Impact of environmental factors on preterm and term-born infants and children

Inaugural dissertation

to

be awarded the degree of

Dr. sc. med.

presented at

the Faculty of Medicine

of the University of Basel

by

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From Geneva, Switzerland

Basel, 2021

Approved by the Faculty of Medicine

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Basel, 21.07.2021

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Acknowledgements

First of all, I would like to thank Prof. Urs Frey, who guided and encouraged me ever since I have started my MD-thesis. Urs, without your support and encouragement to do a PhD, I would not have accomplished all my achievements. Furthermore, your knowledge is and has been invaluable and has helped take my work to a higher level. I am thankful for all the support you have given me during my MD-PhD and the doors you have opened for me with it.

I would like to acknowledge the work and support from Prof. Pablo Sinues. The interdisciplinary work with you was a pleasure and I always appreciated your precise feedback with your view from a different angle, as it helped me gain further skills and sharpen my thinking. Your chemical and statistical knowledge and your kind manner treating colleagues was and is incredibly inspiring.

I would like to thank Prof. Matthias Kopp and Prof. Sven Schulzke. Your acceptance to either review my thesis and attain my defense or as the chair of the defense will elevate both the thesis and defense to a higher academic level.

My colleagues from the BILD cohort from Basel and Bern and from the Sinues lab have substantially contributed to my thesis, in terms of critical discussions, replacement for measurements or even in relation to PhD- or real-life-related issues. I would like to highlight Amanda Gisler and Olga Gorlanova here. You are both amazing statisticians and lovely people. Further, I would like to thank all the people who have read through and commented on this thesis; and a special thanks to Fiona Beck for the editing.

I would also like to mention my colleagues from the Swiss Tropical Public Health Institute: Danielle Vienneau, Kees de Hoogh and Martin Röösli. Without your air pollution data work,

your statistical feedback, and knowledge, the aims of my PhD-thesis would not have been achieved.

Furthermore, a big thank you goes to the families participating in the BILD cohort, the study nurses, and all the prior doctoral and PhD-students, without whom there wouldn't have been a cohort study to write a thesis about.

In addition, I would like to thank my whole family: my parents, brothers and their wives, and my grandparents. You respected my choice of completing a PhD and provided very valuable support, especially in the last fast steps of its finalization. The same gratitude goes to my in-laws, who have celebrated every successful step with me, despite the distance and COVID-situation.

I want to express my gratitude to my husband for his help. He has been the driving force to complete my thesis. You supported me throughout and especially during these last tough weeks even though you had one of the busiest and most stressful time periods yourself. You encouraged me at every step and are still promoting my success. Thank you!

And of course, I want to mention our lovely son and his sibling-to-be. Having you in our lives has changed them for the better and helped me to be more focused and efficient in terms of finalizing my PhD and achieving my goals.

Abbreviations

BILD cohort = Basel–Bern infant lung development cohort

BPD = bronchopulmonary dysplasia

CLD = chronic lung disease of infancy

cPAP = continuous positive airway pressure

CV = coefficient of variation

EBC = exhaled breath condensate

ERS/ATS = European Respiratory Society/American Thoracic Society

ETS = environmental tobacco smoke

$FEF_{25-75\%}$ = forced expiratory flow at 25–75% of FVC

FeNO = fraction of exhaled nitric oxide

FEV_1 = forced expiratory volume per 1 second

FRC = functional residual capacity

FVC = forced vital capacity

GA = gestational age

GINA = Global Initiative for Asthma

LRTI = lower respiratory tract infection

MS = mass spectrometry

NO = nitrogen oxide

NO_2 = nitrogen dioxide

On-line = **real-time** = direct exhalation into mass spectrometer and simultaneous analysis

Off-line = breath collection and analysis by mass spectrometer divided into ≥ 2 steps

O_3 = ozone

PAH = polycyclic aromatic hydrocarbon

PCA = postconceptional age

$PM_{2.5}$ = particulate matter with an aerodynamic diameter ≤ 2.5 µm

PM_{10} = particulate matter with an aerodynamic diameter ≤ 10 µm

PTEF = peak tidal expiratory flow

PTR-MS = proton transfer reaction-mass spectrometry

ROS = reactive oxygen species = free radicals and oxidants

RR = respiratory rate

SESI-HRMS = secondary electrospray ionization-high resolution mass spectrometry

SIFT-MS = selected ion flow tube-mass spectrometry

TBFVL = tidal breathing flow volume loops

t_E = expiratory time

$t_{PTEF/tE}$ = ratio of peak tidal expiratory flow and expiratory time

V_E = minute ventilation

VOCs = volatile organic compounds

V_T = mean tidal volume

4-HNE = 4-Hydroxynonenal

Summary

English summary

Background: Survival rates of prematurely born infants are increasing throughout high-income countries, resulting in around 10% preterm births at present. Preterm infants are a very heterogenous group in terms of respiratory morbidity and mortality and have impaired capacity to deal with oxidative stress in the perinatal period, making them more susceptible to environmental stimuli than their healthy term peers. However, it is unknown, whether this vulnerability is associated with an increased oxidative stress response and low-level inflammation during infancy and childhood, which can be measured in exhaled breath.

Aim: The aim of this thesis was to investigate the effects of air pollution on lung function and inflammatory and metabolic profiles in infancy and childhood. Therefore, the primary aim was to directly compare pre- and postnatal exposure to low-to-moderate air pollution with lung function at 44 weeks of postconceptional age (PCA) and at six years of age in preterm and term–born children. The secondary aim was to develop and standardize a mass spectrometry technique to retrieve metabolic information from exhaled breath of preterm and term infants and to correlate oxidative stress markers with lung function parameters and fraction of exhaled nitric oxide (FeNO).

Methods: All the studies of this thesis were performed within the prospective Basel–Bern infant lung development (BILD) cohort (SNF 182871/1). Information on perinatal risk factors was collected from medical records and by standardized questionnaires. For each individual child and window of exposure, air pollution levels were calculated using space-hybrid models for nitrogen dioxide (NO_2), particulate matter with a diameter ≤ 10 µm (PM_{10}), and ozone (O_3). To assess airway abnormalities and inflammation, lung function and FeNO measurements were performed at 44 weeks of PCA and at six years of age. For the development and standardization

of the off-line technique for metabolic profiling in infants, secondary electrospray ionization-high resolution mass spectrometry (SESI-HRMS) was used.

