnosed with COVID-19?

If so,

- *is number and size of exhaled particles $<5\mu\text{m}$ associated with detected viral load in aerosol?*
- *is detected viral load in aerosol associated with that of oro/nasopharyngeal swab?*
- *does the breath explor (BE) method detect viral load similarly as PExA?*

3 STUDY PARTICIPANTS AND METHODS

Study participants, study design, methods and outcomes in each paper (I-IV) is presented in the following section and summarized in Table 2. For further details see Papers I-IV. All studies are approved by the Regional Ethical Review Board in Gothenburg.

Table 2. Study design, study participants and methods in paper I-IV.

	PAPER I	PAPER II	PAPER III	PAPER IV
Study design	Repeated measure design	Cross-sectional	Cross-sectional	Cross-sectional
Exposure	Birch pollen		Cigarette smoke	SARS-CoV-2
Study participants	13 Birch pollen sensitized adult asthmatics, 12 healthy controls	72 adult asthmatics	37 current smokers, 36 former smokers, 29 never smokers	10+25 with acute COVID-19, 11 healthy controls
Statistical methods	Wilcoxon exact test and Wilcoxon signed rank test, two-sided paired t-test	Mann-Whitney U test, Spearman rank correlation, Quantile regression	Kruskal-Wallis test, Spearman rank correlation, Quantile regression	Mann-Whitney U test, chi-square test
Methods to sample contents from small airways	FENO at multiple flows (50, 100 and 270 mL/s)	FENO at multiple flows (30, 50, 100 and 300 mL/s) PExA after BD	PExA after BD	PExA

3.1 STUDY DESIGN

Paper I is based on repeated measures study design of effects on biomarkers potentially reflecting inflammatory response in central and peripheral airways, in birch pollen sensitized asthmatic subjects and in controls, in and outside pollen season. Study participants were examined in Gothenburg, Sweden, during pollen season between 2011-04-29 and 2011-06-02, and outside pollen season either during the autumn 2010 or 2011.

Paper II is a cross-sectional study exploring associations between small airway ventilation inhomogeneity and surface active lipids from small airways, as well as NO produced in alveolar region, in subjects diagnosed with asthma. Examinations were conducted at the Respiratory research Laboratory at Skaraborgs Hospital Skövde, Sweden, between 2011-2014.

Paper III is a cross-sectional study exploring associations between major component in small airways surfactant, analyzed in exhaled particles, in current smokers compared to former and never smokers. Examinations were conducted at the unit of occupational and environmental medicine at the University of Gothenburg, Sweden, between 2014-2016.

Paper IV explores the possibility to detect traces of SARS-CoV-2 in aerosol particles $<5\mu\text{m}$ sampled during a few minutes of breathing and coughing, from subjects newly diagnosed with COVID-19. Examinations were carried out at Sahlgrenska University Hospital, Gothenburg, Sweden, in a first sub-study between September-October 2020 and in a second sub-study between April-May 2021.

3.2 STUDY PARTICIPANTS

Paper I

Birch pollen sensitized asthmatic subjects were included based on history and presence of specific IgE against birch pollen. They were instructed to withdraw from long acting β -2 stimulators two days prior to the clinical examination. Controls were recruited based on no history of lung disease and no intake of medication. All subjects were non-smokers and were without respiratory tract infections three weeks prior to the clinical examination.

The study group of Paper I consisted of 13 birch pollen allergic non-smoking asthmatic subjects (26-61 years) and 12 healthy non-smoking control subjects (34-67 years) with no history of lung disease.

All participating subjects received oral and written study information prior to giving their written informed consent. The regional ethics board at Gothenburg University (application number 138-10) approved the study protocol.

Paper II

The study group consisted of 72 asthmatic subjects (26-61 years), all of whom participated in a larger asthma study recruited from primary care centers across Skaraborg County in West Sweden (Kjellberg, Houltz, Zetterstrom, Robinson, & Gustafsson, 2016). Study participants were included on a physician diagnosis of asthma which was based on a history of recurrent dyspnea, cough or wheeze, reversible airflow obstruction proven by spirometry and responding to bronchodilator and/or ICS. Subjects with instable asthma, known cardiac disease, systemic disease, or on current medication potentially affecting response from bronchodilation, were excluded.

Eligible for inclusion in the study on which paper II was based on were subjects whom had managed to perform SF6 Multiple breath washout (SF6 MBW) post bronchodilation in the larger asthma study. Participants were instructed to withhold their asthma medication for at least 12 hours prior to their participation in the study.

The classification of subjects with small airways ventilation inhomogeneity was based on SF6 MBW derived indices S_{acin} and/or $S_{cond} > ULN$, whereas classification of subjects with asthma but without small airways ventilation inhomogeneity was based on S_{acin} and $S_{cond} \leq ULN$.

Healthy controls were recruited by letters to randomly selected subjects via the Swedish population register (SPAR, i.e. statens personadressregister) to generate reference equations to measurement SF6 MBW. They were included if they had no known respiratory or chronic or recurrent respiratory symptoms.

All participating subjects received oral and written study information prior to giving their written informed consent. The regional ethics board (application numbers 779-10 and 309-12) approved the study protocols.

Paper III

102 subjects, aged 39-83 years, represented by 37 current smokers, 36 former smokers and 29 never smokers, were included. Study participants were recruited from a population based cohort study, previously described (Mehlig et al., 2017). All current smokers were invited to participate meanwhile former and never smokers were invited randomly based on convenience selection.

Eligible for inclusion in the study on which paper III was based on were subjects whom had managed to perform spirometry in the cohort study and had reported their smoking history. Subjects with ongoing respiratory tract infection, myocardial infarction past month and/or pregnancy in the last trimester were excluded.

The classification of current, former and never smoker was based on smoking history. Current smokers had smoked cigarettes on a daily basis for at least one year at the time of the clinical examination; former smokers had smoked on a daily basis but stopped smoking for one year or more prior to the clinical examination; never smokers reported to never had smoked on a regular basis.

All participating subjects received oral and written study information prior to giving their written informed consent. The regional ethics board at Gothenburg University (application number 626-13) approved the study protocol.

Paper IV

The first sub-study group of Paper IV consisted of ten subjects with newly diagnosed COVID-19, whereas the second sub-study group consisted of 25 subjects newly diagnosed with COVID-19.

Eleven controls with negative antigen test and without any symptoms were included.

Eligible for inclusion were hospital health care workers testing positive for COVID-19 according to the routine testing of hospital staff used at the time for the study inclusion. The first sub-study group were diagnosed with sample retrieved from a combined oropharyngeal and nasopharyngeal (oro/nasopharyngeal) swab and with reverse transcription real-time polymerase chain reaction (RT-PCR) analysis. The second sub-study group were initially diagnosed with a Rapid COVID-19 Antigen Test (CLINITEST) (Siemens, Healthineers, Erlangen, Germany) on a nasopharyngeal swab sample, which was confirmed by a oro/nasopharyngeal swab and RT-PCR the same day.

Recruited subjects received oral and written study information and gave their written informed consent prior to participation.

All participating subjects received oral and written study information prior to giving their written informed consent. The Swedish Ethical Review Authority (application number 2020-03048) approved the study protocol.

3.3 METHODS

In this section, a summary of the included methods in each study is presented, followed by a presentation of each method separately.

Paper I consisted of examinations both in and out of pollen season with FENO measurements in duplicates at three exhalation flows within $\pm 10\%$ (50, 100 and 270 mL/s). Spirometry before and after bronchodilation followed after FENO measurements.

Paper II included a day one visit with a physician based interview, Self-reported Asthma Control Test (ACT) (Schatz et al., 2006), blood sampling for measurement of eosinophils, spirometry and sampling of PEx, and, a day two visit with FENO measurements at different flows (30, 50, 100 and 300 mL/s) and SF₆ MBW. Spirometry and SF₆ MBW were performed pre and post inhalation of 400 mcg salbutamol (Ventoline, GlaxoSmithKline, Uxbridge, UK) via pressurized metered dose inhaler and a non-electrostatic holding chamber (Vortex, Pari Medical GmbH, Starnberg, Germany). FENO was performed pre bronchodilation (BD) and PEx was performed post BD. Skin prick test (SPT) was performed and used for defining perennial allergy (a mean wheal of ≥ 3 mm) on either furred pets (cat, dog horse), mold allergens and/or house dust mites (Solupric SQ; ALK, Copenhagen, Denmark).

Paper III consisted of FENO50 measurement, spirometry, sampling and chemical analysis of PEx, VC N2SBW, blood sampling for measurement of C-reactive protein, and completion of questionnaires. FENO was performed pre BD, spirometry pre and post BD, VC N2SBW pre BD and sampling of PEx post BD.

Paper IV and the aerosol sampling procedure in the first sub-study was performed several days after testing positive for COVID-19 whereas sampling in the second sub-study was performed the same day as testing positive for COVID-19 with antigen test and sampling for RT-PCR analysis. The participation in the study included filling in a short questionnaire with questions regarding symptoms, aerosol sampling with the PExA method during relaxed breathing, during deep breathing with breath-hold and, during cough, and finally, aerosol sampling with BE during relaxed breathing.

3.3.1 PARTICLES IN EXHALED AIR (PEXA) (PAPER II, III AND IV)

Instrumental setup

Particle sampling with PEXA, originally described by Almstrand et al. (Almstrand et al., 2009), was achieved with an in-house built version of the PEXA instrument (PEXA 1.0) (Paper II) and a commercially available version of the PEXA instrument (PEXA 2.0) (PEXA AB, Gothenburg, Sweden) (Paper III and IV).

In brief, the design of the PEXA instrument is based on a cascade impactor for particle collection

- a 3-stage inertial impactor (3-stage PM 10 Impactor, Dekati Ltd., Tampere, Finland) (Paper II)
- a modified inertial impactor with 2 stages (Figure 6) (Paper III and IV)

A thin membrane of hydrophilic polytetrafluorethylene (PTFE) (FHLC02500, Millipore, Billerica, MA, USA) covered the lower plate in the impactor where particles between 0.4-5 μm in aerodynamic diameter impact. In Paper IV, the upper plate where also covered with a PTFE for sampling of particles with a diameter above approximately 5 μm .

The sampling of particles were controlled by

- a vacuum pump with a constant flow of 230 mL/s (Paper II)
- a rotary vane pump with a constant flow of 230 mL/s (Paper III and IV)

Counting and size-fractionating of these particles was simultaneously conducted by an optical particle counter (OPC) (Grimm 1.108, Grimm Aerosol Technik GmbH, Ainring, Germany), sampling a fraction of the exhaled air with a constant flow of 20 mL/s. Also, a flowmeter (OEM Flow Sensor Spiroson-AS, ndd Medical Technologies, Zürich, Switzerland) was used for simultaneous registration of flow and volume.

A valve system allowing inhalation of particle free air through a HEPA filter and exhalation either into the room or into the instrument was also used.

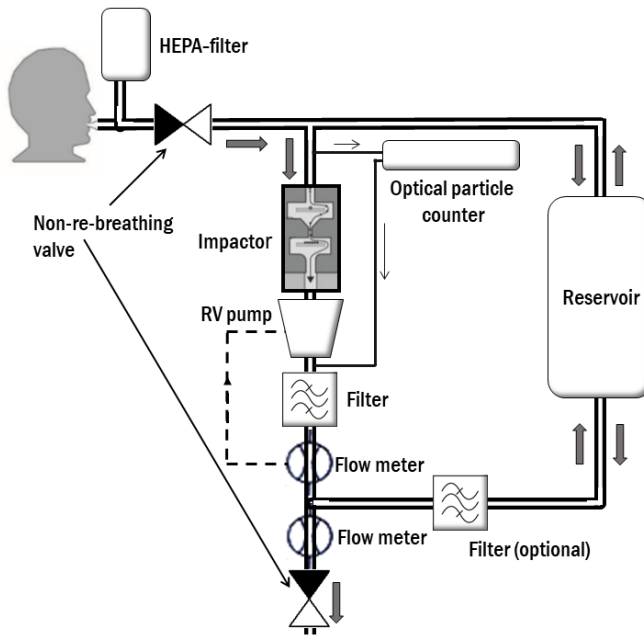


Figure 6. The PExA 2.0 instrument set-up at collection. Illustration adopted with permission from PExA AB.

Sampling procedure (Paper II, III and IV)

The sampling procedure of exhaled particles was conducted

- after bronchodilation (Paper II and III)
- without notice to medication (Paper IV)

The procedure started with participating subjects breathing particle free air in a relaxed manner, connected to the instrument

- for 2 minutes (Paper II)
- for 3 breaths (Paper III and IV)

Thereafter, they were instructed to repeatedly perform an airway opening maneuver (Figure 7) in where particles were being sampled. The maneuver involved a deep exhalation to residual volume (RV), a 5 seconds breath hold at RV followed by a rapid inhalation to total lung capacity (TLC) and finally a relaxed exhalation to RV. In between maneuvers, subjects were instructed to breathe in a relaxed manner through the mouthpiece. Subjects wore a nose clip throughout the sampling procedure.

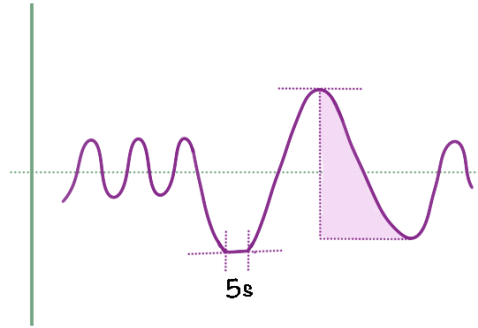


Figure 7. *Airway opening maneuver. A deep exhalation to RV, a 5 s breath-hold at RV, a rapid inhalation to TLC, and finally, a relaxed exhalation to RV.*

The sampling was continued until

- 120 L of exhaled air had been sampled (Paper II)
- 120 ng of particles were sampled (Paper III)

In Paper IV, the sampling where performed in three different setups, as illustrated in Figure 8.

- *Relaxed breathing* for 20 breaths in a row with particles sampled continuously
- *Airway opening maneuver* for 10 breaths, with relaxed breaths and no sampling in between maneuvers
- *Cough maneuvers*, including a deep inspiration to TLC and a forceful expel of the air, repeated 3 times and with relaxed breaths in between where no particles were being sampled

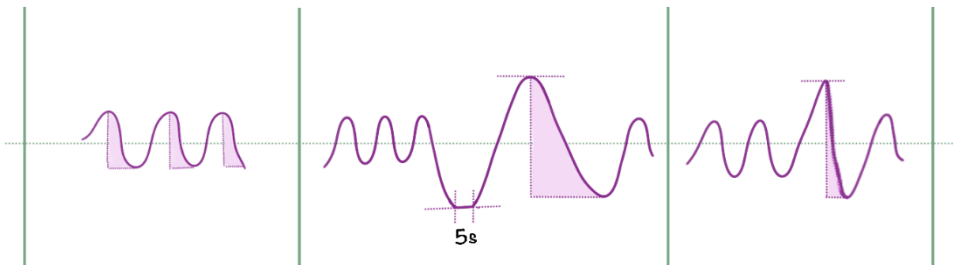


Figure 8. *Illustration of breathing maneuvers used in paper IV. Relaxed breathing (left), airway opening maneuver with 5 s breath-hold (middle) and cough (right). Sampling of PEx occurs at the shaded parts.*

Calculations of amount PEx (Paper II, III and IV)

The OPC counts exhaled particles according to size in eight size intervals. From recalculations, where the different refractive index of the particles have been taken into account, the measured particle sizes were between 0.41-4.55 μm .

Chemical analyses of phospholipids in PEx (Paper II and III)

Chemical analyses of the two major surface active lipids in PEx (DPPC and POPC) were performed with a triple quadrupole mass spectrometer (Sciex API3000, AB Sciex, Canada), equipped with an electrospray ion source operating in positive mode, according to previously described protocol (Larsson, Bake, et al., 2017). In brief, internal standards were added to each sample before extraction. Extracted samples were introduced to the ion source with a flow gradient method, but without chromatography separation.

In Paper II, the lipids were spiked with internal standards and extracted from the PTFE collection membrane using 160 μl of MeOH:chloroform:40 mM Ammonium acetate in the volumetric ratios 6:3:2. Samples were introduced using a direct infusion method. Lipids were detected using multiple reaction monitoring method targeting the phosphatidycholine fragment 184.1 m/z.

The quantification of DPPC and POPC was based on

- In Paper II, using a standard curve using seven calibration levels in the range between 0.005-0.500 μM , run at the start and end of each run. From standards, a linear regression model using $1/x$ weighing where

$y = \text{standard_area} / \text{internal_standard_area}$ and

$x = \text{standard_concentration} / \text{internal_standard_concentration}$

was constructed and used for calculating amount in unknown samples.

- In Paper III, a calibration curve with a linear regression model. In each run PTFE substrates spiked with 25 pico mol DPPC and POPC standards (an amount representative of the study samples) were analyzed to monitor method performance. Based on 22 measurements distributed throughout the study the average recovery was 81% and 104% for DPPC and POPC respectively. The reproducibility RSD% was 6.4 and 8.3 for DPPC and POPC respectively.

Chemical analyses of proteins in PEx (Paper III)

Concentration of Surfactant protein A (SP-A) and Albumin in PEx were analyzed with enzyme-linked immunosorbent assays (ELISAs), according to a previously described protocol (Kokelj et al., 2020). In brief, PEx were extracted from the PTFE membrane samples by adding extraction buffer. Levels of SP-A and albumin in extracted PEx samples were determined with a human SP-A ELISA kit (Lot nr; E-15-108, Product nr; RD191139200R, BioVendor, Brno, Czech Republic) and a human albumin ELISA kit (Lot nr; 19, Part number; E-80AL, Immunology Consultant Laboratory, Newberg, OR, USA), according to the manufacturer's instructions, with minor modifications.

RNA extraction and detection of SARS-CoV-2 in PEx (Paper IV)

Prior to study start, the extraction of SARS-CoV-2 RNA from PTFE filters were tested by spiking patient sample in different dilutions.

The PTFE membranes on each impactor plate with sampled particles were transferred to separate, 1.5 mL SC microtube PC-PT cryotubes (Sarstedts, Numrecht, Germany). The sampled aerosol particles were stored at -80, prior to RNA extraction. The SARS-CoV-2 PCR analysis, from which the cycle threshold (Ct) values were obtained, was performed in a Cobas® 6800 (Roche Diagnostics, Mannheim, Germany) according to manufacturer's instructions at the Virology unit at the department of Clinical Microbiology, Sahlgrenska University Hospital.

PTFE membranes were incubated overnight in 2 ml of lysis buffer, and this volume was used for extraction of total nucleic acid in an EasyMag instrument (Biomerieux, Marcy l'Etoile, France) eluted in 110 µl. Real-time PCR was performed in duplicates in a QuantStudio 6 (Applied Biosystems, Foster City, United States) instrument by using a 50-µl reaction volume including 10 µl of purified nucleic acid, primers, and probe targeting SARS-CoV-2 polymerase gene (RdRP) (Yilmaz et al., 2021) and Taqman Fast Virus 1-step Mastermix (Applied Biosystems). After a reverse transcription step at 46°C for 30 min followed by 10 min of denaturation at 95°C, 45 cycles of two-step PCR was preformed (15 s at 95°C, 60 s at 58°C).