Results: Pre- and postnatal exposure to low-to-moderate air pollution levels showed clear associations with impaired lung function in infancy and at school-age. These effects were most pronounced in children with the highest exposure and when higher air pollution levels occurred during a time windows of accelerated lung development: the second trimester of pregnancy, as well as the first and second year of life. Furthermore, aggravated impairment of lung function in infancy was seen in moderate to late preterm infants (born 32 – 37 weeks of gestational age).

We have found evidence of effects of air pollution on FeNO, however FeNO is influenced by, but not specific for measuring oxidative stress. Oxidative stress markers can potentially be measured in exhalomics, however, techniques are not yet available for use in infants. Thus, as a first step, we developed and tested a new methodology, which showed performance characteristics comparable to the gold standard of SESI-HRMS measurements. For the first time, the successful deployment of this technique enabled the measurement of specific metabolites suggestive of oxidative stress (e.g. 4-Hydroxynonenal) in infants. Additionally, the novel method of capturing information on metabolic profiles from infant breath could provide further biological background information on metabolic responses to environmental stimuli, such as exposure to air pollution, supporting our findings.

Conclusions: Air pollution levels even below currently recommended thresholds are associated with impaired lung function in healthy children and are even more pronounced in preterm infants. As a proof of concept and novelty, these results support the hypothesis of increased time- and dose-dependent vulnerability of children to environmental stimuli and increased susceptibility of preterm infants. Prevention in utero and early life are of utmost importance as lung function deficits in childhood have been shown to trace through life with the potential for

respiratory morbidity in adulthood. Therefore, more stringent policies to reduce air pollution levels and consequently the burden of disease should be taken.

Deutsche Zusammenfassung

Hintergrund: In den vergangenen Jahrzehnten ist die Rate der überlebenden Frühgeborenen in industrialisierten Ländern stetig angestiegen. Der Anteil der Frühgeborenen beträgt weltweit aktuell etwa 10% aller Geburten. Frühgeborene, eine heterogene Population bezüglich respiratorischer Morbidität und Mortalität, haben perinatal eine erhöhte Anfälligkeit gegenüber oxidativem Stress. Folglich sind sie, im Vergleich zu Termingeborenen, anfälliger für die negativen Einflüsse von Umweltfaktoren. Es ist jedoch nicht klar, ob diese Vulnerabilität mit einer gewissen Inflammation im Säuglings- und Kindesalter einhergeht, welche theoretisch in der Ausatemluft gemessen werden kann.

Ziele: Das Ziel dieser Dissertation war es, den Effekt von Luftschadstoffen auf die Lungenfunktion und das inflammatorische und metabolische Profil im Säuglings- und Kindesalter zu untersuchen. Das primäre Ziel war die Untersuchung der Assoziation von prä- und postnataler geringer bis moderater Luftverschmutzung mit der Lungenfunktion im Alter von 44 Wochen postkonzeptionell und 6 Jahren in Früh- und Termingeborenen. Das sekundäre Ziel war die Entwicklung und Standardisierung einer Methode für die Messung von Stoffwechselprodukten in der Ausatemluft von früh- und termingeborenen Kindern mittels Massenspektrometrie und die Korrelation damit gemessener oxidativer Stressmarker mit Lungenfunktionsparametern und exhaliertem Stickstoffoxid (FeNO).

Methoden: Alle Studien dieser Dissertation wurden im Rahmen der prospektiven Basel-Bern infant lung development (BILD) Kohortenstudie (SNF 182871/1) durchgeführt. Informationen über perinatale Risikofaktoren wurden mittels medizinischer Berichte sowie standardisierter Fragebögen erfasst. Die individuelle Exposition gegenüber Luftschadstoffen wurde für unterschiedliche Zeitfenster anhand von räumlich-hybriden Modellen für Stickstoffdioxid (NO_2), Feinstaub mit einem Durchmesser $\leq$ 10 µm (PM_{10}) und Ozon (O_3) berechnet. Lungenfunktionstests und FeNO wurden für die Einschätzung der Lungenfunktion und einer

allfälligen Inflammation der Atemwege im Alter von 44 Wochen postkonzeptionell und 6 Jahren durchgeführt. Für die Entwicklung und Standardisierung der Methode der Messung metabolischer Profile von Kindern wurde das sekundäre Elektrospray Ionisierung-hoch Resolution Massenspektrometer (SESI-HRMS) verwendet.

Resultate: Die prä- und postnatale Belastung mit geringer bis moderater Luftverschmutzung war mit einer verminderten Lungenfunktion im Säuglings- und Kindesalter assoziiert. Die Effekte waren am ausgeprägtesten bei Kindern mit der höchsten Exposition und im Falle einer hohen Luftverschmutzung während Zeitfenstern beschleunigter Lungenentwicklung: das zweite Trimester sowie das erste und zweite Lebensjahr. Des Weiteren zeigte sich bei den moderat bis spät Frühgeborenen (geboren 32 – 37 Gestationswochen) eine zusätzliche Verschlechterung der Lungenfunktion im Alter von 44 Wochen postkonzeptionell.

Wir konnten zudem einen Effekt von Luftverschmutzung auf FeNO aufzeigen, wobei FeNO von oxidativem Stress beeinflusst wird, nicht aber als spezifischer Marker oxidativen Stress gewertet wird. Marker von oxidativem Stress können theoretisch in der Ausatemluft gemessen werden, jedoch ist aktuell keine Messmethodik für Säuglinge verfügbar. Folglich haben wir in einem ersten Schritt eine Messmethodik entwickelt und standardisiert, welche eine gute Vergleichbarkeit zu der aktuell benutzten und etablierten on-line Messmethode mittels SESI-HRMS zeigte. Die Messung spezifischer Marker von oxidativem Stress (bspw. 4-Hydroxynonenal) konnten damit zum ersten Mal in der Ausatemluft von Säuglingen gemessen werden. Die Entwicklung der neuen Messmethode von Metaboliten in der Ausatemluft von Säuglingen hat das Potenzial zusätzliche biologische Hintergrundinformationen zur metabolischen Antwort auf Umweltfaktoren, wie die Luftschadstoffbelastung, zu liefern.