Viral load refers to Ct values, a proxy for inverse viral concentration of detected SARS-CoV-2 RNA. All samples only positive for SARS-CoV-2 in one reaction with Ct > 35 were re-analyzed to confirm the result.

3.3.2 BREATH EXPLOR (BE) (PAPER IV)

Particle sampling in exhaled breath was sampled by impaction using the hand-held device Breath Explor (BE) (Munkplast AB, Uppsala, Sweden), illustrated in Figure 9. The BE device have been described in detail previously by Seferaj et al (Seferaj et al., 2018). Sampling was performed according to the manufacturers instructions (VER: 2019-05-06) with minor modifications. In brief, subjects

inhaled a relaxed breath of room air followed by a relaxed exhalation into the BE device. This maneuver was repeated 20 times.



Figure 9. The breath explor (BE) device. Subject inhale room air and exhale at the inlet (larger hole) of the device.

RNA extraction and detection of SARS-CoV-2 in aerosol sampled with BE

The aerosol particles sampled with BE were stored at -80, prior to RNA extraction.

The SARS-CoV-2 PCR analysis, from which the cycle threshold (Ct) values were obtained, was performed in a Cobas® 6800 (Roche Diagnostics, Mannheim, Germany) according to manufacturer's instructions at the Virology unit at the department of Clinical Microbiology, Sahlgrenska University Hospital.

BE membranes were thoroughly washed with a volume of 1.2 mL of buffered sodium hydroxide by pipetting and vortexing, and 1 mL volume was used for nucleic acid extraction by a MagNA Pure LC instrument (Roche Diagnostics, Mannheim, Germany) using the Large Volume Total Nucleic Acid isolation kit. The total nucleic acid was eluted in 50- μ L volume.

Real-time PCR was performed in duplicates in a QuantStudio 6 (Applied Biosystems, Foster City, United States) instrument by using a 50- μ L reaction volume including 10 μ L of purified nucleic acid, primers, and probe targeting SARS-CoV-2 polymerase gene (RdRP) (Yilmaz et al., 2021) and Taqman Fast Virus 1-step Mastermix (Applied Biosystems). After a reverse transcription step at 46°C for 30 min followed by 10 min of denaturation at 95°C, 45 cycles of two-step PCR was preformed (15 s at 95°C, 60 s at 58°C).

Viral load refers to Ct values, a proxy for inverse viral concentration of detected SARS-CoV-2 RNA. All samples only positive for SARS-CoV-2 in one reaction with Ct >35 were re-analyzed to confirm the result.

3.3.3 ALVEOLAR NO (PAPER I AND II)

Instrumental setup

FENO was measured as per ATS/ERS guidelines ("ATS/ERS recommendations for standardized procedures for the online and offline measurement of exhaled lower respiratory nitric oxide and nasal nitric oxide, 2005," 2005);

- using a NIOX system (NIOX; Aerocrine AB, Stockholm, Sweden) (Paper I)
- using a chemiluminescence NO analyzer (CLD88, EcoMedics AG, Dürnten, Switzerland) (Paper II)

Sampling procedure

FENO measurements were conducted prior to spirometry. The sampling procedure of FENO is illustrated in Figure 10. Participants were instructed to inhale NO-free air and exhaled at pre-determined target flow rates:

- 50, 100 and 270 mL/s (Paper I)
- 30, 50, 100 and 300 mL/s (Paper II)

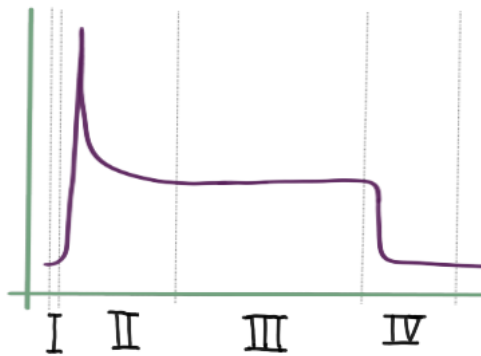


Figure 10. Sampling procedure of FENO. The registered FENO value is adopted in phase III.

Measurements were performed at a minimum of 2 times per flow from where plateau values and the corresponding exhalation flow rates were registered. A mean value within 10% of the target value was reported.

Calculations of alveolar NO production

Calculations of production of NO in the alveolar region (CANO) were performed according to the linear model of Pietropaoli et al. (Pietropaoli et al., 1999) (Paper I) and according to the linear model of Tsoukias et al. (Tsoukias & George, 1998) (Paper II).

3.3.4 SPIROMETRY (PAPER I, II AND III)

Instrumental setup

Dynamic Spirometry was performed by all participants pre and post BD, according to ATS/ERS criteria (M. R. Miller et al., 2005);

- using a Spirare device (SPS3110 sensor and Spirare 3 software; Diagnostica AS, Oslo, Norway) (Paper I and III)
- using Jaeger Masterscreen system (CareFusion, Würzburg, Germany) (Paper II).

Breathing maneuver

Participants were instructed to inhale as much as possible via a mouthpiece and to exhale as forcefully as possible and for as long as possible through the mouthpiece immediately after full inhalation. The exhalation was considered completed when a plateau in the volume/time graph showed less than 25 mL of volume change during the last second of the exhalation.

Derived numerical variables were mainly FEV1 and FVC from where the ratio FEV1/FVC was calculated.

Predicted normal values were based on;

- ECCS/ERS reference equations (Quanjer et al., 1993) (Paper I)
- Swedish reference values reported by Hedenström et al. (Hedenstrom, Malmberg, & Agarwal, 1985; Hedenstrom, Malmberg, & Fridriksson, 1986) (Paper II)
- local reference values from Brisman et al. (Brisman, Kim, Olin, Toren, & Bake, 2016, 2017) (Paper III)

The results are presented as;

- percent predicted (%pred) (Paper I)
- %pred, the z-score (z) or the Lower Limit of Normality (LLN) set to -1.96 SD (Paper II)
- z or LLN set to -1.645 SD (Paper III)

3.3.5 MULTIPLE BREATH WASHOUT (MBW) (PAPER II)

Instrumental setup

The inert gas multiple breath washout (MBW) was performed according to current international consensus standards (Robinson et al., 2013), using an AMIS 2000 respiratory mass spectrometer (Innovision AS, Odense, Denmark) for gas analysis and a Fleisch no.1 pneumotachometer (Metabo SA, Lausanne,

Switzerland) for flow measurement and a PC with dedicated software. The respiratory mass spectrometer was calibrated prior to testing with a calibration gas containing 4.0% SF₆, 4.0% He, 7.0% CO₂, 20.9% O₂ and 64.1% N₂ (certified 1.0% relative accuracy, AGA Specialgas, Enköping, Sweden). Custom hardware (except the mass spectrometer and pneumotachometer) and software (based on LabView package, National Instruments Corporation Ltd, Newbury, UK) were used. The sample line from the mass spectrometer was inserted between the mouthpiece and the pneumotachometer, with a sample flow of less than 20 mL/min and with signals updated at 33 Hz. The pneumotachometer was connected to a by-pass system containing air with added inert gases Sulphur hexafluoride (SF₆) and Helium (He) at a concentration of 4% each.

The sampling procedure

During an initial wash-in phase the 4% SF₆ inert gas mixture was inhaled via a by-pass system until equilibrium between inspired and expired gas concentrations occurred, at which point, the washout phase was started by disconnecting the tracer gas by-pass during an expiration. Washout during room air breathing continued until end-tidal SF₆ concentration remained below 1/40th of starting concentration (i.e. 0.10%) over three successive breaths. End-tidal gas concentration was defined as mean gas concentration over 90-95% of expired volume.

Calculations of small airways ventilation inhomogeneity

A linear regression line between 50-95% of exhaled volume was used on each exhalation expirogram to identify the phase III slope (Robinson et al., 2013), as illustrated in Figure 11. Concentration normalized Slope III (Sn_{III}) analysis of the breaths during the MBW was performed to assess the indices S_{cond}, representing ventilation inhomogeneity in the small conducting airways, and S_{acin}, representing ventilation inhomogeneity in or near the acinar zone.

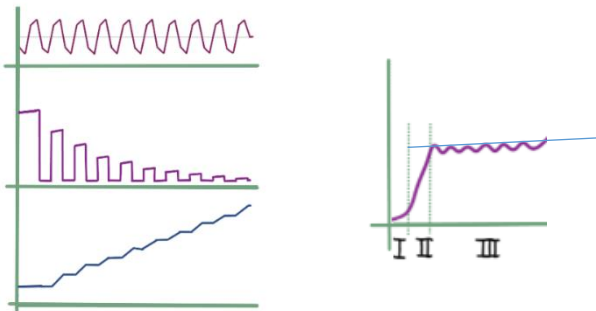


Figure 11. Illustration of MBW washout (left), with tidal breathing (upper), decreasing inert gas concentration (middle) and cumulative exhaled volume (lower), and, the Sn_{III} analysis (right).

Predicted normal values were based on reference equations from contemporary collected healthy control cohort for MBW indices (APPENDIX).

Small airways ventilation inhomogeneity was defined based on S_{cond} and/or S_{acin} >1.96 z-score, post BD.

3.3.6 SINGLE BREATH WASHOUT (SBW) (PAPER III)

Instrumental setup

The inert gas vital capacity single breath washout (VC SBW) was performed according to current international consensus standards (Robinson et al., 2013), using nitrogen (N_2) as a marker gas and the device Exhalyzer D (Eco Medics AG).

Breathing maneuver

In brief, each trial included a full expiration to residual volume (RV), followed by a slow inspiration (maximum 500 mL/s) of 100% oxygen to total lung capacity (TLC), and finally, a slow exhalation (maximum 500 mL/s) from TLC to RV (Figure 12). The inspiratory and expiratory flow was kept below the threshold of 500 mL/s with the help of instructions from the operator and by a resistor placed between the bacterial filter and the flow head.

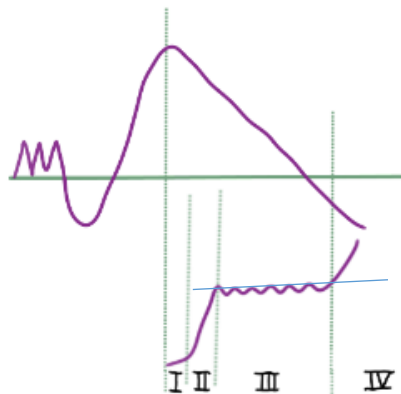


Figure 12. Illustration of the sampling procedure (upper curve) at N_2 SBW and the resulting expirogram (lower curve).

The target was three technically acceptable trials. Trials were acceptable when the coefficient of variation in the expiratory VC was less than 10% between trials. The mean value of the accepted trials was used in the analyses.

Calculations of alveolar nitrogen slope

A linear regression of data between 25–75% of the exhaled volume was performed to obtain the alveolar nitrogen slope (N_2 -slope).

3.3.7 ASTHMA CONTROL TEST (ACT) (PAPER II)

Self-reported asthma control was assessed using the Asthma Control Test (ACT) (Schatz et al., 2006). An ACT-score below 16 indicated poorly controlled asthma.

3.3.8 SYSTEMIC INFLAMMATORY VARIABLES (PAPER I, II AND III)

Blood sampling was performed for

- testing presence of specific IgE against birch-pollen (Pharmacia & Upjohn Diagnostics, Uppsala, Sweden) (Paper I)
- for analyzing blood-eosinophils (Paper II)
- for analyzing C-reactive protein (CRP) (Paper III).

In Paper II, perennial allergy was tested and defined with skin prick test (SPT) (Soluprick SQ; ALK, Copenhagen, Denmark). A positive reaction was determined to a mean wheal of ≥ 3 mm on either furred pets allergens (cat, dog, horse), mould allergens and/or house dust mites.

3.3.9 STATIONARY BIRCH POLLEN MEASUREMENT (PAPER I)

Pollen grains were collected using a 7-day recording volumetric spore sampler (Burkard Scientific Ltd, Uxbridge, Middx, UK), in which they were deposited on a spore capturing sticky tape and later on counted.

3.4 OUTCOMES

Table 3. Primary outcomes

Outcomes	Paper I	Paper II	Paper III	Paper IV
CANO (nL/L)	X (Pietropaoli et al., 1999)	X (Tsoukias & George, 1998)		
Number PEx		X	X	X
DPPC (wt%)		X	X	
POPC (wt%)		X	X	
SP-A (wt%)			X	
Albumin (wt%)			X	
SARS-CoV-2 RNA in PEx				X

3.5 STATISTICAL METHODS

Statistical analyses were performed with

- SAS version 9.3 (SAS Institute, NC, USA) (Paper I)
- IBM SPSS software, version 26.0 (SPSS, Chicago, IL) (Paper III)
- IBM SPSS software, version 28.0 (SPSS, Chicago, IL) (Paper II and IV)

Wilcoxon exact test and Wilcoxon signed rank test were used for comparison of paired observations. Paired t-test (two-sided) were used for comparison of spirometric variables between seasons (Paper I).

Non-parametric tests were used due to skewed distributions of variables from PExA and IGW (Paper II, III and IV). Mann-Whitney's U test and Kruskal-Wallis were used for comparison of continuous data between groups and chi2 test using Fischer's exact significance (two-sided) for comparison of categorical data.

The spearman rank correlation coefficient was used to test associations (Paper II and III).

Quantile regression was performed to assess associations between predictors and different quantiles of the distribution of outcome variables, with age and sex as confounder factors (Paper II and III). Quantile regression is a method suitable for skewed distribution of outcome data, with the advantage to explore associations in the outer parts of the quantiles of the outcome. Each outcome was analyzed in independent models. Predictor in Paper II was small airway ventilation inhomogeneity and in Paper III smoking status. Outcome variables in Paper II: number of PEx per breath and weight percent of DPPC and of POPC and the distribution of alveolar NO ($C_{A\text{NO}}$), and, in Paper III: number PEx per liter exhaled air and weight percent of SP-A, Albumin, DPPC and POPC in PEx.

4 RESULTS

In this section, results from all papers included in the thesis are presented based on the primary outcomes. The intention with this way of presenting is to give a summarized view over the findings from the perspective of the methods used to assess exposure effects in small airways. For further details, please see the included papers and appendix.

4.1 ALVEOLAR NO AND BIRCH POLLEN EXPOSURE (PAPER I)

Summary:

Birch pollen sensitized asthmatic subjects had significantly higher FENO50 and higher alveolar NO production (CANO) than controls, out of season. FENO, but not CANO, increased significantly in the asthmatic subjects during pollen exposure. However, a few asthmatic subjects were found to have markedly increased CANO in season. Controls were unaffected by season. Spirometry remained unchanged.

Exposure levels of Birch pollen during pollen season 2011 in Gothenburg are illustrated in Figure 13.

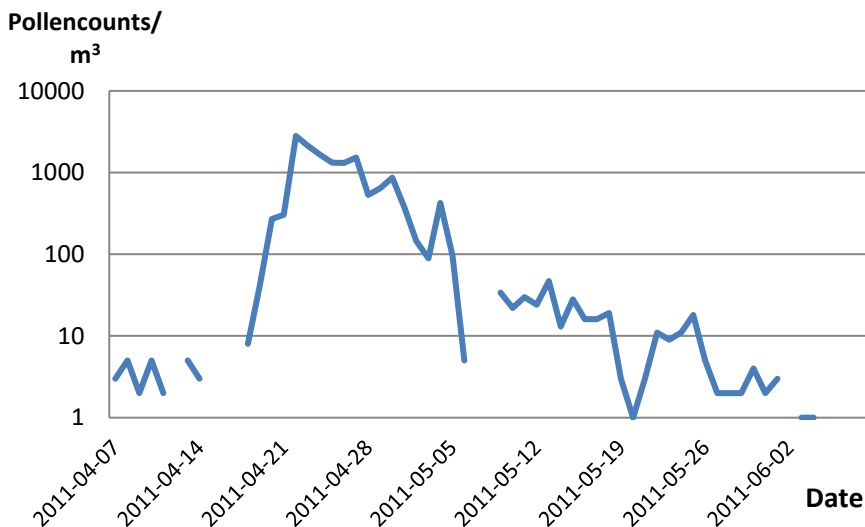


Figure 13. Pollen counts (counts/ m^3) during the pollen season 2011.

Most of the birch pollen sensitized subjects had mild asthma according to lung function assessed with spirometry, as seen in Table 4. The response to bronchodilation was also small.

Table 4. Baseline spirometry and the effect of bronchodilation, expressed as a percentage of the baseline value.

	Subjects with asthma			Controls			Subjects with asthma versus controls, p*	
	Out of season	Season	p#	Out of season	Season	p#	Out of season	Season
FVC (%pred)	102±10	103±9	0.78	109±13	108±13	0.11	0.30	0.50
FEV1 (%pred)	95±14	95±12	0.78	104±13	103±14	0.78	0.17	0.20
ΔFEV1 (%)	6.2±6.5	6.6±6.4	0.7	1.1±3.8	1.5±2.6	0.9	0.07	0.02

p* values refer to two sided Wilcoxon exact test.

p# values refer to two sided paired t-test

Birch pollen sensitized subjects had increased J_{aw}NO but unchanged CANO in pollen season, as seen in Table 5.

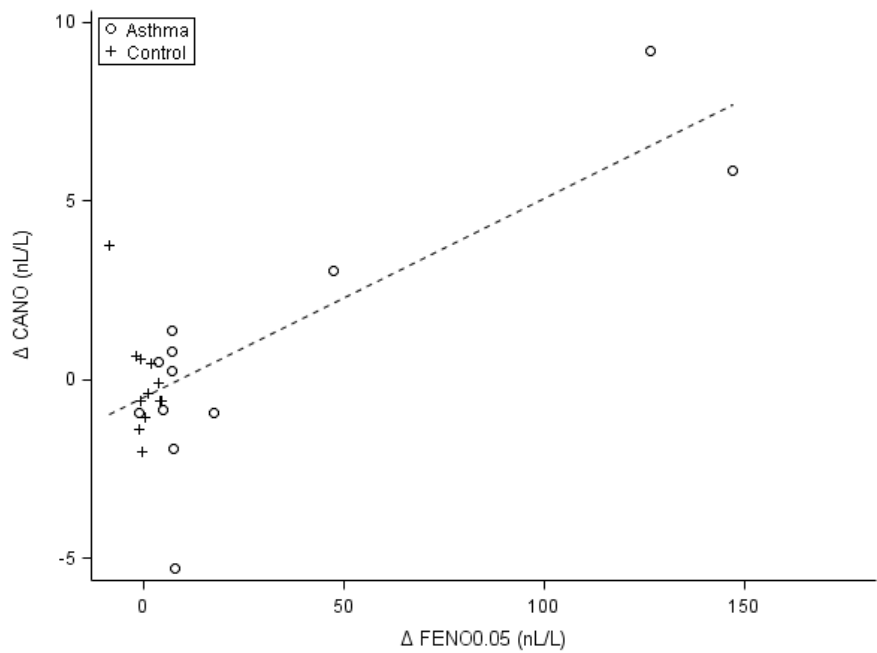
Table 5. NO from airways (J_{aw}NO), from alveolar region (CANO) and CANO corrected for back diffusion (CANO_{corrected}). Median and interquartile range is presented.