Schlussfolgerung: Unsere Resultate belegen, dass eine Luftverschmutzung mit Werten, die unterhalb der aktuell international geltenden Richtlinien liegen, mit einer Verschlechterung der Lungenfunktion in gesunden Kindern und besonders in frühgeborenen Säuglingen assoziiert

ist. Die Erkenntnisse dieser Dissertation unterstützen die Hypothese einer zeit- und dosis-abhängigen Empfindlichkeit der Lunge von Kindern gegenüber Umweltfaktoren sowie eine besonders erhöhte Vulnerabilität von Frühgeborenen. Somit erscheint eine Prävention in utero und in den ersten Lebensjahren enorm wichtig, da aufgezeigt werden konnte, dass sich Defizite der Lungenfunktion im frühen Kindesalter bis ins Erwachsenenalter ziehen und somit für eine respiratorische Morbidität im Lebensverlauf verantwortlich sein können. Eine mögliche Konsequenz, um dieser Entwicklung entgegenzuwirken, könnten strengere Richtlinien für die Reduktion der Luftverschmutzung sein.

1 Introduction

Over the last decades, the global prevalence of respiratory diseases in childhood has significantly risen (1). Respiratory diseases, with asthma being the most common chronic disease in children, represent a substantial issue for health care and families, especially because the increasing number of hospitalizations and long-term medication lead to a high financial burden (2, 3). Studies investigating the long-term outcome of asthmatic children into adulthood have provided evidence that chronic respiratory disease later in life might be determined primarily in early childhood (4, 5), as infants with premorbid lung function might not reach predicted peak lung function during early adult life (6).

However, the development of respiratory disease throughout life is complex and has various contributing factors. Host and environmental factors (7, 8), such as sex, atopy of the mother, prematurity, and exposure to environmental tobacco smoke or air pollution are just a few to mention. Besides the increasing prevalence of asthmatic children, the numbers of infants born prematurely (< 37 weeks of gestational age [GA]) has risen due to substantial progress in obstetric and neonatal care (9). Preterm infants represent a vulnerable subgroup that need special attention. They are known to have impaired capacity to deal with oxidative stress perinatally (10-13) due to various factors augmenting oxidative stress or impairing antioxidative response (14). Subsequently, preterm-born children develop more respiratory symptoms than term-born children (15-17) and have impaired lung function throughout childhood until adolescence and adulthood (18-20).

To date, it is unknown whether preterm infants have persistent low-level airway inflammation as an altered oxidative stress response. This may be reflected in altered metabolic profiles of the lung which, in principle, can be measured in exhaled breath, as it potentially offers a non-invasive window to monitor such processes (21). Additionally, it is not yet well understood, whether exposure to oxidative stress, such as pre- and postnatal exposure to air pollution leads

to aggravated impairment of lung function in infancy and school-age in preterm compared to term-born children. The aim of this thesis was therefore to address these major knowledge gaps.

1.1 The complexity of host–environment interaction and respiratory disease in infancy and childhood

The development of respiratory disease is likely influenced by multiple factors. Host factors, such as sex and maternal atopic disease may lead to the child having a higher susceptibility to respiratory symptoms, and environmental factors, such as environmental tobacco smoke (ETS) exposure, air pollution and infections may contribute to the severity and recovery of respiratory symptoms (22, 23). Interestingly, most of these determinants or risk factors have a small effect size by themselves, but become relevant due to a complex interplay among several factors (8).

It has been hypothesized that these early life factors and especially their interplay modulate the properties of the respiratory system, which include lung growth and development, immune development, and inflammatory and repair response to oxidative stress and respiratory infections (8). These mechanisms are often referred to as “early life programming” (24). This hypothesis assumes that in early life, the various components of the respiratory system inherit a certain plasticity, which, under normal conditions, enable adaptation to the child’s environment. It is likely that inflammation, the oxidative stress response, and lung growth are highly interlinked during early life, during a phase of rapid lung growth and development with fast cell replication (8). Therefore, the relative impact of each contributing subsystem may be age-dependent and dynamic in nature. For example, several prospective studies have investigated the age-dependent effect of air pollution on lung function in infancy (25-27), childhood (28-30) and adolescence (31). However, there were conflicting conclusions, and open questions remained. For instance, no clear agreement has been reached on time windows of

increased vulnerability to exposure to air pollution (28, 32, 33). Moreover, the effects of air pollution on lung function in infancy or even in vulnerable populations (e.g. boys, asthmatics or preterm infants) have not yet been fully answered (25, 34, 35).

1.2 Effects of air pollution on the respiratory system

Air pollution, a mixture of various gases and particles from natural sources (e.g. dust, organic compound emissions from plants) and from human activity (e.g. fossil fuel burning), primarily impacts barrier organs, such as the respiratory system (36). Nonetheless, due to the heterogenous composition of air pollution and its oxidative activity (37, 38), the effects are not just limited to immediate and local responses, but secondarily trigger systemic responses, activating the immune system beyond the immediate location of the insult, activating metabolic functions, and altering organ-to-organ signaling and autonomic nervous system control (39). Besides, continued and persistent environmental exposures are considered to induce apoptosis and necroptosis (40). Specifically, fine particulate matter, a mixture of transition metals, polycyclic aromatic hydrocarbons (PAHs), soot, and other oxygen radical-producing substances (37), contributes through these pathways to the loss of functional lung epithelium and the subsequent development of chronic obstructive lung disease (36).

Furthermore, air pollution leads to oxidative stress in the airways (36), which is defined as an imbalance between the production of free radicals or oxidants and antioxidant defence mechanisms (41), leading to tissue injury and cell death (42). Although the body has many antioxidant mechanisms to fight against reactive oxygen species (ROS) (41), there are certain populations, such as preterm infants, which are particularly vulnerable to the oxidative damage because of an imbalance in ROS production and their immature antioxidant systems with lower possibility to scavenge excess ROS (43, 44). For preterm infants, this is especially due to higher

exposure to supplementary oxygen, inflammation, and occurrence of free iron in tissues and simultaneously poorly developed intracellular antioxidant defence systems (43).

The effects of air pollution on the respiratory system can therefore be clinically measured by various methods, such as lung function testing (e.g. tidal breathing flow volume loops in infancy, or spirometry and body plethysmography) (25, 45), fraction of exhaled nitric oxide (FeNO) as an inflammatory marker (46), occurrence and severity of respiratory symptoms including wheeze (22), specific metabolites suggestive of oxidative stress (e.g. 4-hydroxynonenal) (47), and many others.

On the one hand, the adverse effects of high air pollution levels on respiratory health in children and adults have been demonstrated (7, 8, 22, 48, 49). On the other hand, it is unclear, whether there are thresholds below which children's respiratory health might not be affected and whether such thresholds depend on the age of the child. Even in low-to-moderate polluted areas (e.g. Sweden), the most recent studies indicate the need for more stringent air pollution reductions, since associations have been described between these air pollution levels and impaired lung function in preschool and school-aged children (45, 50, 51).

There is some evidence suggesting that the timing of susceptibility and dose-response might vary for different pollutants during lung tissue development (52). Additionally, due to the manifold mechanisms behind the adverse effects of air pollution on respiratory health (e.g. endocrine dysfunction, toxicity, impaired development, and epigenetics) (53), prospective cohort studies with precise pre- and postnatal risk factor and lung function assessment at different time points throughout infancy and childhood are needed to evaluate whether there are time- and dose-dependent effects of air pollution in low-to-moderate polluted areas, such as Switzerland.

Moreover, since children starting with impaired lung function are especially at risk for chronic lung disease in adulthood and respiratory mortality (6, 54), the impact of exposure to air pollution on more vulnerable subgroups, such as asthmatics or preterm-born children should be investigated. For example, preterm infants, who are particularly susceptible to oxidative stress shortly after birth (11), might be more vulnerable to such environmental stimuli. Unfortunately, studies on the effects of air pollution on respiratory health in this population are lacking. Such investigations, however, are of utmost importance for the determination and early prevention of populations at risk for later respiratory disease (6).

1.3 Susceptibility of premorbid groups of children for air pollution effects: The special situation of preterm infants

Due to advances in obstetric and especially in neonatal care over the last decades, the rate of prematurely born infants has risen throughout high-income countries (9). Consequently, around 7% of infants in Switzerland (55) and around 15 million infants worldwide are born prematurely each year (56). This subgroup of new-born infants is quite heterogenous in terms of risk of respiratory morbidity (18) and mortality (57), due to the interrupted development of the lungs at birth at different maturation phases. Premature birth leads to incompletely developed alveoli, inefficient gas exchange and an immature surfactant system (58), resulting in smaller and less compliant airways (59).

Many studies have investigated the long-term outcome of former preterm children, especially extremely (< 28 weeks of GA) and very early (29 – 31 weeks of GA) preterm infants (19, 60). These trials showed strong correlations of low lung function from early childhood until adolescence and adulthood with preterm infants never reaching optimal lung function in early adulthood (20, 59, 61-65). However, studies on respiratory outcomes in moderate to late

preterm infants (32 – 37 weeks of GA) are conflicting (66-69), and studies directly comparing preterm and term infants and the effects of air pollution on lung function are lacking. Such investigations are important to better understand differences in the susceptibility of infants born prematurely compared to term infants, which can only be answered in well-controlled, direct comparisons. Moderate to late preterm infants further represent the epidemiologically largest group of preterm births, accounting for around 6% of total births (55). However, assessing the special susceptibility of preterm infants is difficult, due to many other factors impacting on lung function with potentially stronger influences (19, 70). Thus, methodological strategies to tackle this issue are: multivariable approach and stratification of preterm infants in subgroups according to their GA at birth (e.g. born before and after 32 weeks of GA). Additionally, it is unknown, whether preterm infants show persistent low-level airway inflammation as an altered oxidative stress response, which might be reflected in altered metabolic profiles of the lung that, in principle, can be measured in exhaled breath.

1.4 Detecting oxidative stress response by breath analysis

There are several ways to detect oxidative stress response and inflammation in humans (71), but most techniques are measured in plasma (72), tissues and/or urine (44, 73) and are not specific for respiratory processes. These methods may be invasive in nature or need extensive sample preparation, which can be both time consuming and expensive. Invasive techniques are not optimal to apply especially in pediatric populations or in research settings, and should ideally be omitted or replaced by alternative approaches. In the last decades, several techniques have been developed with the aim of non-invasively capturing information on oxidative stress response and inflammation (46) of the respiratory system.

Much attention has been given to FeNO measurements as a non-invasive marker of primarily eosinophilic respiratory inflammation, in particular in asthma (46). However, it has not been included in the Global Initiative for Asthma (GINA) guidelines for asthma diagnosis mainly due to its low discriminating power (specificity and sensitivity) (74). Other studies, however, indicated the power of FeNO in phenotyping asthma and monitoring of corticosteroid treatment response (75). FeNO has been shown to be influenced by environmental stimuli, such as maternal smoking (76) or air pollution (77), and viruses (78) and allergens (79) have shown direct induction of inducible nitric oxide synthase. Therefore, FeNO levels should always be carefully interpreted within the context of environmental factors, as it reflects a mixture of low-grade inflammation and oxidative stress response with different cell types influencing FeNO levels.

Almost simultaneously to the development of FeNO measurements, a second approach evolved, with various studies investigating exhaled breath condensate (EBC) to detect possible correlations between oxidative stress response and the development of respiratory disease (80, 81). The drawbacks of EBC include the dependency on the sampling procedure (e.g. normalisation to sample size), as well as time-consuming collection (5 to 15 min for every 1 ml of EBC) and analysis of condensates. In addition, the method is prone to dilution due to the exhaled vapor, requiring sensitive analytical tools for the detection of various markers occurring in very low concentrations (80, 82). Despite broad attempts to make EBCs more reproducible and less dependent on environmental factors (e.g. humidity, temperature), this method only remains available for research purposes and has not been implemented clinically.