	OUT OF POLLEN SEASON			POLLEN SEASON			p
	Median	Q1	Q3	Median	Q1	Q3	
Subjects with asthma							
J _{aw} NO (nL/s)	0.84 ^S	0.66	1.49	1.84 ^S	0.94	5.69	0.001
CANO (nL/L)	3.51 ^S	2.87	4.33	4.67 ^S	2.67	8.07	0.68
CANO _{corrected} (nL/L)	1.61 ^{NS}	1.44	2.31	1.01 ^{NS}	0.33	2.00	0.012
Controls							
J _{aw} NO (nL/s)	0.53	0.43	0.75	0.72	0.40	0.87	0.27
CANO (nL/L)	2.60	2.08	3.25	2.09	1.55	3.36	0.38
CANO _{corrected} (nL/L)	1.61	1.15	2.30	1.14	0.77	2.11	0.27

p values are two sided Wilcoxon signed rank of differences between season and out of season.

^S and ^{NS} denotes a significantly/non-significantly higher value for subjects with asthma compared to controls (one sided Wilcoxon exact test).

All but one study participant sensitive to birch pollen had increased levels of FENO50 during spring 2011 in comparison to the value registered during autumn 2010, as seen in Figure 14. The majority of asthmatic subjects had minor changes, but four showed markedly increased FENO50. Three of these four subjects also had elevated CANO. Ten subjects, five asthmatics and five controls, also showed a decrease in CANO during pollen season.



4.2 ALVEOLAR NO AND SMALL AIRWAYS VENTILATION INHOMOGENEITY (PAPER II)

Summary:

The calculated alveolar NO production (C_{ANO}) was found increased in subjects with small airway disease, but with a wide spread.

Lung function and FENO outcomes, with study participants subdivided based on small airways ventilation are presented in Table 6.

Table 6. FENO and Lung function in asthma cohort, subdivided based on S_{cond} and S_{acin} ≤ and vs S_{acin} and/or S_{cond} >ULN. Data displayed as median (Q1; Q3). P-values from Mann-Whitney U test between groups.

Variable	S _{acin} and S _{cond} ≤ ULN	S _{acin} and/or S _{cond} > ULN	p-value
N	45	27	
F _E NO ₅₀ , ppb	16.6 (9.8; 30.3)	31.1 (17.5- 67.2)	0.006
C _A NO, ppb	1.1 (0.3; 2.0)	2.3 (1.4- 3.1)	0.003
FEV ₁ post BD, z	0.32 (-0.89; -1.36)	-1.59 (-2.13; 0.07)	0.001
Reversibility of FEV ₁ , %	4.6 (2.2; 8.6)	12.0 (6.4; 22.4)	<0.001
FVC post BD, z	0.32 (-0.63; 1.18)	-0.94 (-1.87; 1.34)	0.05
FEV ₁ /FVC post BD, %	-0.75 (-1.37; -0.14)	-1.87 (-3.05; -1.33)	<0.001

The subject with the highest C_ANO (6.7 ppb) also had a markedly increased F_ENO (158 ppb), an association also indicated by the correlation between FENO₅₀ and C_ANO (r_s=0.55, p<0.01) illustrated in Figure 15.

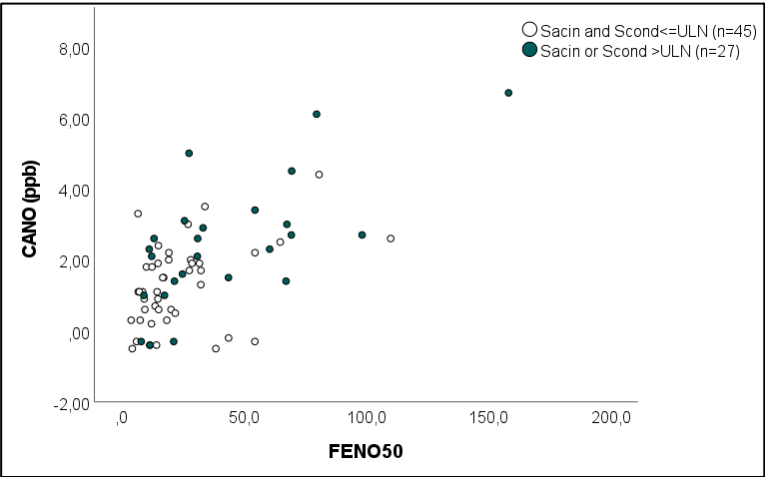


Figure 15. Scatter plot of $CANO$ (ppb) by $FENO50$, in subjects with compared to without small airways ventilation inhomogeneity.

In the multiple regression analysis (Figure 16), adjusted for age and sex, alveolar NO ($CANO$) was found increased in subjects with compared to without small airways ventilation inhomogeneity. The confidence interval however, did overlap in the higher quartiles.

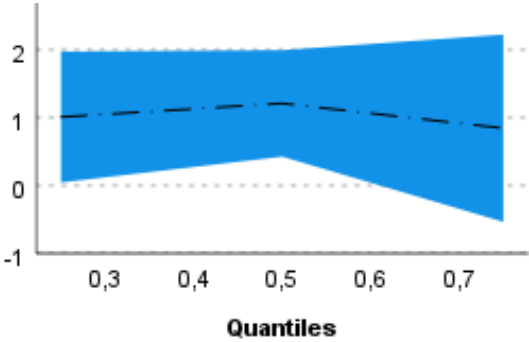


Figure 16. Levels of $CANO$ (ppb) in asthmatic subjects with compared to without small airways ventilation inhomogeneity. Plots illustrating sex- and age-adjusted quantile regression estimates (black dotted line with blue confidence interval) from subjects with small airways ventilation inhomogeneity ($Sacin$ and/ or $Scond > ULN$). Subjects without small airways ventilation inhomogeneity ($Sacin$ and $Scond \leq ULN$) equals parameter estimates of zero (set as a reference). Quantiles on x-axis refers to the distribution of $CANO$.

4.3 AMOUNT OF PEX AND SMALL AIRWAYS VENTILATION INHOMOGENEITY (PAPER II)

Summary:

Subjects with asthma and with small airways ventilation inhomogeneity, assessed with S_{acn} and/or $S_{cond} > ULN$, was associated with lower levels of number exhaled particles in subjects with asthma, in comparison to subjects with asthma without small airways ventilation inhomogeneity.

In a multiple regression analysis, adjusted for sex and age, the entire distribution of number PEx per breath were significantly less in subjects with small airways ventilation inhomogeneity in comparison to those without (Figure 17).

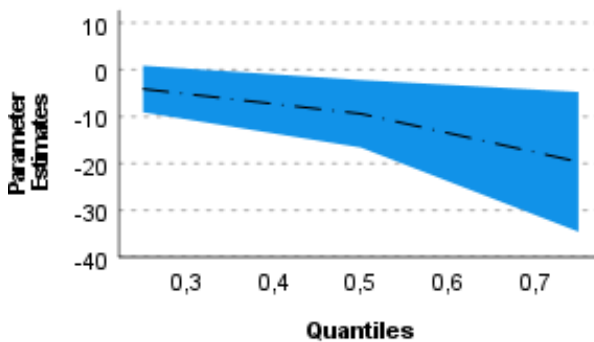


Figure 17. Levels of number of PEx (kn/ breath) in asthmatic subjects with compared to without small airways ventilation inhomogeneity. Plots illustrating sex- and age-adjusted quantile regression estimates (black dotted line with blue confidence interval) from subjects with small airways ventilation inhomogeneity (S_{acn} and/or $S_{cond} > ULN$). Subjects without small airways ventilation inhomogeneity (S_{acn} and $S_{cond} \leq ULN$) equals parameter estimates of zero (set as a reference). Quantiles on x-axis refers to the distribution of number of PEx.

Particle fraction of the smallest particle sizes were found in higher amounts in subjects with small airway ventilation inhomogeneity (Figure 18).

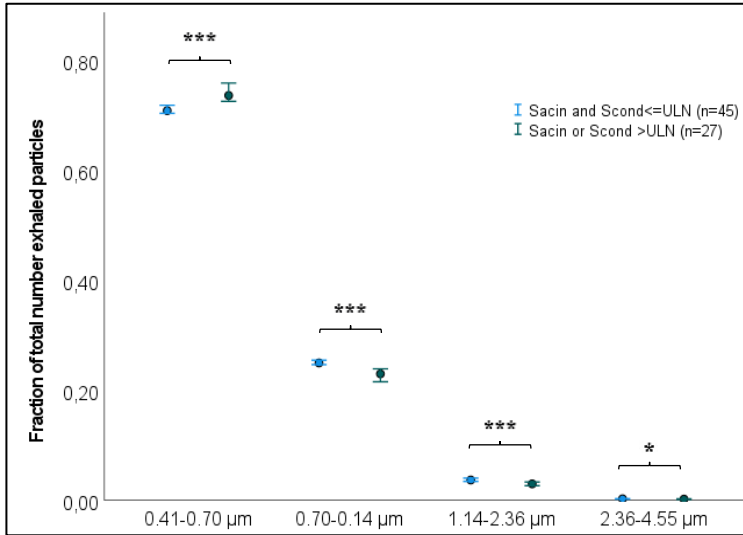


Figure 18. Fraction of the total numbers of exhaled particles in size intervals: 0.41-0.70 μm ; 0.70-1.14 μm ; 1.14-2.36 μm and 2.36-4.55 μm of total number exhaled particles, by small airway ventilation indices (S_{acin} and S_{cond}), post BD, above and below ULN. * $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$ from Mann-Whitney U test between groups.

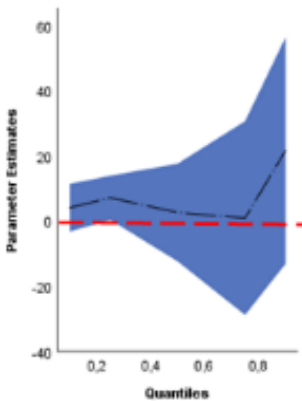
4.4 AMOUNT OF PEX AND ACTIVE SMOKING (PAPER III)

Summary:

Higher levels of number PEx (kn/L) was found, in current compared to never smokers, among subjects with impaired lung function according to spirometry.

In multiple regression analysis, adjusted for age and sex, separate analyses were performed on study participants with A) normal and B) impaired lung function, as seen in Figure 19. Current smokers were associated with higher levels of PEx, among subjects with impaired lung function.

A) Normal lung function



B) Impaired lung function

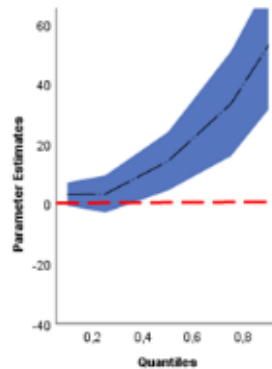


Figure 19. Amount PEx (PEX/L) among current- compared to never-smokers at normal and abnormal lung function. Plots illustrating age- and sex-adjusted quantile regression estimates of current smokers to never smokers (the red dotted line), stratified on normal and abnormal lung function ($n=35$ and $n=31$, respectively). Parameter estimates from current smokers compared to never smokers presented at normal lung function ($n=19$ and $n=16$, respectively) and abnormal lung function ($n=18$ and $n=13$, respectively). Sub-groups were analyzed separately. Quantiles on x-axis refers to the distribution of PEx/L. Estimates from quantile regression denoted by black dotted line with blue confidence intervals.

4.5 AMOUNT OF PEX AND SARS-COV-2 INFECTION (PAPER IV)

Summary:

Subjects with COVID-19 exhaled less amount of PEx than healthy controls during normal breathing and airway opening maneuvers.

No association between amount PEx and viral load in aerosol.

No obvious associations was found between neither PEx/breath and detection of viral load in any of the breathing maneuvers, nor between detection of viral load in aerosol and viral load from oro/nasopharyngeal swab, as seen in Figure 20.

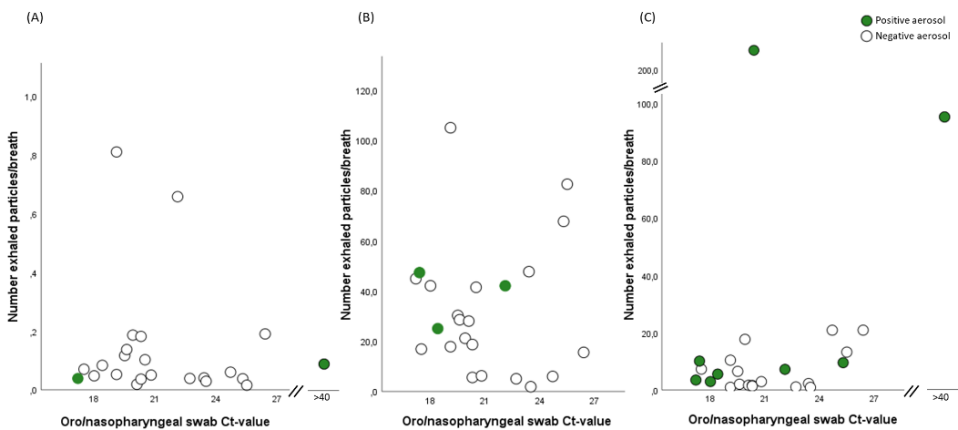


Figure 20. Number of exhaled particles, expressed as per breath, collected with the PExA method from (A) 20 relaxed breaths, (B) 10 airway opening maneuvers with breath-hold, and from (C) 3 coughing maneuvers, in subjects with COVID-19.

Subjects with COVID-19 exhaled on average less particles per breath than that of controls, at relaxed breathing and at the breathing maneuver including deep breaths with breath-hold, as seen in Figure 21.

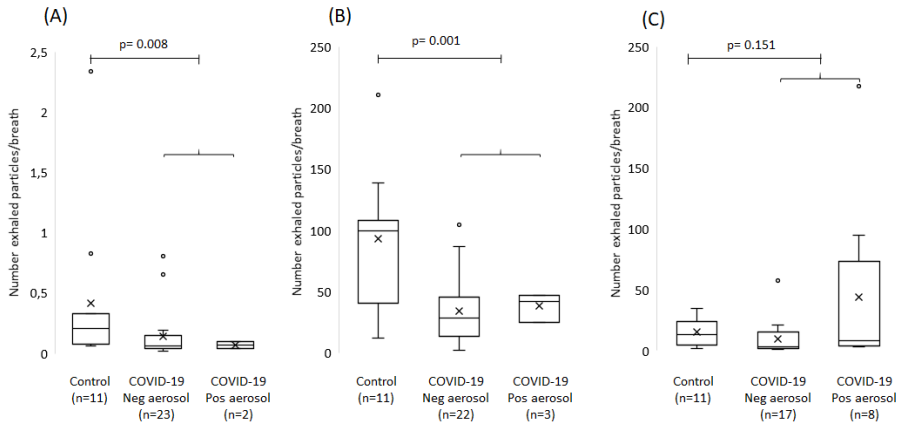


Figure 21. Box plots of particle number concentrations among study participants subdivided in control subjects and subjects with clinically confirmed COVID-19 with or without positive aerosol sample.

The two outliers with highest number of exhaled particles/breath of all sampled aerosols, and with detected viral load in aerosol, also exhaled 100 times as many particles $< 1.1 \mu\text{m}$ than that of the other study participants at cough, as seen in Figure 22.

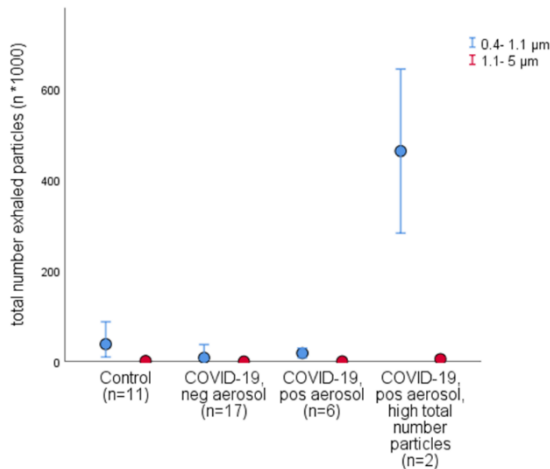


Figure 22. Cough samples with total number of exhaled particles $< 5 \mu\text{m}$ presented, from control subjects, and in subjects with COVID-19 infection. 2 subjects with extreme high total number of Pex are presented separately. Median (dots) and 95%CI (error bars) presented.

4.6 MAJOR SURFACE ACTIVE LIPIDS IN PEX AND SMALL AIRWAYS VENTILATION INHOMOGENEITY (PAPER II)

Summary:

Small airways ventilation inhomogeneity, assessed with S_{acin} and/or $S_{cond} > ULN$, was not associated with altered levels of the surfactant lipids DPPC and POPC in exhaled aerosol particles.

Levels of surface active lipids in PEx (POPC and DPPC) were highly correlated to each other (r_s 0.67, $p < 0.01$) in the full asthma cohort ($n=72$).

No difference in levels of surface active lipids (DPPC, POPC) in PEx in subjects with compared to without small airways ventilation inhomogeneity was found in the multiple regression analysis, adjusted for age and sex (Figure 23).

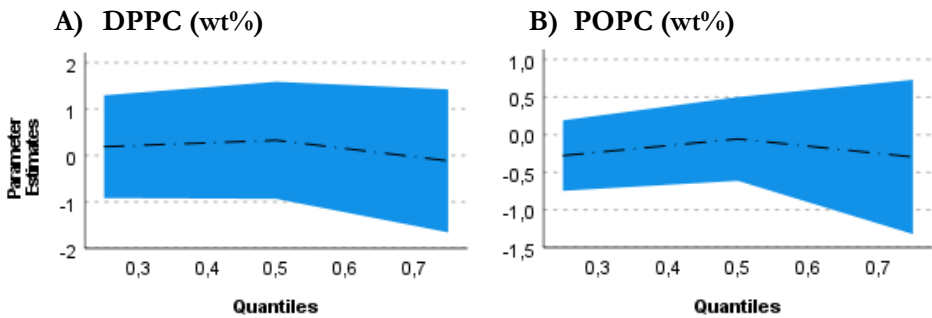


Figure 23. Levels of A) DPPC (wt%) and B) POPC (wt%) in asthmatic subjects with compared to without small airways ventilation inhomogeneity. Plots illustrating sex- and age-adjusted quantile regression estimates (black dotted line with blue confidence interval) from subjects with small airways ventilation inhomogeneity (S_{acin} and/or $S_{cond} > ULN$). Subjects without small airways ventilation inhomogeneity (S_{acin} and/or $S_{cond} \leq ULN$) equals parameter estimates of zero (set as a reference). Quantiles on x-axis refers to the distribution of the outcome-variable studied (DPPC, POPC).

4.7 MAJOR SURFACE ACTIVE LIPIDS IN PEX AND CURRENT EXPOSURE TO TOBACCO SMOKE (PAPER III)

Summary:

Increased levels of major surface-active lipids in PEx, in current compared to never smokers, among subjects with normal lung function according to spirometry.

Among current smokers ($n=37$), no association was found between surface active lipids in PEx (DPPC and POPC in wt%) and ventilation inhomogeneity assessed with N₂ SBW (N₂-slope in %N₂/L) (r_s 0.206 and 0.032, respectively). Packyears in current smokers did not correlate with either DPPC (r_s -0.033 $p=0.855$) as illustrated in Figure 24, nor with POPC (r_s -0.094, $P=0.597$).

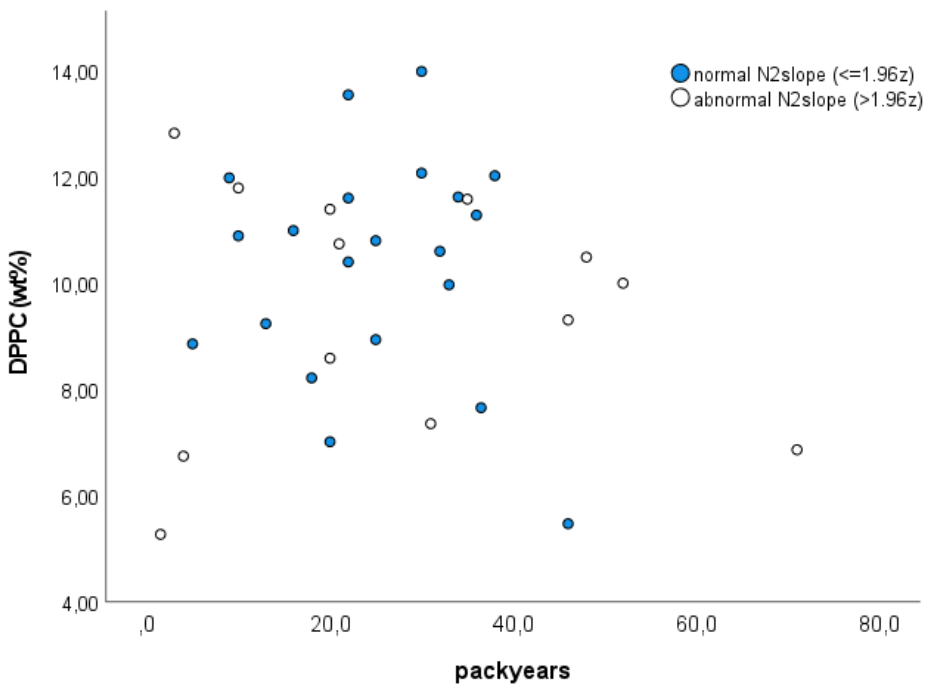


Figure 24. scatterplot of DPPC (wt%) by packyears, in current smokers subdivided on normal or impaired ventilation assessed with N₂ SBW.

In crude regression analysis (Table 7) current smokers had higher concentration of DPPC and POPC in PEx than never smokers.

Table 7. DPPC and POPC in PEx, in study population, subdivided according to smoking category. Data are presented as median with interquartile range (Q1-Q3).

	Never smokers	Former smokers	Current smokers	p-value
DPPC (wt %)	10.3 (8.5- 11.7)	10.6 (8.6- 11.6)	11.3 (10.2- 12.9) ^a	0.025
POPC (wt %)	2.9 (2.5- 4.0)	3.1 (2.4- 3.6)	3.7 (3.2- 4.2) ^{a,b}	0.008

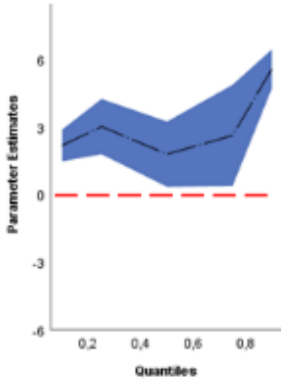
Note: P-values based on Kruskal-Wallis test followed by Bonferroni's multiple comparisons tests of significant difference ($p < 0.05$) between:

^acurrent smokers and never smokers;

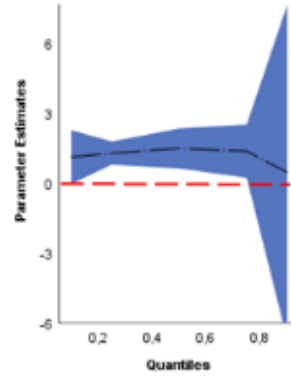
^bcurrent smokers and former smokers

In the multiple regression analyses among subjects with normal lung function (FEV_1 , FVC and $FEV_1/FVC \geq LLN$) and adjustments for age and sex, current smokers were associated with higher levels of DPPC and POPC in PEx, in comparison to never smokers (Figure 25 A). When restricting the analysis to subjects with impaired lung function (FEV_1 and/or FVC and/or $FEV_1/FVC < LLN$), current and never smokers were not significantly different with respect to levels of surface active lipids (Figure 25 B).

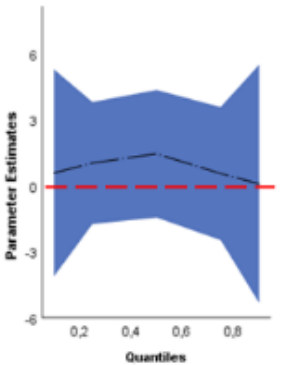
A. DPPC (wt%)
normal lung function



POPC (wt%)
normal lung function



B. DPPC (wt%)
impaired lung function



POPC (wt%)
impaired lung function

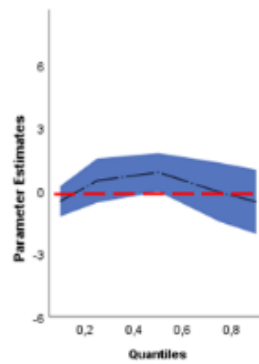


Figure 25. Major surface active lipids in PEx among current- compared to never-smokers at normal and impaired lung function. Plots illustrating age- and sex-adjusted quantile regression estimates of current smokers to never smokers (the red dotted line), stratified on normal and impaired lung function ($n=35$ and $n=31$, respectively). A) DPPC (wt%) and B) POPC (wt%) were analyzed separately, as were the sub-groups. Quantiles on x-axis refers to the distribution of the PEx-variable studied. Estimates from current smokers compared to never smokers presented at normal lung function ($n=19$ and $n=16$, respectively) and impaired lung function ($n=18$ and $n=13$, respectively). Estimates from quantile regression denoted by black dotted line with blue confidence intervals.

4.8 SURFACTANT PROTEIN A IN PEX (III)

Summary:

SP-A in PEx was found increased at impaired lung function, in current compared to never smokers.

Multiple regression analysis with adjustments for age and sex was performed separately for SP-A in PEx among subjects with normal lung function according to one or several spirometry indices, and, among subjects with impaired lung function (Figure 26). Current smokers, in comparison to never smokers, was associated with higher levels of SP-A in PEx, at impaired lung function, significant mainly in the higher quantiles of SP-A. The trend was similar at normal lung function but less pronounced. Albumin was unaffected by smoking status (for results on albumin, see paper III).

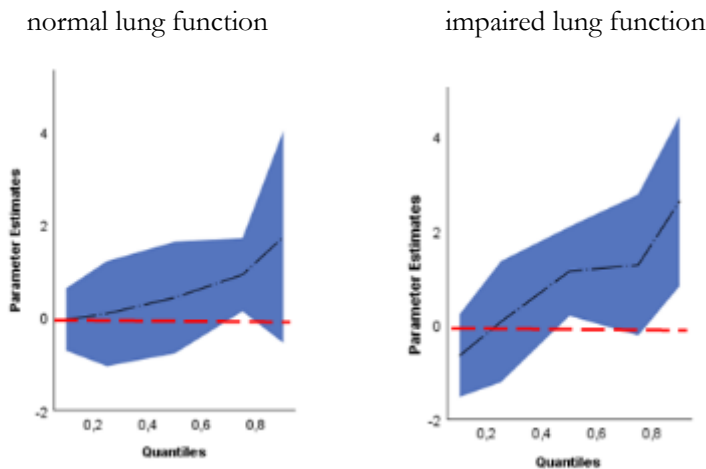


Figure 26. SP-A in PEx among current- compared to never-smokers at normal and impaired lung function. Plots illustrating age- and sex-adjusted quantile regression estimates of current smokers to never smokers (the red dotted line), stratified on normal and impaired lung function ($n=35$ and $n=31$, respectively). Sub-groups were analyzed separately. Quantiles on x-axis refers to the distribution of SP-A (wt%). Estimates from current smokers compared to never smokers presented at normal lung function ($n=19$ and $n=16$, respectively) and impaired lung function ($n=18$ and $n=13$, respectively). Estimates from quantile regression denoted by black dotted line with blue confidence intervals.

4.9 VIRAL RNA IN PEX (PAPER IV)

Summary:

Detection of COVID-19 is possible in a few exhalations and coughs, sampled with PEXA and BE in individuals early in disease course of COVID-19.

SARS-CoV-2 RNA detection in different breathing maneuvers

Sampling of aerosol particles from subjects with acute COVID-19, using PEXA and BE, resulted in total, in 10 out of 25 subjects with detectable viral load in aerosol (Table 8). Sampling with PEXA by 20 relaxed breaths, 10 deep breaths with breath-hold, and 3 coughs resulted in detection of SARS-CoV-2 RNA in 13 samples (2 from relaxed breaths, 3 from deep breaths and 8 from coughs), found in 9 out of 25 subjects with confirmed COVID-19. Sampling of aerosol by 20 relaxed breaths using the BE instrument resulted in detection of SARS-CoV-2 RNA in 2 out of 25 subjects. The positive aerosol findings with each method were from different subjects. The detected viral load in aerosol was low in comparison to that of oro/nasopharyngeal swab.

Table 8. Viral load at clinical test, aerosol sampling with PExA at three different breathing maneuvers, and, aerosol sampling with BE at relaxed breathing. Subjects are numbered according to rising Ct-value from clinical test with oro/nasopharyngeal swab.

	Clinical test	PExA (<5 µm)						BE
	swab	Normal breaths		Airway opening maneuvers		Coughs		Normal breaths
ID	Ct	Ct	PEx /breath	Ct	PEx /breath	Ct	PEx /breath	Ct
1	17.2	33.8	0.0	-	44.9	35.4	3.5	-
2	17.4	-	md	33.4	47.3	36.2	10.1	37.7
3	17.5	-	0.1	-	16.9	-	7.3	-
4	18	-	0.0	-	42.0	36.5	2.9	-
5	18.4	-	0.1	31.8	25.0	34.5	5.5	-
6	19.1	-	0.8	-	104.9	-	10.4	-
7	19.1	-	0.1	-	17.8	-	0.9	-
8	19.5	-	0.1	-	30.2	-	6.5	37.5
9	19.6	-	0.1	-	28.5	-	2.0	-
10	19.9	-	0.2	-	21.1	-	17.7	-
11	20.1	-	0.0	-	28.0	-	1.7	-
12	20.3	-	0.2	-	18.6	-	1.6	-
13	20.3	-	0.0	-	5.6	-	1.4	-
14	20.5	-	0.1	-	41.4	29.5	217.4	-
15	20.8	-	0.0	-	6.2	-	2.9	-
16	22.1	-	0.7	36.8	42.0	36.4	7.2	-
17	22.7	-	0.0	-	5.0	-	1.1	-
18	23.4	-	0.0	-	47.7	-	2.3	-
19	23.5	-	0.0	-	1.9	-	0.9	-
20	24.7	-	0.1	-	6.0	-	20.9	-
21	25.3	-	0.0	-	67.6	35	9.5	-
22	25.5	-	0.0	-	82.4	-	13.2	-
23	26.4	-	0.2	-	15.5	-	20.9	-
24	-	-	0.1	-	29.5	35.8	94.8	-
25*	-	36.8	0.1	-	86.7	-	57.5	-

Note: Exhaled particles (PEx) expressed as n*1,000 (kn) per breath.

Abbreviations: Ct; cycle threshold; md; missing data.

*ID 25 also had a positive PCR in sample of particles >5 µm (Ct value = 37), collected with PExA at normal breaths.

Symptom patterns as well as symptom duration were similar between subjects with COVID-19, with compared to without detected viral load in any of the aerosol samples collected, as seen in Table 9.

Table 9. Demographical characteristics of study participants, with COVID-19 subjects subdivided according to positive or negative aerosol PCR.

Variable	Pos aerosol-PCR (n=9)	Neg Aerosol-PCR (n=16)	Controls (n=11)	p-value
Females	5 (56)	12 (75)	7 (64)	0.593
Age, years	48 (33-61)	42 (23-58)	42 (23-67)	0.463
Current smoker	0	1 (7)	1 (9)	
Swab, Ct-value from RT-PCR	18,4 (17,2-25,3)	20,3 (17,5-26,4)	-	0.175
Symptom duration, days	2 (1-9)	2 (0-9)	0 (0)	0.834
<i>Symptoms</i>				
shortness of breath at rest	1 (11)	2 (13)	0 (0)	1.000
cough	8 (89)	9 (56)	0 (0)	0.182
fever	5 (56)	5 (31)	0 (0)	0.397
runny nose	7 (67)	11 (69)	0 (0)	1.000
nasal congestion	2 (22)	11 (69)	0 (0)	0.017
sore throat	6 (67)	7 (44)	0 (0)	0.411

Note: analyses on symptom differences was only performed between subjects diagnosed with COVID-19 with or without positive aerosol sample.

5 DISCUSSION

The overall quality of the studies included in this thesis can be addressed by trying to evaluate the validity and reliability of the studies (Gunnarsson, 1999). Good quality is desirable and achieved by high validity and high reliability. This evaluation may also help to decide whether or not to go further with the findings from the included studies. The use of methods with good validity and reliability is needed if the results from the measurements were to be made generalizable for others than those being studied.

Alveolar NO, amount PEx and major contents in PEx as well as SARS-CoV-2 in PEx, used in this thesis to explore small airways impairment in different exposure settings, will here be touched upon based on the following validity and reliability aspects, adopted from Gunnarsson (Gunnarsson, 1999):

Validity- *To measure what is relevant in the context, that is, striving at measuring what you actually want to measure*

Can be further explained by:

Content validity- *The selected method is suitable for the purpose of the measurement*

Coherence validity- *results gained is consistent with other studies*

Construct validity- *measurements are in agreement with related concepts*

Communicative validity- *the availability of the research process*

Pragmatic validity- *the usefulness of the results*

Reliability- *To measure in a reproducible way, that is the accuracy of an instrument.*

Can be further explained by:

Inter-rater reliability- *the agreement between results gained from different operators*

Test-retest reliability- *the effect of time on the results*

Internal consistency reliability- *the agreement with response in a questionnaire addressing the same phenomenon*

5.1 VALIDITY AND RELIABILITY ASPECTS OF ALVEOLAR NO (PAPER I, II)

VALIDITY

Research questions in Paper I and II aimed at assessing if there is an increase in alveolar NO in subjects with asthma sensitized to pollen, and if there is an association with small airways ventilation inhomogeneity. Further, we wanted to compare modeling of alveolar NO with clinical outcomes.

Exhaled nitric oxide is a measure of type-II inflammation in the airways, and is performed in a non-invasive and easy way, minimizing the discomfort and the risk for a study participant, wherefore we considered it highly justifiable to use in this context.

Models for alveolar NO (Paper I and II)

The modelling of alveolar NO was at the time of the studies, a novel attempt to try to pick an inflammatory signal from the smallest airways, as it was lacking non-invasive methods to achieve this. Different models for alveolar NO are still being used, and there is no generally agreed upon model accepted. Modelling of alveolar NO in Paper I was performed according to Pietropaoli et al. (Pietropaoli et al., 1999), but also recalculated using the method of Tsoukias et al. (Tsoukias & George, 1998), which is one of the methods recommended by the ERS-task force (Horváth et al., 2017). Anyhow, there is yet no common conformity regarding the use of either a linear (Tsoukias & George, 1998) or a non-linear (Högman et al., 2002) mathematical method to calculate the alveolar NO. In addition, the added clinical value of alveolar NO is also yet to be established (Lehtimäki et al., 2020).

Alveolar NO and exposure to birch pollen (Paper I)

The lack of increase of alveolar NO during pollen season in subjects with asthma sensitized to pollen remains unexplained. Some of the subjects with asthma included in the study were also sensitized to perennial allergens, but did not report any respiratory symptoms outside pollen-season. Despite this, they might have had subclinical inflammation in small airways. We could neither confirm nor exclude this, as no other methods were used to assess small airway function. An alternative explanation is that the Bet v 1 allergens do not reach small airways. The pollen grain is large and for the allergen to reach small airways, the grain needs to burst and the allergens needs to be inhaled. Using pollen-exposure to assess the validity of modelled alveolar NO should therefore preferably have been done with a parallel method to assess small airway involvement.

Concerning the validity of exposure assessments in Paper I, the individual exposure levels to pollen were unknown. The registration of pollen counts were

performed at the roof of Eastern Hospital in Gothenburg, but pollen exposure may vary a lot in the Gothenburg region. Most days the registered pollen counts were above 15 per m³, the limit likely to give rise to symptoms in many sensitized subjects (Frenz, 2000). The individual exposure levels to pollen were, however, probably highly variable within the studied group. There is also a risk that subjects with severe pollen-allergy avoid exposure, for example by staying indoors. In addition, the exposure to airway irritants outside pollen season are unknown. Also, the unknown small airways impairment in present study might bias the results.

The aim of the study was also to validate a model including corrections for back-diffusion of NO and its effect on CANO (CANO_{corrected}). In our hand this approach does not seem adequate, as it resulted in lower CANO during pollen season, that we judged highly unlikely. The unrealistic decrease in CANO during pollen season have also been seen in subjects with rhinitis (Tufvesson et al., 2007). In addition, similar results were found by Verbanck et al. (Verbanck et al., 2008) who concluded that the suggested correction for back-diffusion overestimates the effect due to constriction of intra acinar airways. In the ERS technical standard for exhaled biomarkers in lung disease (Horváth et al., 2017), it is postulated that the correction for backdiffusion on CANO in subjects with decreased small airways may lead to overcorrection resulting in negative values, wherefore the use of correction factors are not recommended. Anyhow, small airways ventilation inhomogeneity, CANO and CANO corrected for back diffusion may together add knowledge to the understanding of the molecular diffusion of NO from small airways into the alveolar compartment.

Alveolar NO and small airways ventilation inhomogeneity (Paper II)

In Paper II, CANO was higher in subjects with small airways ventilation inhomogeneity, as assessed by multiple breath washout test, in a cross-sectional setting. There was however no correlation between CANO and S_{acin} or S_{cond} , respectively. The association between CANO and small airways ventilation inhomogeneity was mainly significant in the lower end of the distribution of CANO (i.e. within normal range of CANO), indicating that higher CANO is determined by other factors than that of small airways ventilation inhomogeneity.

Coherence with other studies (Paper I and II)

In the literature, there are contradictory results on CANO during pollen season. As in Paper I, Lopez et al found no increase of CANO during pollen season (Lopez et al., 2012). Tufvesson et al., on the other hand, found subjects with rhinitis and asthma to have increased CANO during pollen-season compared to out of season (Tufvesson et al., 2007).

Up to now, published studies include relatively few subjects why the results must be interpreted with caution. Small airway inflammation give rise to few symptoms (Mead, 1970), and exposure to perennial allergens present outside pollen season might differ between studies. If there is an enrichment of subjects with allergic responses also outside pollen season, exposure to birch pollen might not further increase the alveolar NO production? This emphasizes the importance of a profound characterization of the participating subjects.