Direct analysis of breath by mass spectrometry (MS) (on-line/real-time breath analysis) however, has gained great momentum in recent years, and has been shown to non-invasively capture profiles of the endogenous metabolism of patients (21, 83). There are various analytical platforms enabling on-line breath analysis, including selected ion flow tube-MS (SIFT-MS),

proton transfer reaction-MS (PTR-MS), and secondary electrospray ionization-high resolution mass spectrometry (SESI-HRMS). All the mentioned techniques report detection limits ranging in the parts-per-trillion range. In contrast to PTR-MS and SIFT-MS, ionization of exhaled metabolites takes place at atmospheric pressure in SESI-MS. This results in a higher ionization probability (84) and allows the use of any pre-existing atmospheric pressure ionization mass analyzer, such as an ultra-high-resolution MS (e.g. Orbitrap), making SESI-HRMS a sensitive and selective on-line analysis tool for measuring the whole metabolome in exhaled breath (85).

The non-invasive nature of SESI-HRMS makes it pivotal for future more personalized medicine (86). However, one of the remaining challenges for the implementation of breath measurements in clinics are non-cooperative patients or patients not capable of performing the necessary maneuveres for on-line breath measurement (87). Therefore, off-line breath collection techniques have been the subject of research (88-90). Off-line techniques can be divided into at least two independent steps of breath collection and analysis, thus, optimizing the potential availability for patients in need of disease diagnosis and monitoring or non-invasive therapeutic drug monitoring.

Currently, three techniques to collect off-line breath measurements are available: EBC as described previously (80), adsorption (91, 92), and sampling in containers or bags (93). Each technique has several advantages and disadvantages, however, for breath measurement of volatile organic compounds (VOCs) mainly bags have been used, due to their affordability, easy use and light weight (93, 94). Nonetheless, the application of off-line techniques in non-cooperative patients has only been studied in intubated adults (95) and issues in the use and storage of off-line samples have occurred (e.g. reaction of metabolites with bag material or between various metabolites present in bag). Therefore, measuring techniques for metabolic profiling by MS in infants and children still remain unknown, and the development and standardization of such collection and analysis platforms is therefore needed.

2 Aims of the thesis

The aim of this thesis was to investigate host–environment interactions and their effects on lung function and metabolic responses in infancy and childhood. The primary aim was therefore to assess the effects of air pollution on lung function in preterm versus term-born infants and children. The secondary aim was to standardize a measuring technique to study the metabolic profile of preterm and term infants. To achieve these goals, I mainly worked in the BILD cohort recruiting children, and performing and analyzing lung function measurements. Simultaneously, within the Sinues Lab we developed and standardized an off-line sampling method to capture metabolic profiles of infants in exhaled breath.

The following specific aims were addressed in this work:

1. To understand the complex network of interactions leading to impairment of lung function development in utero, in early life, and in later childhood:
    a. to comprehensively summarize extant literature on lung function development and airway disease-related lung function trajectories throughout childhood
2. To assess the effect of low-to-moderate pre- and postnatal exposure to nitrogen dioxide (NO_2), ozone (O_3), and particulate matter with an aerodynamic diameter of ≤ 10 µm (PM_{10}):
    a. with lung function at 44 weeks of postconceptional age (PCA)
    b. with lung function of healthy formerly term–born children at school-age
    c. to investigate specific windows of enhanced vulnerability pre- and postnatally
    d. to investigate the potential susceptibility of subgroups, such as preterm infants
3. To enable metabolic profiling in infant breath by SESI-HRMS analysis:
    a. to develop and standardize a collection and analysis platform for metabolic information retrieval of uncooperative patients in exhaled breath by SESI-HRMS

b. to prove feasibility of the off-line measuring technique by i) measuring key metabolites in comparison to the gold standard method; ii) investigating short-term repeatability; iii) investigating the in-/dependency of the collection method on breathing patterns; iv) exploring an exemplary relationship between oxidative stress markers measured off-line by SESI-HRMS and an independently measured, less specific marker of oxidative stress and inflammation (FeNO)

Our group has previously shown (22) that premorbid conditions may make certain groups of infants more vulnerable to the deleterious effect of air pollution. One large group with premorbid alterations and potential susceptibility are infants born prematurely, which are particularly susceptible to oxidative stress shortly after birth. Unfortunately, studies on the effects of air pollution on respiratory health in this population are lacking. With around 10% of infants worldwide being born prematurely, this population reflects a large and heterogeneous population. Such investigations are therefore of utmost importance for the determination and early prevention of populations at risk for later respiratory disease.

3 Methods

3.1 The Basel–Bern infant lung development cohort

The Basel–Bern infant lung development (BILD) cohort (SNF 182871/1) was initiated in 1999 in Bern and in 2011 in Basel to study early lung development and the environmental and genetic factors influencing it from infancy through childhood.

Infants are therefore recruited antenatally and standardized interviews and questionnaires are used to assess potential risk factors for respiratory morbidity during childhood. To assess genetic and immune response information, cord blood samples are collected at birth. Furthermore, the urine of infants is collected at several time points during the first year of life for the analysis of metabolic markers. To monitor the respiratory health of the participants, weekly phone interviews are made by study nurses during the first year of life and a lung function test and collection of exhaled breath at 44 weeks of PCA are conducted.

At six years of age, participants are invited for a follow-up visit, which includes a variety of lung function tests, the collection of exhaled breath, and the assessment of asthma and allergic disease by standardized interviews and prick test.

Figure 1 illustrates the current study outline. The study has been described elsewhere in detail (96). The Ethics Committees of Bern and Basel, Switzerland approved the study and informed consent was obtained from parents.