Most of the subjects with birch pollen sensitization in Paper I had normal and unchanged spirometry in pollen season. Inflammatory responses usually occur prior to onset of clinical symptom or disease, wherefore symptoms and lung function decline might not be present at the same time as elevated FENO is detected. This has recently been further highlighted with elevated CANO prior to onset of symptoms and clinical changes (Koskela et al., 2021; Sprio et al., 2022). Koskela et al. reported a high CANO at baseline in foundry workers to be associated with an accelerated decline in lung function, validating the measurement of alveolar NO in the exposure setting. Spiro et al. reported a higher CANO one month prior to exacerbation in subjects with asthma and with experiences of recurrent exacerbations, suggesting the use of CANO as a marker of impending exacerbations. CANO has also been shown to be increased in silica exposed workers (Sauni et al., 2012) and asbestos exposed workers (Lehtimäki et al., 2010).

RELIABILITY

FENO was performed according to ATS/ERS guidelines ("ATS/ERS recommendations for standardized procedures for the online and offline measurement of exhaled lower respiratory nitric oxide and nasal nitric oxide, 2005," 2005) in both Paper I and II. This implies that each subject performed at least two measurements on each exhalation flow to reach plateau phase values that agree within 10% of each other.

In contrast to present recommendations (Horváth et al., 2017), suggesting three flow-rates of 100 mL/s and above, three flowrates were used in paper I, but only two of 100 and above (50, 100 and 270 mL/s). Also, the model of Pietropaoli et al. (Pietropaoli et al., 1999) was used, which is not one of the recommended models today. Similarly, only two flow-rates of 100 and above were used in Paper II (100 and 300 mL/s), however with the model of Tsoukias et al. (Tsoukias & George, 1998) recommended as one of two to use today.

The agreement between results gained from different operators for FENO was not tested in either Paper I or Paper II, but is generally considered to be high, as the exhalation flow is required to be within certain limits to be accepted by the

instrument. The performance of instructions between examiners may however vary, and hence the number of subjects that succeed in performing the test.

The clinical examination in Paper I was only performed once in season and once outside season, based on limited resources. More frequent examinations during each season had increased the reliability. FENO at multiple flows in Paper II could have been addressed at several occasions due to the recurrent visits by the study participants. Anyhow, that was unfortunately not the design of the study.

Neither the measurement of FENO in Paper I nor in Paper II was performed with any regard to time of day.

THE USEFULNESS OF THE RESULTS (Paper I and II)

Based on our results in Paper I, we cannot with certainty identify any added value of CANO on group level in birch pollen sensitized subjects with asthma during pollen season. CANO was increased both during- and outside the pollen-season in the group with asthma, in comparison to healthy controls. FENO50 was also increased, and the subjects that increased most in FENO50 during pollen season also increased most in alveolar NO. On group level, FENO50 however, increased during pollen-season suggesting an increased inflammation in more central airways. The additive value of modelling CANO to FENO50 is not obvious neither in Paper I nor Paper II, due to the conformity between the two outcomes. Even though elevated CANO was found in some subjects with asthma, these subjects also had elevated FENO50, in both Paper I and II. Anyhow, the notion that inflammation might be present in peripheral as well as central airways may contribute to the choice of treatment, in comparison to only targeting the central airways.

The method to correct alveolar NO for back diffusion (CANO_{corrected}) seems incorrect in Paper I. Anyhow, back-diffusion may influence CANO but more information on small airways constriction and alveolar volume would be of interest and need further investigations, for instance with outcomes from inert gas washout techniques and body plethysmography. Our results suggest that inflammation in small airways may increase during pollen-season, in a subset of subjects.

High values of CANO in Paper II did not separate subjects with asthma with or without small airways ventilation inhomogeneity. Current exposure to allergens as well as individual levels of specific IgE was not addressed in Paper II.

Findings on alveolar NO in this thesis therefore indicate a potential value of CANO on individual level, depending on context, but also the need for more data on individual exposure to compare exposure effects between subjects.

5.2 VALIDITY AND RELIABILITY ASPECTS OF AMOUNT PEX (PAPER II, III AND IV)

VALIDITY

Research questions in Paper II, III and IV targeted at exploring whether amount PEx is altered in subjects currently or previously exposed to inhalable irritants with potential to harm the small airways.

The standardized breathing maneuver, used in all three Papers, have shown to increase the mass of exhaled particles with 100-1000 times in comparison to slow exhalations (Larsson, Bake, et al., 2017), in line with findings in Paper IV. Overall, there is a large and unexplained inter-individual variability in number of exhaled particles that hampers its use as biomarker (Bake, Ljungström, et al., 2017; Morawska, 2009; Schwarz, Biller, Windt, Koch, & Hohlfeld, 2010). Also, at different aerosol generating breathing patterns such as singing and talking, there is a large variation (S. L. Miller et al., 2021).

There were associations between amount PEx and small airways ventilation inhomogeneity and smoking status, respectively, in the crude regression analysis in Paper II and III. These associations were further examined with quantile regression and adjustments for age and sex. Quantile regression is a method suitable for skewed distribution of outcome data and further presents the opportunity to explore associations in the outer parts of the quantiles of the outcome. This was warranted in Paper II and III, as the associations were suspected not to be linear. Asthma is a heterogeneous disease with varying severity, as are the varying effect of smoking in different subjects. We suspected that small airways ventilation inhomogeneity in subjects with asthma, as well as the effect of active smoking, were unequal distributed among included subjects. The outcome PEx may be both increased and decreased in different stages of a disease progression, which we wanted to explore using the quantile regression.

Amount PEx and impaired lung function (Paper II and III)

Inflammation, mucus plugging, epithelial disruption and remodeling of airways causes narrowing of the airways. Obstruction in the small airways may also be due to parenchymal destruction leading to loss of lung elastic recoil. Obstructed small airways have little effect on lung mechanics, due to its low contribution to the total airway resistance. At the same time, small airways obstruction has major effect on ventilation distribution (Robinson et al., 2009).

Amount PEx and cigarette smoke exposure (Paper III)

Cigarette smoke is known to cause great damage in the small airways (Ghio et al., 2008), but there is yet no method to detect early pathological processes. Whether increase/decrease in PEx reflects exposure effects such as increased closing volume is not known. In previous studies, number of PEx have been associated with lung function (FVC and FEV₁), but its actual association with closing volume has not been examined so far. The closing volume, i.e. the lung volume during an expiration when a substantial number of airways close, has previously been reported as increased in current smokers (Buist & Ross, 1973; McCarthy et al., 1972). This is why we wanted to examine if the amount of PEx is affected in smoking. The findings of current smokers associated with higher amount PEx, in comparison to never smokers, may be interpreted as increased number of small airways that close and re-open, but unfortunately closing volume was not measured in those subjects. Why there is an increase in number of exhaled particles we can only speculate: the composition of the lining fluid may alter, alternatively the diameter of small airways decrease and imply that a larger number of airways close and re-open.

Coherence with other studies (Paper II and III)

Subjects with asthma, in comparison to lung healthy controls, have previously been reported with less amount PEx, though sampled during forced exhalations (Almstrand et al., 2012). Small airways dysfunction, assessed with impulse oscillometry and multiple breath washout, was associated with lower number of exhaled particles in subjects with asthma in a study from Soares et al. (Soares et al., 2018), in line with findings in Paper II. In a pilot-study of COPD, by Lärstad et al. (Larstad et al., 2015), lower number of particles were also associated with impaired lung function (FVC and FEV₁). Subjects with asthma sensitized to birch pollen have also been shown with decreased number of PEx at exposure to birch pollen, in comparison to healthy controls (Larsson, Larstad, et al., 2017), indicating an exposure effect on the number of PEx at affected airways. Kokelj et al. (Kokelj et al., 2021) found increased number of exhaled particles in female smokers, but not in male smokers. In that study 6 out of the 21 male smokers had COPD, that may have influenced the number of particles.

Amount PEx at SARS-CoV-2 infection (Paper IV)

Research questions in Paper IV addressed if the number of PEx, using different breathing maneuvers, were affected at acute COVID-19 infection. SARS-CoV-2 mainly infects the upper airways, but have also the potential to cause great damage in the small airways (Bernheim et al., 2020). When acutely infected, the number of PEx decreased. There seemed however, not to be any association between viral load and number of PEx, using any of the different breathing maneuvers. The present study was however too small to draw any firm conclusions. The amount

PE_{Ex} at different breathing maneuvers and its association to viral load is of extra interest to identify super-spreaders of viral respiratory infections. Here, one observation was that cough caused a large increase in the number of the smallest size fraction of PE_{Ex}, in two out of the eight subjects at cough with SARS-CoV-2 positive aerosol sample.

SARS-CoV-2 infection may change the properties of the lining fluid, such as its viscosity, which could in turn influence the formation of the particles originating from small airways. Additionally, the inflammation caused by SARS-CoV-2 in the small airways may reduce their diameter, resulting in the reduction of the number of small airways that open and close during breathing.

Coherence with other studies (Paper IV)

Amount PE_{Ex} has not previously been studied in the context of COVID-19 infection in an early stage of disease, wherefore the results in Paper IV cannot be directly compared. Furthermore, the knowledge about the effect of respiratory infection on the amount of exhaled particles is scarce. A study comparing subjects with symptoms of a respiratory infection and healthy controls has reported that symptomatic subjects emit more particles during coughing than the controls, especially of the size $<1\ \mu\text{m}$ (Hersen et al., 2008). We have observed that the number of PE_{Ex} was decreased in those with confirmed COVID-19 during normal breathing and using the standardized breathing maneuver, but no difference in the amount of exhaled particles was observed between cases and controls during coughing. The disagreement between our findings and the results previously reported by Hersen et al. could be explained by the difficulties in standardizing coughing across all subjects and therefore the results could be influenced by how coughing is performed. Furthermore, the location in the airways where the particles are formed differs between coughing and normal breathing. In a study that was made at our department where subjects with confirmed COVID-19 were examined with the PE_{ExA} method in the acute stage of the disease, using a standardized breathing maneuver only, decreased amount of PE_{Ex} was observed in cases as compared to healthy controls (unpublished data).

RELIABILITY

There was neither any inter-rater reliability testing of the PE_{ExA} instrument performed in Paper II or III, nor any possibilities to adjust for different operators in the analysis due to lack of information on operator responsible for the sampling. On the other hand, the operators were all well trained biomedical analysts accustomed to examining lung function with other methods than the PE_{ExA} method, wherefore one may assume a fairly reproducible sampling procedure for each operator. In Paper IV, one operator performed all the PE_{Ex} sampling and one operator performed all the handling of the sampled aerosol material, minimizing the risk of influence on the results from different operators.

Unfortunately, Paper II, III and IV only include measurements at one occasion, meaning that no reproducibility testing have been performed. Neither has the sampling of PEx been planned to a certain time of day. Amount PEx in healthy subjects have shown not to be influenced of time at day, but with rather high variation of repeated measurements (Kokelj et al., 2020). The effect of time of day, as well as repeated measurements, on amount PEx in subjects with asthma and in smokers is unknown. In Paper IV, it was not possible to consider time of day for sampling of PEx due to logistic reasons. It would have been desirable to re-examine the study participants in Paper IV, but that is beyond the scope of this thesis.

THE USEFULLNESS OF THE RESULTS (Paper II, III and IV)

The lower amount PEx in subjects with ongoing SARS-CoV-2 infection may potentially be explained by changes in the lining fluid where the particles are formed, and/or due to increased small airways.

Attempts trying to address changes in amount PEx however, needs to be taken with caution due to known intra- and inter-individual variation, low number of study participants and unknown data on potential secondary exposures among subjects in the control groups.

Even though the PExA method is non-invasive, patient friendly and provides an on-line measure of the amount PEx in different size fractions, the usefulness of amount PEx as a biomarker needs further validation in the context of assessing acute and chronic effects of exposure affecting small airways.

5.3 VALIDITY ASPECTS OF CONTENT IN PEX (PAPER II, III AND IV)

VALIDITY

Research questions in Paper II and III targeted at exploring whether surface active changes in the small airways lining fluid were associated with small airways ventilation inhomogeneity and active smoking. Furthermore, Paper III also aimed at exploring whether Surfactant protein A, participating in the immune defense in the small airways lining fluid, was associated with active smoking. In addition, research question in Paper IV targeted at explore the possibility to detect SARS-CoV-2 RNA in PEX at different breathing maneuvers in subjects with acute COVID-19 infection.

Other methods to assess RTLF from small airways, such as BAL fluid, has the downfall of unknown and variable dilution, implying difficulties in reproducing the results. The possibility to quantify the sampled material with the PEXA method is a central feature in the chemical analysis of exhaled particles and opens up for studies on the composition of RTLF in different settings. The counting and size-fractioning of exhaled particles with the PEXA method enables estimations of the concentration of analyte in exhaled particles, implying that the PEXA method offers a quantifiable amount of analytes from small airways RTLF (Almstrand et al., 2009; Larsson et al., 2012). The sampling of particles in exhaled breath, instead of sampling in the airways as with biopsy, is a potential way of indirectly assessing the small airways RTLF. Studies have shown no contamination of saliva in the retrieved PEX samples (Bredberg et al., 2012), supporting the notion of an undiluted sample of the RTLF. Furthermore, sampling and chemical analysis of SP-A in PEX have been compared to that of Exhaled breath condensate (EBC), showing the PEX method to be far more efficient in terms of detecting the protein (Larsson et al., 2012), validating the use of the PEXA method for non-invasive studies of SP-A. Therefore, we considered the PEXA method as a valid method in this context.

The associations found in the crude regression analysis between content in PEX and small airways ventilation inhomogeneity (Paper II) as well as active smoking (Paper III), were further tested with quantile regression and adjustments for age and sex. This, in order to explore associations also in the outer parts of the quantiles of the outcome.

Major surface active lipids in PEX (Paper II and III)

Subjects with asthma and associations to alterations in surface active lipids in PEX if small airways ventilation inhomogeneity is present or not, has previously not been explored. The “negative” finding, that surface active lipids in PEX did not

separate between subjects with or without small airways ventilation inhomogeneity, is therefore difficult to compare to previous literature. In a small study on subjects with asthma and lung healthy controls, it was possible to discriminate between the groups based on time-of-flight-secondary ion mass spectrometry and multivariate analyses of phospholipids (Almstrand et al., 2012). In a cohort study by Hussain-Alkhateeb et al. (Hussain-Alkhateeb et al., 2021), no significant changes in concentration of DPPC and POPC in PEx was found in subjects with asthma compared to healthy controls. In the same cohort-study, increased content of DPPC and POPC in PEx in current compared to never smokers was found, consistent with findings in Paper III.

In a study on subjects with asthma by Wright et al. (Wright et al., 2000), DPPC in BAL fluid was found in lower levels compared to controls. The more severe asthma, the lower the levels of DPPC. In Paper II, there was unfortunately no control-group, so it is difficult to judge if the levels were lower in asthma than in healthy controls. If comparing to healthy controls in the European Respiratory Community Health Survey (Hussain-Alkhateeb et al., 2021) the levels were lower; 11.8 wt% in PEx in healthy subjects compared to 9.7-9.8 wt% in subjects with asthma in Paper II.

Surfactant protein A in PEx and coherence with other studies (Paper III)

The results of SP-A in PEx, with a tendency to be increased in current smokers, is in line with findings from Kokelj et al (Kokelj et al., 2021) reporting a significant impact of active smoking on 81 proteins, where those most clearly discriminating between current and never smokers were found less abundant in current smokers. This indicates a measurable effect in PEx content of active smoking on proteins in small airways lining fluid.

Broncho-alveolar lavage (BAL) is the matrix that most resemble PEx-samples, even though it is diluted and possibly contaminated with small amount of substances from blood. Analysis of protein content in PEx have previously shown a high degree of overlap between to proteins found in BAL (Bredberg et al., 2012). Bredberg et al. concluded that the findings strongly supported the theory that PEx originates from the RTLF and that it also reflects undiluted RTLF. Comparison of levels of SP-A and albumin in PEx with that found in BAL fluid and in Bronchial wash (BW) fluid has been examined in a study on 15 healthy non-smoking subjects, showing albumin and albumin corrected SP-A to be associated between BAL and PEx but not to BW (Behndig, Mirgorodskaya, Blomberg, & Olin, 2019).

SARS-CoV-2 in PEx (Paper IV)

Study participants in Paper IV were recruited and examined at the very beginning of symptom debut, when virus load is expected to be at the highest.

Common methods used for collection of endogenous material for later chemical analyses to determine presence of viral RNA, is by swab-sampling in the oropharyngeal and/or the nasopharyngeal area. When presence of viral load in the alveolar region is of interest, the invasive method of BAL may instead be used. The detection of SARS-CoV-2 RNA in exhaled aerosol particles using the PExA method highlight the possibility with non-invasive sampling in the context.

Coherence with other studies (Paper IV)

The detection of SARS-CoV-2 virus in exhaled particles $<5\ \mu\text{m}$ has been reported previously (Alsved et al., 2022; K. K. Coleman et al., 2021; Wang et al., 2021; Zhang et al., 2021). The viral RNA was detectable in aerosols from 40% of the subjects in our study, which is somewhat lower as compared to other studies, where 59% (K. K. Coleman et al., 2021) and 50% of subjects (Alsved et al., 2022) had detectable SARS-CoV-2 RNA in exhaled particles. However, both these studies have used longer sampling time, 15-30 min and 10 min, respectively, in comparison with our study, where the sampling time ranged between 1 and 5 minutes. The presence of virus RNA in our study was almost exclusively detected in particles $<5\ \mu\text{m}$ as compared to $>5\ \mu\text{m}$, which goes in line with previous findings (Alsved et al., 2022; K. K. Coleman et al., 2021; Zhang et al., 2021). It has been suggested that SARS-CoV-2 RNA is enriched in aerosols of the size $<5\ \mu\text{m}$, further supporting this finding (Wang et al., 2021). In agreement with other studies, we have also observed a lower viral load in aerosol samples as compared to the oro/nasopharyngeal samples (K. K. Coleman et al., 2021; Malik, Kunze, Bahmer, Herget-Rosenthal, & Kunze, 2021; Milton, Fabian, Cowling, Grantham, & McDevitt, 2013).

RELIABILITY

There was neither any inter-rater reliability testing of the PExA instrument performed in Paper II, III or IV, nor any possibilities to adjust for different operators in the analysis due to lack of information on operator responsible for the sampling. On the other hand, the operators were all well trained biomedical analysts accustomed to examine lung function with other methods than the PExA, wherefore one may assume a fairly reproducible sampling procedure for each operator.

The intra- and inter-individual variation in healthy subjects, of SP-A in PEx within one day and between days, have been shown to be rather high but not influenced by the time intervals between measurements (Kokelj et al., 2020), wherefore the content of SP-A in PEx ought to be interpreted with caution. No studies on variation in healthy subjects and content of surface active lipids in PEx have been presented yet. Unfortunately, Paper II, III and IV only includes measurements at

one occasion meaning that no reproducibility testing have been performed. Nevertheless, the content on analyte in PEx in Paper II and III is expressed as a mass of the total volume of PEx sampled, divided on either the total lung volume exhaled or the number of exhalations performed. This means that an average quantity of analyte is being presented

THE USEFULNESS OF THE RESULTS (Paper II, III and IV)

The findings in Paper II and III needs to be addressed in a replication cohort as well as in a longitudinal setting prior to addressing the usefulness of the results in a clinical setting. Also, there was comparatively small sample sizes in all of the included studies, wherefore the altered levels of PEx content between subgroups need to be interpreted with caution. The intra- and inter-individual variation of major surface active lipids in PEx, which has not been established today, also needs to be addressed. However, measurable affected small airways linked to current tobacco exposure within subjects with otherwise normal lung function, may have the potential to distinguish subjects in a risk of developing more serious lung damage if continuing being exposed.