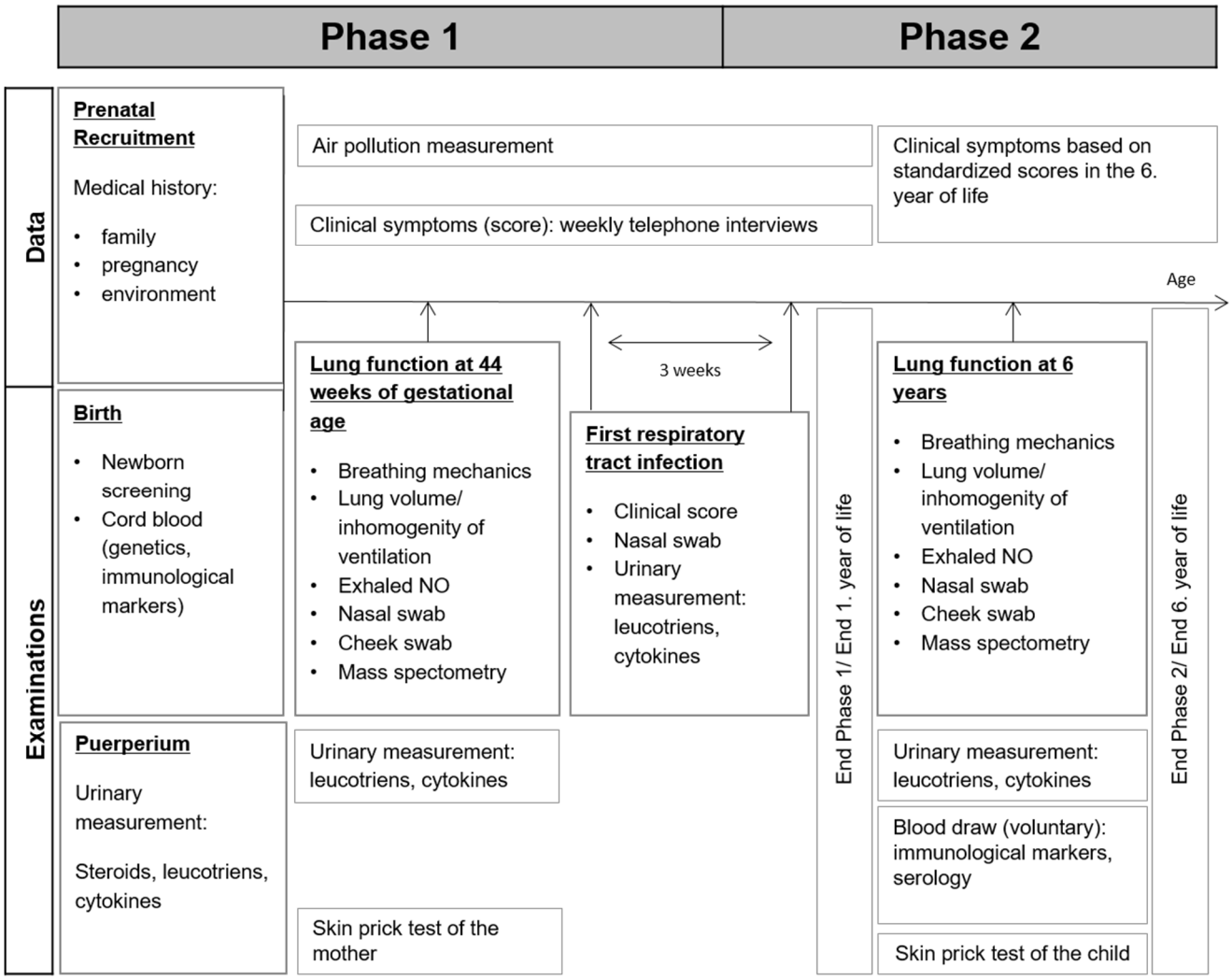

Figure 1. Schematic layout of the BILD cohort study. Phase 1 includes lung function test and exhaled breath measurement at 44 weeks of postconceptional age and weekly telephone interviews during the first year of life. Phase 2 includes lung function tests, exhaled breath measurement, prick test and questionnaires/interviews for atopic outcome definition. Exhaled NO: fraction of exhaled nitric oxide (FeNO)

3.2 Exposures

3.2.1 Assessment of risk factor and modifier

Information on possible covariates and confounders for respiratory morbidity were gathered from medical records (e.g. birth weight, duration of supplementary oxygen), and standardized questionnaires and interviews after birth and at follow-up visits.

Risk factors could be divided in prenatal/host, perinatal and postnatal factors. With atopic disease of the mother, active smoking during pregnancy, ETS during pregnancy, and education status of the mother being prenatal/host factors. Perinatal influences included gestational age at birth, birth weight and length, APGAR, supplementary oxygen and mechanical ventilation duration, and bronchopulmonary dysplasia (BPD) classification, where BPD was defined according to treatment at 36 weeks' GA, irrespective of prior or current oxygen therapy (97). No BPD was defined as no support, mild/grade I was defined as nasal cannula ≤ 2 L/min, moderate/grade II as nasal cannula > 2 L/min or non-invasive positive airway pressure, severe/grade III as invasive mechanical ventilation (97). Postnatal factors included catch-up weight gain until lung function measurement, weight and length at lung function, and ETS.

Risk factors were included into regression models based on previous literature (22, 98, 99) and statistical approaches (e.g. backward selection) were used to check for significance.

3.2.2 Air pollution assessment

Air pollution data included daily mean levels of NO_2, O_3 and PM_{10}. For the calculation of individual exposures, space-hybrid models were used and residential or hospital addresses were geocoded using a reference file from the Swiss Federal Statistical Office (Neuchâtel).

For NO_2 exposure estimates time-spaced hybrid models were used in order to capture seasonal air pollution variations during the entire study period and spatial variation in different study

areas. This model was based on high-quality information on land use, population density, traffic, road network, dispersion models, meteorological data, and air quality from a fixed measurement station (i.e. Payerne), trained with 28,849 NO_2 biweekly and monthly passive sampler measurements. For external validation of the model, observations were consecutively collected over more than ten years at 146 locations (100). PM_{10} exposure was estimated using a simplified spatial-temporal model in which the temporal variation from Payerne was superimposed on the annual dispersion model from Pollumap (101) which provided the spatial contrast in exposures.

3.3 Clinical outcomes

3.3.1 Respiratory symptoms during the first year of life

Children's health status, respiratory symptoms, and lower respiratory tract infections (LRTI) were assessed by weekly telephone interviews using a standardized respiratory symptom score, which groups symptoms into four levels according to severity (**Appendix Table E1**) (102). LRTIs were defined, as previously described (103), as more than two consecutive days with wheeze, cough, breathing difficulties and upper respiratory tract symptoms (e.g. rhinitis) and fever (> 38.0°C). Parents reported the duration of the LRTI as the number of days the child presented symptoms.