The detection of SARS-CoV-2 RNA with the PExA instrument in Paper IV strengthens impaction based methods like PExA to be used in future studies to detect respiratory viral infections and to analyze the spread of viral infections in the airways, non-invasively. Furthermore, studies on viral transmission through sampling of aerosol particles in exhaled breath in comparison to sampling of oro/nasopharyngeal material may also be considered legitimate when focusing on aerosol transmission of respiratory viral infections. However, aerosol sampling by impaction for detection of virus infection needs to go through optimization and standardization of the sampling procedure. In addition, to strengthen the theory of possible viral transmission trough aerosol particles, detection of viable viruses in the sampled aerosol is warranted. From that, guidelines for high and low viral load in exhaled aerosol may be somewhat different from that of sampled oro/nasopharyngeal material in terms of potential contagiousness.

6 CONCLUSION

In this thesis, we have explored the possibility to assess effects of current and/or chronic exposure to airway irritants affecting small airways using breath analyses. More precisely, we examined the effects of exposure to birch-pollen and tobacco smoke by analyses of exhaled droplets (i.e. particles) and alveolar nitric oxide (CANO). In addition, we examined if we could detect SARS-CoV-2 virus in small airways by analyses of breath using different breathing maneuvers. The long term goal is to identify easy and patient friendly methods for early detection of adverse effects of airborne exposures. With valid methods, we hope to contribute to the understanding of exposure effects in small airways.

Breath analyses are convenient as they are non-invasive and therefore possible to reproduce without any significant discomfort for the subject. Nevertheless, the analyses also need to be reproducible, and above all, reflecting what is strived to be measured. Due to the complexity of the airway geometry, it is not possible today to perform pathophysiological studies in the preacinar/acinar region without studying lung biopsies or donated lungs. Anyhow, a PEx sample represents far more a sample from the area of interest, possibly also alveolar NO production, in comparison to analysis of systemic effects in blood, the most common matrix in the search for pulmonary biomarkers.

Methods to assess exposure effects strive to quantify the response in the target organ. Without knowing the exact location in the small airways from where PEx and CANO originates, the results in this thesis suggests that these methods can be used to detect exposure-related effects in the small airways, and that they to some extent are associated with small airways ventilation inhomogeneity. Furthermore, we have proven that it is possible to detect SARS-CoV-2 deposited in the airways, by analyses of exhaled air with the PExA method.

Before any of these methods can be used in a clinical setting, they need further validation and need to be applied in larger studies.

The separate conclusions for the use of alveolar NO and PEx in the different settings they have been used to explore small airways involvement in are as follows:

Alveolar NO (Paper I and II)

There seemed to be no added value of sampling FENO at multiple flows to model CANO, in comparison to sampling FENO at one flow (FENO50), except for the notion that inflammation also may occur in small airways.

CANO did not increase during pollen season in comparison to outside pollen season, but was markedly elevated in some subjects. This can be interpreted as a response due to different subject characteristics or differences in the levels of exposure. Individual exposure levels, both within as well as outside pollen season, would need to be addressed in order to better explore effects of birch pollen exposure on CANO.

CANO was higher in some, but not all, subjects with asthma with small airway ventilation inhomogeneity. This may indicate that small airway obstruction is not necessarily associated with inflammation, and may be a result of structural changes, such as remodeling. Alternatively, other types of inflammation may be present- not reflected by CANO. In parallel, some subjects had increased alveolar NO but no measurable airway inhomogeneity.

Amount PEx (Paper II, III and IV)

Number of exhaled particles might add value to the interpretation of small airway dysfunction and small airways ventilation inhomogeneity.

Increased number of PEx in active smokers, as well as decreased number of PEx in subjects with acute COVID-19 infection, may be due to altered composition of the lining fluid, alternatively decreased diameter of small airways, or, may just be a random finding.

No obvious association was found between PEx/breath and detection of viral load in any of the breathing maneuvers used to sample aerosol particles with the PExA method.

Surface active lipids (DPPC, POPC) and surfactant protein A (SP-A) in PEx (Paper II and III)

Levels of DPPC, POPC and SP-A in PEx might be used as markers of ongoing exposure to airway irritants with potential to deposit in the small airways, more research is however needed. An improved individual risk-assessment with signs of exposure linked ongoing inflammation in the small airways may help people to quit smoking.

The subjects with asthma, taking ICS and regarded as well controlled, may have less of a barrier damage in the small airways and un-altered levels of major surfactant lipids in PEx, regardless of small airway ventilation inhomogeneity.

SARS-CoV-2 in PEx (Paper IV)

It is possible to detect SARS-CoV-2 infection in exhaled breath in an early stage of COVID-19 infection, where the PExA method further indicate the particle sizes with detected viral load to be related to that of aerosol transmission.

7 FUTURE PERSPECTIVES

Many questions remains to be addressed regarding the validity of alveolar NO and amount PEx as well as content in PEx as markers of small airway impairment in different exposure settings. Before any of these methods can be used in a clinical setting, they need further validation regarding technical enhancements. Thereafter, they need to be better validated from reproducibility aspects such as the influence of different operators on the results as well as the within- and between day variation of the results. Larger studies are also warranted.

Suggested follow-up studies from this thesis

Alveolar NO

- Further explore alveolar NO in the context of small airways impairment and birch pollen exposure, with well characterized subjects and individual exposure levels
- Further explore the modeling of alveolar NO to assess its potential clinical relevance
- Further explore the impact on back-diffusion on alveolar NO, in the context of small airways impairment

PEx

- Addressing the uncertainty regarding the reproducibility of amount PEx
- Addressing intra- and inter-individual variation in content of major surface active lipids in PEx
- Further explore suitable breathing maneuver to standardize the sampling
- Simplified methodology to minimize potential operator bias

Respiratory viruses and PEx

- Further explore the use of PEx to detect other respiratory viral infections
- analyze the spread of viral infections in the airways by analyzing viral load in PEx, sampled at different breathing maneuvers
- follow-up studies on disease progression

ACKNOWLEDGEMENT

Detta avhandlingsarbete bygger på en gemensam drivkraft i en mångfacetterad forskargrupp att vilja bidra till utvecklingen av patientvänliga metoder att studera exponeringseffekter i små luftvägar. Samt, ca 400 lungfunktionsundersökningar gjorda på vuxna göteborgare och skaraborgare. Förutom ett varmt TACK till alla studiedeltagare så önskar jag även få rikta några extra innerliga TACK till:

Anna-Carin, min huvudhandledare, för att du för drygt 10 år sedan såg till att denna resa började i och med att du erbjöd mig jobb på Arbets- och miljömedicin. Och för att du har sett till att jag också har slutfört detta projekt! Tack för alla oförglömliga äventyr du har bjudit på däremellan, stora som små! Jag har dig även att tacka för att jag har fått lära känna alla dessa underbara kollegor som nämns här: Mina bihandledare **Jeong-Lim** och **Lotta**, för att ni så välvilligt delat med er av era egna erfarenheter och ert kunnande; **Per G**, som i begynnelsen var den som introducerade mig till forskningsområdet ”små luftvägar i utandningsluften”; mina inofficiella handledare **Björn** och **Per L**, som alltid tycks finnas till hands för kloka råd, stöttning och vägledning; min inofficiella mentor och vän **Marianne**, som har funnits vid min sida från dag ett på AMM; min oersättliga rumskamrat **Spela**, för bollplank och för all hjälp med avhandlingen, men också för alla skratt, och för fin vänskap; mina övriga bundsförvanter **Hatice**, **Sanna och Susanna**, för all stöttning i både vått och torrt, för skratt, och för att ni ser till att sätta saker i rätt perspektiv; hela forskargruppen **Luftvägar och hälsa**, där idéerna till samt författandet av de ingående delarbetena gjorts, liksom planerandet och utförandet av studierna; **Johan Westin med kollegor**, för möjligheten att genomföra COVID-19 studien med er, vilket ju blev till en jättefin resa; alla tidigare kollegor i forskargruppen, **Katya**, **Anna**, **Laith**, **Annica**, **Helen**, **Göran** och **Hanne**, för att ni alla verkligen var precis sådana där goa kollegor som man önskar att få ha; alla nuvarande kollegor på AMM för, bland annat, det så viktiga småpratet om allt och inget.

Tack också alla kära vänner utanför arbetet, som stöttat och påmint mig om livet på andra sidan av denna avhandling. Tack för tålamodet mina underbara älskade busungar **Agnes**, **Otto** och **Sonja**, som nog ser fram emot tiden efter avhandlingen minst lika mycket som jag. Ett innerligt tack också till min härliga familj, som jag är så stolt över att tillhöra och som lär mig så mycket om mig själv.

Allra mest Tack till min älskade **Tobias**, mitt livs kärlek, som har hållit mig sällskap och peppat mig genom skrivandet och som även har ritat alla illustrationer i denna avhandling. Jag älskar dig 3000!

NU är jag redo för livets nästa kapitel!

8 PAPERS ASSOCIATED TO THIS WORK NOT INCLUDED IN THE THESIS

Ericson, P. A., E. Mirgorodskaya, O. S. Hammar, **E. A. Viklund**, A. R. Almstrand, P. J. Larsson, G. C. Riise, and A. C. Olin. "Low Levels of Exhaled Surfactant Protein a Associated with Bos after Lung Transplantation." *Transplant Direct* 2, no. 9 (Sep 2016): e103.
<https://dx.doi.org/10.1097/txd.0000000000000615>.

Kjellberg, S., **E. Viklund**, P. D. Robinson, O. Zetterstrom, A. C. Olin, and P. Gustafsson. "Utility of Single Versus Multiple Breath Washout in Adult Asthma." *Clin Physiol Funct Imaging* (Jan 24 2018).
<https://dx.doi.org/10.1111/cpf.12503>.

Ljungkvist, G., H. Tinnerberg, J. Löndahl, T. Klang, **E. Viklund**, J. L. Kim, L. Schiöler, N. Forsgard, and A. C. Olin. "Exploring a New Method for the Assessment of Metal Exposure by Analysis of Exhaled Breath of Welders." *Int Arch Occup Environ Health* (Jan 23 2022).
<https://dx.doi.org/10.1007/s00420-022-01833-z>.

Ljungkvist, G., S. Ullah, A. Tinglev, K. Stein, B. Bake, P. Larsson, A. C. Almstrand, **E. Viklund**, O. Hammar, S. Sandqvist, O. Beck, and A. C. Olin. "Two Techniques to Sample Non-Volatiles in Breath-Exemplified by Methadone." *J Breath Res* 12, no. 1 (Dec 8 2017): 016011.
<https://dx.doi.org/10.1088/1752-7163/aa8b25>.

Soares, M., E. Mirgorodskaya, H. Koca, **E. Viklund**, M. Richardson, P. Gustafsson, A. C. Olin, and S. Siddiqui. "Particles in Exhaled Air (Pexa): Non-Invasive Phenotyping of Small Airways Disease in Adult Asthma." *J Breath Res* 12, no. 4 (Sep 14 2018): 046012. <https://dx.doi.org/10.1088/1752-7163/aad9d1>.

Tinglev Å, D., S. Ullah, G. Ljungkvist, **E. Viklund**, A. C. Olin, and O. Beck. "Characterization of Exhaled Breath Particles Collected by an Electret Filter Technique." *J Breath Res* 10, no. 2 (Mar 17 2016): 026001.
<https://dx.doi.org/10.1088/1752-7155/10/2/026001>.

REFERENCES

- Agudelo, C. W., Kumley, B. K., Area-Gomez, E., Xu, Y., Dabo, A. J., Geraghty, P., . . . Garcia-Arcos, I. (2020). Decreased surfactant lipids correlate with lung function in chronic obstructive pulmonary disease (COPD). *PloS one*, 15(2), e0228279. doi:10.1371/journal.pone.0228279
- Almstrand, A. C., Bake, B., Ljungstrom, E., Larsson, P., Bredberg, A., Mirgorodskaya, E., & Olin, A. C. (2010). Effect of airway opening on production of exhaled particles. *Journal of applied physiology*, 108(3), 584-588. doi:10.1152/jappphysiol.00873.2009
- Almstrand, A. C., Josefson, M., Bredberg, A., Lausmaa, J., Sjoval, P., Larsson, P., & Olin, A. C. (2012). TOF-SIMS analysis of exhaled particles from patients with asthma and healthy controls. *The European respiratory journal*, 39(1), 59-66. doi:10.1183/09031936.00195610
- Almstrand, A. C., Ljungstrom, E., Lausmaa, J., Bake, B., Sjoval, P., & Olin, A. C. (2009). Airway monitoring by collection and mass spectrometric analysis of exhaled particles. *Analytical chemistry*, 81(2), 662-668. doi:10.1021/ac802055k
- Alsved, M., Nygren, D., Thuresson, S., Medstrand, P., Fraenkel, C. J., & Löndahl, J. (2022). SARS-CoV-2 in exhaled aerosol particles from covid-19 cases and its association to household transmission. *Clin Infect Dis*. doi:10.1093/cid/ciac202
- ATS/ERS recommendations for standardized procedures for the online and offline measurement of exhaled lower respiratory nitric oxide and nasal nitric oxide, 2005. (2005). *American journal of respiratory and critical care medicine*, 171(8), 912-930. doi:10.1164/rccm.2004406-710ST
- Bake, B., Ljungstrom, E., Claesson, A., Carlsen, H. K., Holm, M., & Olin, A. C. (2017). Exhaled Particles After a Standardized Breathing Maneuver. *Journal of aerosol medicine and pulmonary drug delivery*, 30(4), 267-273. doi:10.1089/jamp.2016.1330
- Bake, B., Ljungström, E., Claesson, A., Carlsen, H. K., Holm, M., & Olin, A. C. (2017). Exhaled Particles After a Standardized Breathing Maneuver. *Journal of aerosol medicine and pulmonary drug delivery*, 30(4), 267-273. doi:10.1089/jamp.2016.1330
- Balbi, B., Pignatti, P., Corradi, M., Baiardi, P., Bianchi, L., Brunetti, G., . . . Malerba, M. (2007). Bronchoalveolar lavage, sputum and exhaled clinically relevant inflammatory markers: values in healthy adults. *The European respiratory journal*, 30(4), 769-781. doi:10.1183/09031936.00112306

- Balzar, S., Wenzel, S. E., & Chu, H. W. (2002). Transbronchial biopsy as a tool to evaluate small airways in asthma. *The European respiratory journal*, 20(2), 254-259. doi:10.1183/09031936.02.00261102
- Barnes, P. J., & Belvisi, M. G. (1993). Nitric oxide and lung disease. *Thorax*, 48(10), 1034-1043.
- Baughman, R. P. (1997). The uncertainties of bronchoalveolar lavage. *The European respiratory journal*, 10(9), 1940-1942. doi:10.1183/09031936.97.10091940
- Behndig, A. F., Mirgorodskaya, E., Blomberg, A., & Olin, A. C. (2019). Surfactant Protein A in particles in exhaled air (PExA), bronchial lavage and bronchial wash - a methodological comparison. *Respiratory research*, 20(1), 214. doi:10.1186/s12931-019-1172-1
- Bernhard, W., Hoffmann, S., Dombrowsky, H., Rau, G. A., Kamlage, A., Kappler, M., . . . Poets, C. F. (2001). Phosphatidylcholine molecular species in lung surfactant: composition in relation to respiratory rate and lung development. *Am J Respir Cell Mol Biol*, 25(6), 725-731. doi:10.1165/ajrcmb.25.6.4616
- Bernheim, A., Mei, X., Huang, M., Yang, Y., Fayad, Z. A., Zhang, N., . . . Chung, M. (2020). Chest CT Findings in Coronavirus Disease-19 (COVID-19): Relationship to Duration of Infection. *Radiology*, 295(3), 200463. doi:10.1148/radiol.2020200463
- Bondesson, E., Jansson, L. T., Bengtsson, T., & Wollmer, P. (2009). Exhaled breath condensate-site and mechanisms of formation. *J Breath Res*, 3(1), 016005. doi:10.1088/1752-7155/3/1/016005
- Bosken, C. H., Wiggs, B. R., Pare, P. D., & Hogg, J. C. (1990). Small airway dimensions in smokers with obstruction to airflow. *The American review of respiratory disease*, 142(3), 563-570. doi:10.1164/ajrccm/142.3.563
- Bredberg, A., Gobom, J., Almstrand, A. C., Larsson, P., Blennow, K., Olin, A. C., & Mirgorodskaya, E. (2012). Exhaled endogenous particles contain lung proteins. *Clinical chemistry*, 58(2), 431-440. doi:10.1373/clinchem.2011.169235
- Bredberg, A., Josefson, M., Almstrand, A. C., Lausmaa, J., Sjovall, P., Levinsson, A., . . . Olin, A. C. (2013). Comparison of exhaled endogenous particles from smokers and non-smokers using multivariate analysis. *Respiration; international review of thoracic diseases*, 86(2), 135-142. doi:10.1159/000350941
- Brisman, J., Kim, J. L., Olin, A. C., Toren, K., & Bake, B. (2016). A physiologically based model for spirometric reference equations in adults. *Clin Physiol Funct Imaging*, 36(1), 77-84. doi:10.1111/cpf.12198
- Brisman, J., Kim, J. L., Olin, A. C., Toren, K., & Bake, B. (2017). Spirometric reference equations for Swedish adults. *Clin Physiol Funct Imaging*, 37(6), 640-645. doi:10.1111/cpf.12349

- Buist, A. S., & Ross, B. B. (1973). Predicted values for closing volumes using a modified single breath nitrogen test. *The American review of respiratory disease*, 107(5), 744-752.
- Caillaud, D. M., Martin, S., Ségala, C., Vidal, P., Lecadet, J., Pellier, S., . . . Evrard, B. (2015). Airborne pollen levels and drug consumption for seasonal allergic rhinoconjunctivitis: a 10-year study in France. *Allergy*, 70(1), 99-106. doi:10.1111/all.12522
- Cañadas, O., Olmeda, B., Alonso, A., & Pérez-Gil, J. (2020). Lipid-Protein and Protein-Protein Interactions in the Pulmonary Surfactant System and Their Role in Lung Homeostasis. *Int J Mol Sci*, 21(10). doi:10.3390/ijms21103708
- Canova, C., Heinrich, J., Anto, J. M., Leynaert, B., Smith, M., Kuenzli, N., . . . Jarvis, D. (2013). The influence of sensitisation to pollens and moulds on seasonal variations in asthma attacks. *The European respiratory journal*, 42(4), 935-945. doi:10.1183/09031936.00097412
- Castillo-Sánchez, J. C., Cruz, A., & Pérez-Gil, J. (2021). Structural hallmarks of lung surfactant: Lipid-protein interactions, membrane structure and future challenges. *Arch Biochem Biophys*, 703, 108850. doi:10.1016/j.abb.2021.108850
- Chroneos, Z. C., Sever-Chroneos, Z., & Shepherd, V. L. (2010). Pulmonary surfactant: an immunological perspective. *Cell Physiol Biochem*, 25(1), 13-26. doi:10.1159/000272047
- Coleman, J. W. (2001). Nitric oxide in immunity and inflammation. *Int Immunopharmacol*, 1(8), 1397-1406. doi:10.1016/s1567-5769(01)00086-8
- Coleman, K. K., Tay, D. J. W., Sen Tan, K., Ong, S. W. X., Son, T. T., Koh, M. H., . . . Wai, T. K. (2021). Viral Load of SARS-CoV-2 in Respiratory Aerosols Emitted by COVID-19 Patients while Breathing, Talking, and Singing. *Clin Infect Dis*. doi:10.1093/cid/ciab691
- Crawford, A. B., Makowska, M., Paiva, M., & Engel, L. A. (1985). Convection- and diffusion-dependent ventilation maldistribution in normal subjects. *Journal of applied physiology*, 59(3), 838-846.
- Dollfuss, R. E. (1967). Regional ventilation of the lung studied with boluses of 133 Xenon. *Respiration physiology*, 2, 234-246.
- Dweik, R. A., Boggs, P. B., Erzurum, S. C., Irvin, C. G., Leigh, M. W., Lundberg, J. O., . . . Taylor, D. R. (2011). An official ATS clinical practice guideline: interpretation of exhaled nitric oxide levels (FENO) for clinical applications. *American journal of respiratory and critical care medicine*, 184(5), 602-615. doi:10.1164/rccm.9120-11ST
- Fijten, R. R. R., Smolinska, A., Drent, M., Dallinga, J. W., Mostard, R., Pachen, D. M., . . . Boots, A. W. (2017). The necessity of external validation in exhaled breath research: a case study of sarcoidosis. *J Breath Res*, 12(1), 016004. doi:10.1088/1752-7163/aa8409

- Fowler, W. S. (1949). Lung function studies; uneven pulmonary ventilation in normal subjects and in patients with pulmonary disease. *Journal of applied physiology*, 2(6), 283-299. doi:10.1152/jappl.1949.2.6.283
- Frenz, D. A. (2000). Interpreting atmospheric pollen counts for use in clinical allergy: spatial variability. *Annals of allergy, asthma & immunology : official publication of the American College of Allergy, Asthma, & Immunology*, 84(5), 481-489; quiz 489-491. doi:10.1016/S1081-1206(10)62506-9
- Fujisawa, T., Yasui, H., Akamatsu, T., Hashimoto, D., Enomoto, N., Inui, N., . . . Chida, K. (2013). Alveolar nitric oxide concentration reflects peripheral airway obstruction in stable asthma. *Respirology*, 18(3), 522-527. doi:10.1111/resp.12031
- Ghio, A. J., Hilborn, E. D., Stonehuerner, J. G., Dailey, L. A., Carter, J. D., Richards, J. H., . . . Pinkerton, K. E. (2008). Particulate matter in cigarette smoke alters iron homeostasis to produce a biological effect. *American journal of respiratory and critical care medicine*, 178(11), 1130-1138. doi:10.1164/rccm.200802-334OC
- Goerke, J. (1998). Pulmonary surfactant: functions and molecular composition. *Biochim Biophys Acta*, 1408(2-3), 79-89. doi:10.1016/s0925-4439(98)00060-x
- Griese, M. (1999). Pulmonary surfactant in health and human lung diseases: state of the art. *The European respiratory journal*, 13(6), 1455-1476.
- Gunnarsson, R. (1999, July 10, 2020). Validitet och reliabilitet [in INFOVOICE.SE].
- Gustafsson, L. E., Leone, A. M., Persson, M. G., Wiklund, N. P., & Moncada, S. (1991). Endogenous nitric oxide is present in the exhaled air of rabbits, guinea pigs and humans. *Biochem Biophys Res Commun*, 181(2), 852-857. doi:10.1016/0006-291x(91)91268-h
- Haslbeck, K., Schwarz, K., Hohlfeld, J. M., Seume, J. R., & Koch, W. (2010). Submicron droplet formation in the human lung. *Journal of Aerosol Science*, 41(5), 429-438. doi:<https://doi.org/10.1016/j.jaerosci.2010.02.010>
- Hedenstrom, H., Malmberg, P., & Agarwal, K. (1985). Reference values for lung function tests in females. Regression equations with smoking variables. *Bulletin europeen de physiopathologie respiratoire*, 21(6), 551-557.
- Hedenstrom, H., Malmberg, P., & Fridriksson, H. V. (1986). Reference values for lung function tests in men: regression equations with smoking variables. *Ups J Med Sci*, 91(3), 299-310. doi:10.3109/03009738609178670
- Hersen, G., Moularat, S., Robine, E., Géhin, E., Corbet, S., Vabret, A., & Freymuth, F. (2008). Impact of Health on Particle Size of Exhaled Respiratory Aerosols: Case-control Study. *Clean (Weinh)*, 36(7), 572-577. doi:10.1002/clen.200700189

- Hetrick, E. M., & Schoenfish, M. H. (2009). Analytical chemistry of nitric oxide. *Annu Rev Anal Chem (Palo Alto Calif)*, 2, 409-433. doi:10.1146/annurev-anchem-060908-155146
- Hoffmeyer, F., Raulf-Heimsoth, M., & Brüning, T. (2009). Exhaled breath condensate and airway inflammation. *Curr Opin Allergy Clin Immunol*, 9(1), 16-22. doi:10.1097/ACI.0b013e32831d8144
- Hogg, J. C., Pare, P. D., & Hackett, T. L. (2017). The Contribution of Small Airway Obstruction to the Pathogenesis of Chronic Obstructive Pulmonary Disease. *Physiol Rev*, 97(2), 529-552. doi:10.1152/physrev.00025.2015
- Holmgren, H. (2010). Size distribution of exhaled particles in the range from 0.01 to 2.0 µm. *Journal of Aerosol Science*, 41, 439-446.
- Horváth, I., Barnes, P. J., Loukides, S., Sterk, P. J., Högman, M., Olin, A. C., . . . Vink, T. J. (2017). A European Respiratory Society technical standard: exhaled biomarkers in lung disease. *The European respiratory journal*, 49(4). doi:10.1183/13993003.00965-2016
- Hou, Y. J., Okuda, K., Edwards, C. E., Martinez, D. R., Asakura, T., Dinnon, K. H., 3rd, . . . Baric, R. S. (2020). SARS-CoV-2 Reverse Genetics Reveals a Variable Infection Gradient in the Respiratory Tract. *Cell*, 182(2), 429-446.e414. doi:10.1016/j.cell.2020.05.042
- Hussain-Alkhateeb, L., Bake, B., Holm, M., Emilsson, Ö., Mirgorodskaya, E., & Olin, A. C. (2021). Novel non-invasive particles in exhaled air method to explore the lining fluid of small airways-a European population-based cohort study. *BMJ Open Respir Res*, 8(1). doi:10.1136/bmjresp-2020-000804
- Högman, M., Drca, N., Ehrstedt, C., & Meriläinen, P. (2000). Exhaled nitric oxide partitioned into alveolar, lower airways and nasal contributions. *Respiratory medicine*, 94(10), 985-991. doi:10.1053/rmed.2000.0872
- Högman, M., Holmkvist, T., Wegener, T., Emtner, M., Andersson, M., Hedenström, H., & Meriläinen, P. (2002). Extended NO analysis applied to patients with COPD, allergic asthma and allergic rhinitis. *Respiratory medicine*, 96(1), 24-30. doi:10.1053/rmed.2001.1204
- Högman, M., Thornadtsen, A., Liv, P., Hua-Huy, T., Dinh-Xuan, A. T., Tufvesson, E., . . . Lehtimäki, L. (2017). Effects of growth and aging on the reference values of pulmonary nitric oxide dynamics in healthy subjects. *J Breath Res*, 11(4), 047103. doi:10.1088/1752-7163/aa7957
- Johnson, G. R., & Morawska, L. (2009). The mechanism of breath aerosol formation. *Journal of aerosol medicine and pulmonary drug delivery*, 22(3), 229-237. doi:10.1089/jamp.2008.0720
- Keen, C., Olin, A. C., Wennergren, G., & Gustafsson, P. (2011). Small airway function, exhaled NO and airway hyper-responsiveness in paediatric asthma. *Respiratory medicine*, 105(10), 1476-1484. doi:10.1016/j.rmed.2011.04.004
- Kerckx, Y., Michils, A., & Van Muylem, A. (2008). Airway contribution to alveolar nitric oxide in healthy subjects and stable asthma patients.

- Journal of applied physiology*, 104(4), 918-924.
doi:10.1152/jappphysiol.01032.2007
- Kharitonov, S. A., & Barnes, P. J. (1997). Nasal contribution to exhaled nitric oxide during exhalation against resistance or during breath holding. *Thorax*, 52(6), 540-544. doi:10.1136/thx.52.6.540
- Khatri, S. B., Iaccarino, J. M., Barochia, A., Soghier, I., Akuthota, P., Brady, A., . . . Hallstrand, T. S. (2021). Use of Fractional Exhaled Nitric Oxide to Guide the Treatment of Asthma: An Official American Thoracic Society Clinical Practice Guideline. *American journal of respiratory and critical care medicine*, 204(10), e97-e109. doi:10.1164/rccm.202109-2093ST
- Kjellberg, S., Houlitz, B. K., Zetterstrom, O., Robinson, P. D., & Gustafsson, P. M. (2016). Clinical characteristics of adult asthma associated with small airway dysfunction. *Respiratory medicine*, 117, 92-102. doi:10.1016/j.rmed.2016.05.028
- Kokelj, S., Kim, J. L., Andersson, M., Runstrom Eden, G., Bake, B., & Olin, A. C. (2020). Intra-individual variation of particles in exhaled air and of the contents of Surfactant protein A and albumin. *PloS one*, 15(1), e0227980. doi:10.1371/journal.pone.0227980
- Kokelj, S., Östling, J., Georgi, B., Fromell, K., Ekdahl, K. N., Olsson, H. K., & Olin, A. C. (2021). Smoking induces sex-specific changes in the small airway proteome. *Respiratory research*, 22(1), 234. doi:10.1186/s12931-021-01825-6
- Koskela, K., Sauni, R., Oksa, P., Uitti, J., Moilanen, E., & Lehtimäki, L. (2021). High alveolar nitric oxide is associated with steeper lung function decline in foundry workers. *J Breath Res*, 15(3). doi:10.1088/1752-7163/abf272
- Larsson, P., Bake, B., Wallin, A., Hammar, O., Almstrand, A. C., Larstad, M., . . . Olin, A. C. (2017). The effect of exhalation flow on endogenous particle emission and phospholipid composition. *Respiratory physiology & neurobiology*, 243, 39-46. doi:10.1016/j.resp.2017.05.003
- Larsson, P., Larstad, M., Bake, B., Hammar, O., Bredberg, A., Almstrand, A. C., . . . Olin, A. C. (2017). Exhaled particles as markers of small airway inflammation in subjects with asthma. *Clin Physiol Funct Imaging*, 37(5), 489-497. doi:10.1111/cpf.12323
- Larsson, P., Lärstad, M., Bake, B., Hammar, O., Bredberg, A., Almstrand, A. C., . . . Olin, A. C. (2017). Exhaled particles as markers of small airway inflammation in subjects with asthma. *Clin Physiol Funct Imaging*, 37(5), 489-497. doi:10.1111/cpf.12323
- Larsson, P., Mirgorodskaya, E., Samuelsson, L., Bake, B., Almstrand, A. C., Bredberg, A., & Olin, A. C. (2012). Surfactant protein A and albumin in particles in exhaled air. *Respiratory medicine*, 106(2), 197-204. doi:10.1016/j.rmed.2011.10.008

- Larstad, M., Almstrand, A. C., Larsson, P., Bake, B., Larsson, S., Ljungstrom, E., . . . Olin, A. C. (2015). Surfactant Protein A in Exhaled Endogenous Particles Is Decreased in Chronic Obstructive Pulmonary Disease (COPD) Patients: A Pilot Study. *PloS one*, 10(12), e0144463. doi:10.1371/journal.pone.0144463
- Lehtimäki, L., Karvonen, T., & Högman, M. (2020). Clinical Values of Nitric Oxide Parameters from the Respiratory System. *Curr Med Chem*, 27(42), 7189-7199. doi:10.2174/0929867327666200603141847
- Lehtimäki, L., Oksa, P., Järvenpää, R., Vierikko, T., Nieminen, R., Kankaanranta, H., . . . Moilanen, E. (2010). Pulmonary inflammation in asbestos-exposed subjects with borderline parenchymal changes on HRCT. *Respiratory medicine*, 104(7), 1042-1049. doi:10.1016/j.rmed.2010.01.019
- Lopez, V., Prieto, L., Perez-Frances, C., Barato, D., & Marin, J. (2012). Natural pollen exposure increases the response plateau to adenosine 5'-monophosphate and bronchial but not alveolar nitric oxide in sensitized subjects. *Respiration; international review of thoracic diseases*, 83(3), 225-232. doi:10.1159/000328750
- Louis, R., Satia, I., Ojanguren, I., Schleich, F., Bonini, M., Tonia, T., . . . Usmani, O. S. (2022). European Respiratory Society Guidelines for the Diagnosis of Asthma in Adults. *The European respiratory journal*. doi:10.1183/13993003.01585-2021
- Macklem, P. T. (1998). The physiology of small airways. *American journal of respiratory and critical care medicine*, 157(5 Pt 2), S181-183. doi:10.1164/ajrccm.157.5.rsaa-2
- Malik, M., Kunze, A. C., Bahmer, T., Herget-Rosenthal, S., & Kunze, T. (2021). SARS-CoV-2: Viral Loads of Exhaled Breath and Oronasopharyngeal Specimens in Hospitalized Patients with COVID-19. *Int J Infect Dis*, 110, 105-110. doi:10.1016/j.ijid.2021.07.012
- McCarthy, D. S., Spencer, R., Greene, R., & Milic-Emili, J. (1972). Measurement of "closing volume" as a simple and sensitive test for early detection of small airway disease. *The American journal of medicine*, 52(6), 747-753.
- Mead, J. (1970). The lung's "quiet zone". *The New England journal of medicine*, 282(23), 1318-1319. doi:10.1056/NEJM197006042822311
- Mehlig, K., Berg, C., Bjorck, L., Nyberg, F., Olin, A. C., Rosengren, A., . . . Lissner, L. (2017). Cohort Profile: The INTERGENE Study. *Int J Epidemiol*, 46(6), 1742-1743h. doi:10.1093/ije/dyw332
- Milic-Emili, J., Torchio, R., & D'Angelo, E. (2007). Closing volume: a reappraisal (1967-2007). *Eur J Appl Physiol*, 99(6), 567-583. doi:10.1007/s00421-006-0389-0
- Miller, M. R., Hankinson, J., Brusasco, V., Burgos, F., Casaburi, R., Coates, A., . . . Wanger, J. (2005). Standardisation of spirometry. *The European respiratory journal*, 26(2), 319-338. doi:10.1183/09031936.05.00034805

- Miller, S. L., Nazaroff, W. W., Jimenez, J. L., Boerstra, A., Buonanno, G., Dancer, S. J., . . . Noakes, C. (2021). Transmission of SARS-CoV-2 by inhalation of respiratory aerosol in the Skagit Valley Chorale superspreading event. *Indoor Air*, 31(2), 314-323. doi:10.1111/ina.12751
- Milton, D. K., Fabian, M. P., Cowling, B. J., Grantham, M. L., & McDevitt, J. J. (2013). Influenza virus aerosols in human exhaled breath: particle size, culturability, and effect of surgical masks. *PLoS Pathog*, 9(3), e1003205. doi:10.1371/journal.ppat.1003205
- Morawska, L. (2009). Size distribution and sites of origin of droplets expelled from the human respiratory tract during expiratory activities. *Aerosol Science*, 40, 256-269.
- Olin, A. C., Bake, B., & Toren, K. (2007). Fraction of exhaled nitric oxide at 50 mL/s: reference values for adult lifelong never-smokers. *Chest*, 131(6), 1852-1856. doi:10.1378/chest.06-2928
- Parra, E., & Pérez-Gil, J. (2015). Composition, structure and mechanical properties define performance of pulmonary surfactant membranes and films. *Chem Phys Lipids*, 185, 153-175. doi:10.1016/j.chemphyslip.2014.09.002
- Pastva, A. M., Wright, J. R., & Williams, K. L. (2007). Immunomodulatory roles of surfactant proteins A and D: implications in lung disease. *Proc Am Thorac Soc*, 4(3), 252-257. doi:10.1513/pats.200701-018AW
- Pietropaoli, A. P., Perillo, I. B., Torres, A., Perkins, P. T., Frasier, L. M., Utell, M. J., . . . Hyde, R. W. (1999). Simultaneous measurement of nitric oxide production by conducting and alveolar airways of humans. *Journal of applied physiology*, 87(4), 1532-1542.
- Pouwels, S. D., Burgess, J. K., Verschuuren, E., & Slebos, D. J. (2021). The cellular composition of the lung lining fluid gradually changes from bronchus to alveolus. *Respiratory research*, 22(1), 285. doi:10.1186/s12931-021-01882-x
- Quanjer, P. H., Tammeling, G. J., Cotes, J. E., Pedersen, O. F., Peslin, R., & Yernault, J. C. (1993). Lung volumes and forced ventilatory flows. Report Working Party Standardization of Lung Function Tests, European Community for Steel and Coal. Official Statement of the European Respiratory Society. *The European respiratory journal. Supplement*, 16, 5-40.
- Queralto, N., Berliner, A. N., Goldsmith, B., Martino, R., Rhodes, P., & Lim, S. H. (2014). Detecting cancer by breath volatile organic compound analysis: a review of array-based sensors. *J Breath Res*, 8(2), 027112. doi:10.1088/1752-7155/8/2/027112
- Ricciardolo, F. L. (2003). Multiple roles of nitric oxide in the airways. *Thorax*, 58(2), 175-182. doi:10.1136/thorax.58.2.175
- Robertson, J. S., Siri, W. E., & Jones, H. B. (1950). Lung ventilation patterns determined by analysis of nitrogen elimination rates; use of mass