3.3.2 Wheeze, atopic disease, and rhinitis at six years of age

Information on clinical outcomes (e.g. wheeze, rhinitis, and atopic dermatitis) at the age of six years was gathered through standardized questionnaires, adapted from the International Study on Asthma and Allergy in Childhood (ISAAC) questionnaire (104). Asthma was defined following GINA guidelines (105) as cough/wheeze/difficult or heavy breathing in the absence of a respiratory tract infection in the last 12 months in combination with an asthma medication

(inhaled corticosteroids or ß-agonists) used in the last 12 months and/or positive past history of allergic conditions (atopic dermatitis and/or rhinitis/rhino conjunctivitis) and/or a positive family history of allergic conditions. Rhinitis/rhino conjunctivitis was defined as parent-reported pro-longed sneezing, runny, or blocked nose accompanied by ocular itching and tearing without a common cold in the last 12 months, according to international standards (106).

Wheeze was obtained from a physician-administered questionnaire and defined as present if the child had at least 1 episode of wheeze during the 12 months before the questionnaire.

3.4 Lung function outcomes

3.4.1 Infant lung function testing

Lung function testing was performed at 44 weeks of PCA using the Exhalyzer D (EcoMedics, Duernten, Switzerland) according to current European Respiratory Society/American Thoracic Society (ERS/ATS) guidelines (107). For analysis we used the first 100 regular breaths during non-rapid eye movement (non-REM) sleep from the total recorded breathing. We excluded sighs and ten breaths before and after a sigh. Mean tidal flows, volume, and flow-volume loop were calculated following ERS/ATS guidelines for infant lung function testing.

The following parameters were investigated: respiratory rate (RR), mean tidal volume (V_T), and minute ventilation (V_E). Ratio of time to peak tidal expiratory flow (PTEF) and expiratory time ($t_{PTEF/tE}$) were used to describe tidal breathing flow volume loops (TBFVL) shapes.

3.4.2 Lung function testing at school-age

During follow-up visits, spirometry was performed using the MasterLab setup (Jaeger, Wurzburg, Germany) according to ERS/ATS guidelines (108). The following lung function

parameters were measured: forced expiratory volume in 1 second (FEV_1), forced vital capacity (FVC), forced expiratory flow at 25–75% of FVC ($FEF_{25-75\%}$), and FEV_1/FVC was calculated. Tests were defined as reproducible if FVC and FEV_1 agreed within 100 mL or 10% between the best two exhalations (108). Data were expressed as z-scores using the Global Lung Function Initiative (GLI) 2012 prediction equation (109).

Body plethysmography measurements were performed to assess the functional residual capacity (FRC_{pleth}), which is the volume of air present in the lungs at the end of passive expiration (108). Three high-quality measurements (coefficient of variation (CV) between measurements < 25%) were used to calculate their mean.

3.4.3 Fraction of exhaled nitric oxide measurements in infants

Concurrent to TBFVL recording at 44 weeks of PCA, the fraction of exhaled nitric oxide (FeNO) was measured online with a chemiluminescence analyzer (CLD 77 AM; EcoMedics AG, Duernten, Switzerland) during the third quartile of expiration and averaged over the 100 breaths used for analysis and adjusted for V_E, as previously described (98). We used air free of nitric oxide (NO) for respiration to prevent contamination of FeNO with ambient NO.

3.4.4 Fraction of exhaled nitric oxide measurements in school-aged children

During the follow-up visits, FeNO was measured by the single-breath method with a rapid-chemiluminescence analyzer (CLD 88 sp; EcoMedics, Duernten, Switzerland). According to ERS/ATS guidelines (110), flow was simultaneously recorded and FeNO values adjusted for.

3.5 Development of breath measurement by mass spectrometry

Metabolic profiles in breath were measured in adults and infants. Healthy adults provided breath on-line and off-line. Infant breath measurements were performed subsequent to the infant lung function measurement at 44 weeks of PCA in unsedated sleep. Off-line breath collection was performed until the Nalophan bags were filled (approximately 2 L for adults and 500 mL for infants) and replicates were attempted if infants were still asleep after the first bag was filled.

After off-line collection of breath in Nalophan bags, the samples were emptied into the ion source (Super-SESI, FIT, Spain). The ion source featured a low-pressure mass flow controller that ensured similar flow conditions through the ion source for the samples.

The on-line breath analysis platform consisted of the Exhalion interface (Exhalion, FIT, Spain) for real-time display of CO_2, flow rate and exhaled volume and an the previously mentioned ion source (Super SESI, FIT, Spain) coupled to a high-resolution MS (Q-Exactive Plus, Thermo Fisher Scientific, Germany) (85, 111). The Q Exactive Plus MS was operated via Q Exactive Tune software in full scan mode, positive mode over the mass range m/z 70-1000 with a resolution of 140,000 for positive mode at m/z 200, 4 microscans, automatic gain control (ACG) target 1×10^6 and maximum injection time 500 ms.

4 Publications

4.1 Article I - Lung functional development and asthma trajectories

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Seminars in Immunopathology (2020) 42:17–27

https://doi.org/10.1007/s00281-020-00784-2

Accepted: 15 January 2020

Lung functional development and asthma trajectories

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Abstract

Early life environmental risk factors are associated with chronic respiratory morbidity in child- and adulthood. A possible mechanism for this sustained effect is their influence on early life lung functional growth and development, a susceptible phase of rapid lung growth with increased plasticity. We summarize evidence of hereditary and environmental ante-, peri- and early postnatal factors on lung functional development, such as air pollution, tobacco exposure, nutrition, intrauterine growth retardation, prematurity, early life infections, microbiome and allergies and their effect on lung functional trajectories. While some of the factors (e.g. prematurity) directly impair lung growth, the influence of many environmental factors is mediated through inflammatory processes (e.g. recurrent infections or oxidative stress). The timing and nature of these influences and their impact results in degrees of impaired maximal lung functional capacity in early adulthood; and they potentially impact future long-term respiratory morbidity such as chronic asthma or chronic obstructive airway disease (COPD). We discuss possibilities to prevent or modify such early abnormal lung functional growth trajectories and the need for future studies and prevention programs.