- spectrometer as a continuous gas analyzer. *The Journal of clinical investigation*, 29(5), 577-590. doi:10.1172/jci102295
- Robinson, P. D., Goldman, M. D., & Gustafsson, P. M. (2009). Inert gas washout: theoretical background and clinical utility in respiratory disease. *Respiration; international review of thoracic diseases*, 78(3), 339-355. doi:10.1159/000225373
- Robinson, P. D., Latzin, P., Verbanck, S., Hall, G. L., Horsley, A., Gappa, M., . . . Gustafsson, P. M. (2013). Consensus statement for inert gas washout measurement using multiple- and single- breath tests. *The European respiratory journal*, 41(3), 507-522. doi:10.1183/09031936.00069712
- Sauni, R., Oksa, P., Lehtimäki, L., Toivio, P., Palmroos, P., Nieminen, R., . . . Uitti, J. (2012). Increased alveolar nitric oxide and systemic inflammation markers in silica-exposed workers. *Occupational and environmental medicine*, 69(4), 256-260. doi:10.1136/oemed-2011-100347
- Schatz, M., Sorkness, C. A., Li, J. T., Marcus, P., Murray, J. J., Nathan, R. A., . . . Jhingran, P. (2006). Asthma Control Test: reliability, validity, and responsiveness in patients not previously followed by asthma specialists. *The Journal of allergy and clinical immunology*, 117(3), 549-556. doi:10.1016/j.jaci.2006.01.011
- Schwarz, K., Biller, H., Windt, H., Koch, W., & Hohlfeld, J. M. (2010). Characterization of exhaled particles from the healthy human lung--a systematic analysis in relation to pulmonary function variables. *Journal of aerosol medicine and pulmonary drug delivery*, 23(6), 371-379. doi:10.1089/jamp.2009.0809
- Schwarz, K., Biller, H., Windt, H., Koch, W., & Hohlfeld, J. M. (2015). Characterization of exhaled particles from the human lungs in airway obstruction. *Journal of aerosol medicine and pulmonary drug delivery*, 28(1), 52-58. doi:10.1089/jamp.2013.1104
- Seferaj, S., Ullah, S., Tinglev, Å., Carlsson, S., Winberg, J., Stambeck, P., & Beck, O. (2018). Evaluation of a new simple collection device for sampling of microparticles in exhaled breath. *J Breath Res*, 12(3), 036005. doi:10.1088/1752-7163/aaaf24
- Shin, H. W., Condorelli, P., Rose-Gottron, C. M., Cooper, D. M., & George, S. C. (2004). Probing the impact of axial diffusion on nitric oxide exchange dynamics with heliox. *J Appl Physiol (1985)*, 97(3), 874-882. doi:10.1152/jappphysiol.01297.2003
- Silkoff, P. E., McClean, P. A., Slutsky, A. S., Furlott, H. G., Hoffstein, E., Wakita, S., . . . Zamel, N. (1997). Marked flow-dependence of exhaled nitric oxide using a new technique to exclude nasal nitric oxide. *American journal of respiratory and critical care medicine*, 155(1), 260-267.
- Silkoff, P. E., Sylvester, J. T., Zamel, N., & Permutt, S. (2000). Airway nitric oxide diffusion in asthma: Role in pulmonary function and bronchial

- responsiveness. *American journal of respiratory and critical care medicine*, 161(4 Pt 1), 1218-1228. doi:10.1164/ajrccm.161.4.9903111
- Smith, M., Jäger, S., Berger, U., Sikoparija, B., Hallsdottir, M., Sauliene, I., . . . van Ree, R. (2014). Geographic and temporal variations in pollen exposure across Europe. *Allergy*, 69(7), 913-923. doi:10.1111/all.12419
- Soares, M., Mirgorodskaya, E., Koca, H., Viklund, E., Richardson, M., Gustafsson, P., . . . Siddiqui, S. (2018). Particles in exhaled air (PExA): non-invasive phenotyping of small airways disease in adult asthma. *J Breath Res*, 12(4), 046012. doi:10.1088/1752-7163/aad9d1
- Sprio, A. E., Bertolini, F., Fucà, R., Levra, S., Carriero, V., Högman, M., & Ricciardolo, F. L. M. (2022). Alveolar Nitric Oxide and Peripheral Oxygen Saturation in Frequent Exacerbators with Asthma: A Pilot Study. *Int Arch Allergy Immunol*, 183(1), 105-115. doi:10.1159/000518320
- Sungnak, W., Huang, N., Bécavin, C., & Berg, M. (2020). SARS-CoV-2 Entry Genes Are Most Highly Expressed in Nasal Goblet and Ciliated Cells within Human Airways. *ArXiv*.
- Taylor, P. E., Flagan, R. C., Miguel, A. G., Valenta, R., & Glovsky, M. M. (2004). Birch pollen rupture and the release of aerosols of respirable allergens. *Clin Exp Allergy*, 34(10), 1591-1596. doi:10.1111/j.1365-2222.2004.02078.x
- Thornton, D. J., Rousseau, K., & McGuckin, M. A. (2008). Structure and function of the polymeric mucins in airways mucus. *Annu Rev Physiol*, 70, 459-486. doi:10.1146/annurev.physiol.70.113006.100702
- Tsoukias, N. M., & George, S. C. (1998). A two-compartment model of pulmonary nitric oxide exchange dynamics. *Journal of applied physiology*, 85(2), 653-666.
- Tsoukias, N. M., Tannous, Z., Wilson, A. F., & George, S. C. (1998). Single-exhalation profiles of NO and CO₂ in humans: effect of dynamically changing flow rate. *J Appl Physiol (1985)*, 85(2), 642-652. doi:10.1152/jappl.1998.85.2.642
- Tufvesson, E., Aronsson, D., Ankerst, J., George, S. C., & Björner, L. (2007). Peripheral nitric oxide is increased in rhinitic patients with asthma compared to bronchial hyperresponsiveness. *Respiratory medicine*, 101(11), 2321-2326. doi:10.1016/j.rmed.2007.06.015
- Usmani, O. S., Biddiscombe, M. F., & Barnes, P. J. (2005). Regional lung deposition and bronchodilator response as a function of beta2-agonist particle size. *American journal of respiratory and critical care medicine*, 172(12), 1497-1504. doi:10.1164/rccm.200410-1414OC
- Verbanck, S., Kerckx, Y., Schuermans, D., Vincken, W., Paiva, M., & Van Muylem, A. (2008). Effect of airways constriction on exhaled nitric oxide. *Journal of applied physiology*, 104(4), 925-930. doi:10.1152/japplphysiol.01019.2007

- Verbanck, S., & Paiva, M. (1990). Model simulations of gas mixing and ventilation distribution in the human lung. *J Appl Physiol* (1985), 69(6), 2269-2279. doi:10.1152/jappl.1990.69.6.2269
- Wang, C. C., Prather, K. A., Sznitman, J., Jimenez, J. L., Lakdawala, S. S., Tufekci, Z., & Marr, L. C. (2021). Airborne transmission of respiratory viruses. *Science*, 373(6558). doi:10.1126/science.abd9149
- Wilson, A. F., Novey, H. S., Berke, R. A., & Surprenant, E. L. (1973). Deposition of inhaled pollen and pollen extract in human airways. *The New England journal of medicine*, 288(20), 1056-1058. doi:10.1056/nejm197305172882006
- Wright, S. M., Hockey, P. M., Enhorning, G., Strong, P., Reid, K. B., Holgate, S. T., . . . Postle, A. D. (2000). Altered airway surfactant phospholipid composition and reduced lung function in asthma. *J Appl Physiol* (1985), 89(4), 1283-1292.
- Yilmaz, A., Marklund, E., Andersson, M., Nilsson, S., Andersson, L. M., Lindh, M., & Gisslén, M. (2021). Upper Respiratory Tract Levels of Severe Acute Respiratory Syndrome Coronavirus 2 RNA and Duration of Viral RNA Shedding Do Not Differ Between Patients With Mild and Severe/Critical Coronavirus Disease 2019. *J Infect Dis*, 223(1), 15-18. doi:10.1093/infdis/jiaa632
- Zhang, C., Guo, Z., Zhao, Z., Wang, T., Li, L., Miao, F., . . . Gao, Y. (2021). SARS-CoV-2 Aerosol Exhaled by Experimentally Infected Cynomolgus Monkeys. *Emerg Infect Dis*, 27(7), 1979-1981. doi:10.3201/eid2707.203948

APPENDIX

Supplemental material to Paper II

1. Inert gas washout reference values

Subjects

122 (57 females) healthy subjects, aged 18.1-60.6 years, performed multiple breath washout (MBW) using sulphur hexafluoride (SF_6) and helium (He) as tracer gases. The same recording devices as described in the main paper were used. Reference values were derived for SF_6 -based LCI, S_{cond} and S_{acin} .

Subjects were included via informative letters sent to randomly selected addresses derived from the Swedish population register called SPAR (“Statens personadressregister”), and via recruitment among staff at the hospital and their relatives. Inclusion criteria’s were no known chronic respiratory disease, absence of cardiac problems or malformations of the chest or the airways, and no developmental problems. Pregnant subjects and subjects with a smoking history of ≥ 10 pack-years were excluded from participation. Subjects were not being tested within a three-week period of an airway tract infection. The study was performed at the Respiratory Research Laboratory at Skaraborg Central Hospital, Skövde, Sweden between October 2009 and February 2017. The participants were asked to carefully read and sign a consent form prior to examination. The study was approved by the Regional Ethics Committee in Gothenburg.

Spirometry

112 (91.8 %) of the healthy controls performed spirometry in triplicates according to ATS/ERS guidelines ¹, Table S1. Spirometry measurements were related to Swedish reference equations ^{2,3} and expressed in z-scores.

Table S1. Demographic data and results for the healthy control subjects. Mean (standard deviation) are given.

Variable	All (n=122)	Females (n=57)	Males (n=65)
Age (years)	40.3 (13.1)	39.8 (12.9)	40.8 (13.3)
Height (cm)	174.9 (9.0)	167.3 (5.3)	181.5 (5.6) ***
Weight (kg)	75.0 (13.8)	66.9 (9.3)	82.1 (13.3) ***
BMI (kg/m ²)	24.4 (3.6)	23.9 (3.3)	24.9 (3.8)
FEV ₁ (z-score)	-0.04 (0.99)	-0.15 (0.89)	0.05 (1.06)
FVC (z-score)	-0.24 (0.97)	-0.22 (1.02)	-0.26 (0.93)
FEV ₁ /FVC (z-score)	0.34 (0.84)	0.12 (0.76)	0.52 (0.87) *
MEF ₅₀ (z-score)	-1.16 (3.18)	-2.51 (4.21)	-0.03 (1.08) ***
MEF ₂₅ (z-score)	-0.33 (1.09)	-0.42 (0.93)	-0.26 (1.22)

BMI, body mass index. FEV₁, forced expiratory volume in 1 second. FVC, forced expiratory volume. MEF₅₀, MEF₂₅, maximal expiratory flow at 50% and 25% of exhaled FVC, respectively. * $p < 0.05$. $p < 0.001$.

Identifying potential predictors

No gender difference was observed for MBW indices, Table S2.

Table S2. Multiple breath washout (MBW) indices in healthy control subjects (n=122) and for females and males, respectively. Mean (standard deviation) are given. No gender difference was observed.

MBW variable	All (n=122)	Females (n=57)	Males (n=65)
LCI	6.73 (0.54)	6.65 (0.53)	6.79 (0.55)
S _{cond}	0.016 (0.006)	0.016 (0.007)	0.016 (0.006)
S _{acin}	0.106 (0.024)	0.105 (0.022)	0.108 (0.025)

LCI, lung clearance index.

Age, weight, height and BMI (body mass index) were used as independent predictors in a bivariate correlation matrix in order to identify potential predictors for MBW indices. Pearson r for correlations is given in Table S3. Because of the strong correlation between weight and BMI in the cohort ($r=0.83$, $p<0.001$), weight was excluded from the subsequent multiple regression analysis if both predictors demonstrated a significant contribution to an MBW index. There was a weak correlation between age and BMI ($r=0.27$, $p<0.01$).

Table S3. Bivariate correlation matrix between independent and dependent variables (age, height, weight and BMI). Pearson r is given.

	Age (years)	Height (cm)	Weight (kg)	BMI (kg/m ²)
LCI	0.62***	<i>ns</i>	0.20*	0.22*
S _{cond}	<i>ns</i>	<i>ns</i>	<i>ns</i>	<i>ns</i>
S _{acin}	0.31***	<i>ns</i>	<i>ns</i>	<i>ns</i>

BMI, body mass index. LCI, lung clearance index. *ns*, non-significant. * $p < 0.05$,

*** $p < 0.001$.

In a hierarchical multiple regression analysis with LCI as dependent variable, age (introduced in step 1) had an R^2 change of 0.387, $p < 0.001$, while BMI introduced in step 2 did not add any extra significant explanation. Since S_{cond} did not correlate with any antropometric variable, and S_{acin} only demonstrated a weak correlation with age ($r = 0.31$; $p < 0.001$), the derived reference equations for the two SnIII-analysis outcomes was simply reported as mean and SD in the healthy control group, Table S2.

Table S4. Predicted values and reference equations for multiple breath washout derived indices. SD or RSD and p-value and F-value are also given, when appropriate.

Variables	Reference equation / Predicted mean	1RSD / 1SD	p-value	F-value
LCI	$5.692 + 0.026 \times \text{age}$	0.424	<0.001	75.68
S _{cond}	0.016	0.006		
S _{acin}	0.106	0.024		

SD, standard deviation. RSD, residual standard deviation. LCI, lung clearance index.

Figures S1-S3 demonstrates LCI, S_{cond} and S_{acin} plotted against age.

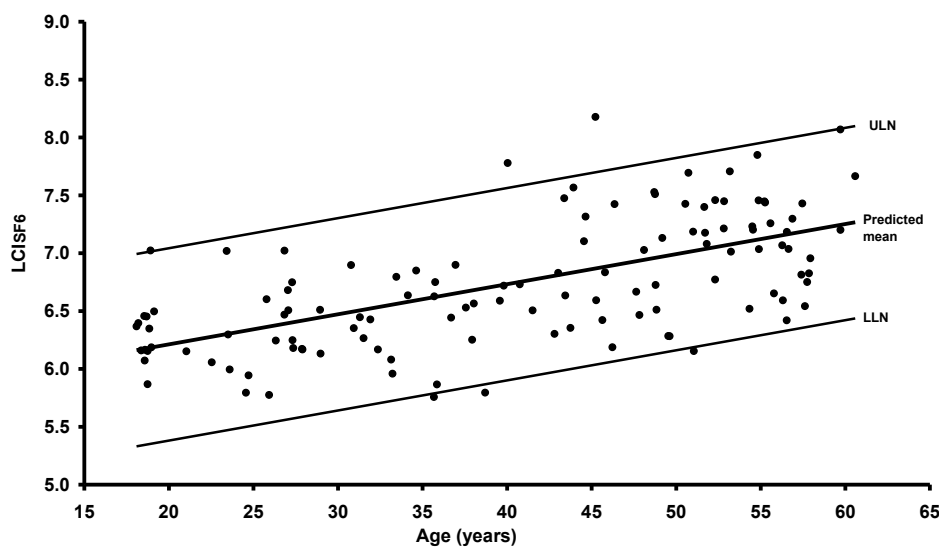


Figure S1. Lung clearance index (LCI) versus age in 122 healthy controls. Predicted mean is given by the equation: $LCI = 5.692 + 0.026 \times \text{age}$. 1 residual standard deviation (RSD) = 0.424. Upper limit of normal (ULN) and lower limit of normal (LLN) is identified by ± 1.96 RSD.

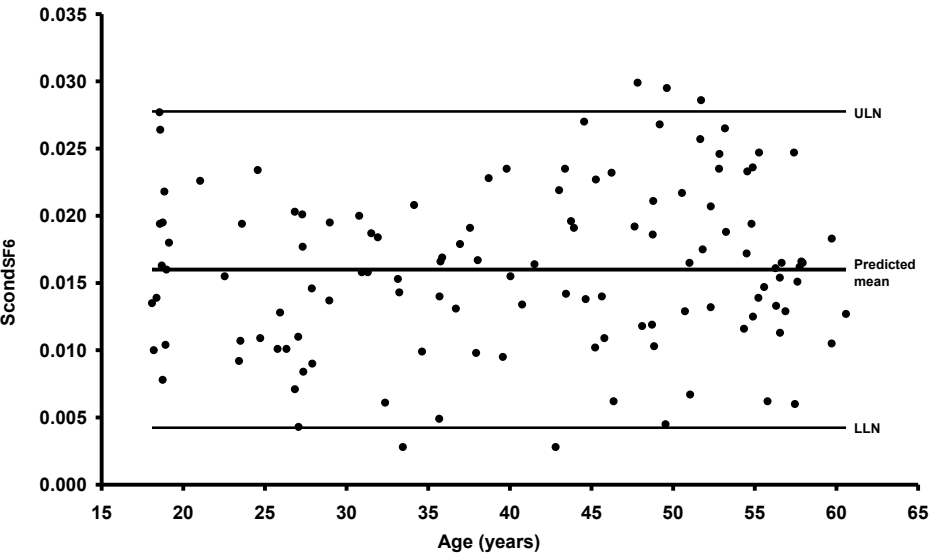


Figure S2. S_{cond} versus age in 122 healthy controls. Predicted mean (standard deviation), 0.016 (0.006). Upper limit of normal (ULN) and lower limit of normal (LLN) is identified by ± 1.96 SD.

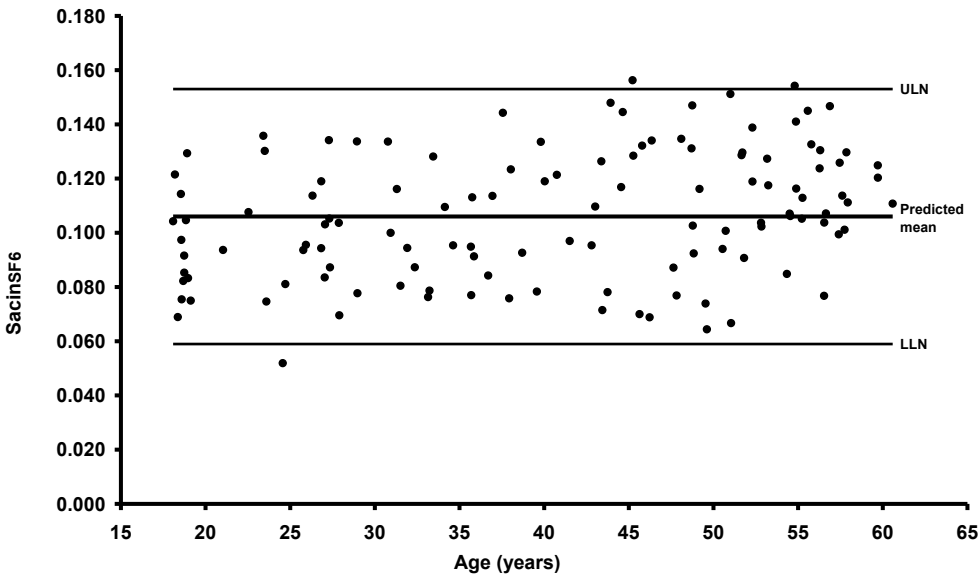


Figure S3. S_{acin} versus age in 122 healthy controls. Predicted mean (standard deviation), 0.106 (0.024). Upper limit of normal (ULN) and lower limit of normal (LLN) is identified by ± 1.96 SD.

References

1. Miller MR, Hankinson J, Brusasco V, et al. Standardisation of spirometry. *Eur Respir J*. 2005;26(2):319-338.
2. Hedenstrom H, Malmberg P, Agarwal K. Reference values for lung function tests in females. Regression equations with smoking variables. *Bulletin europeen de physiopathologie respiratoire*. 1985;21(6):551-557.
3. Hedenstrom H, Malmberg P, Fridriksson HV. Reference values for lung function tests in men: regression equations with smoking variables. *Upsala journal of medical sciences*. 1986;91(3):299-310.