Keywords: Review, Lung growth, Asthma, children, development, environmental factor

Literature search strategy

We searched PubMed for articles published in English from 2000 to 2019 with terms: “lung function/ trajectories/lung growth/air pollution/environmental tobacco smoke/” and associated terms. We identified relevant articles published before 2000 through a search via Google Scholar and through reviewing the articles and their relevant references cited. This was by no means an exhaustive search.

Acknowledgments

The authors thank Karine Bell (University Children's Hospital □asel) for her critical reading of the manuscript.

Funding This work was supported by the Swiss National Science Foundation grant 320030B_182871.

Competing interest The authors have no conflicts of interest to declare.

Contributions FD and UF conceived the study. FD drafted the manuscript; all authors have revised and approved the final manuscript.

Introduction

Asthma is one of the most common causes of morbidity and hospitalizations in childhood in the industrialized world. Over the past decades, there is increasing evidence that asthma is a complex and dynamic disease entity, whereby multiple environmental factors and multiple genetic polymorphisms determine the various phenotypes of asthma (e.g. (1)). Interestingly enough, most of these determinants or risk factors have a small effect size and it is likely that no single mechanism explains the complex development of asthma during childhood. There are also complex interactions between some of these factors. There is increasing evidence that early life risk factors play a critical role in the development of asthma in adulthood and even in chronic airway disease of the elderly. How exactly these early life factors influence the development of adult asthma disease is still unclear. It has been hypothesized that these early life factors modulate the properties of the respiratory system, including lung growth and development, immune development, as well as the inflammatory and repair response to oxidative stress and infections. These mechanisms are often referred to as 'early life programming' (2). This hypothesis is based on the assumption, that in early life, the various components of the respiratory system inherit a certain plasticity, which under normal conditions allows for adaptation to the child's environment. However, given that we do not yet know whether deviation from normal growth and development is preventable or treatable, we cannot yet say whether early life interventions have an asthma modifying effect.

It is likely, that inflammation, the oxidative stress response and lung growth are highly interlinked during early life, during a phase of rapid lung growth and development (3) (**Figure 1**). The relative impact of each contributing subsystem may be age-dependent and dynamic in nature. It is also still not very well known how early life inflammatory and immunological events affect the long-term development of the immune system into adulthood (**Figure 1**, right panel). However, there is increasing evidence that early life impairment of lung functional

growth could be central to the promotion of early life environmental risk factors and triggers leading to the development of asthma in adulthood (**Figure 1**, left panel). A significant number of longitudinal studies have suggested that recurrent respiratory symptoms are associated with poor lung function throughout life, (e.g. (4, 5)). Similarly, there is evidence suggesting that impaired lung function at birth and early life persists into adulthood, (e.g. (5, 6)), highlighting the importance of the vulnerable phase in early life ('window of opportunity'). However, the most convincing evidence has come from recent longitudinal studies showing the close relationship between respiratory senescence and early life respiratory morbidity and lung function impairment (3, 4, 6-9).

While we do not yet understand the complex network of interactions, which lead to impairment of lung functional development in utero, early life and later childhood, in this review, we aim to summarize extant literature on lung functional development and airway disease related lung functional trajectories.

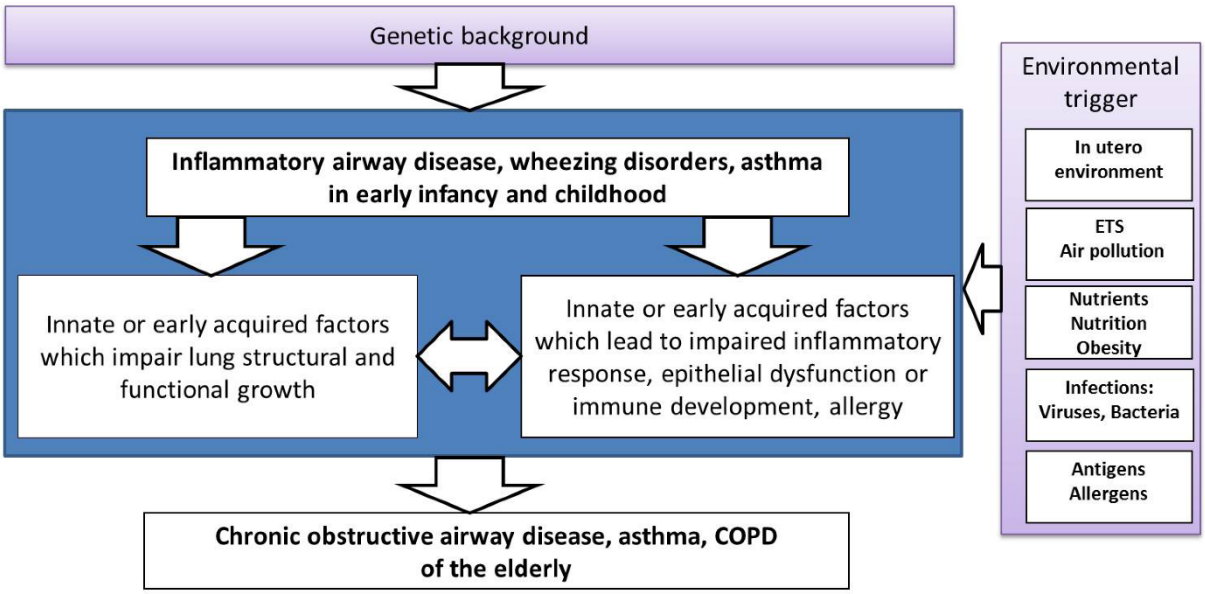

Figure 1. Simplified interaction model of intrinsic and extrinsic stimuli on lung growth and development of chronic respiratory disease in the elderly.

Mechanisms and factors impacting lung function trajectories

Lung development begins around the fifth gestational week and continues in stages into early adulthood, when lung function peaks (10). Lung function plateaus for a few years before it begins to slowly decline with lung aging (11). Several mechanisms are known to influence lung growth and development. A large variety of genetic and growth factors are known to influence alveolarization, airway branching and differentiation in utero (9). Some of these growth factors are modified by intrauterine mechanical forces (e.g. breathing movements), hydramnion, geometric constraints of the thoracic walls (e.g. diaphragmatic hernia, skeletal, obesity) as well as nutritional toxins and prematurity. A second category of factors are mediated through a process of chronic, low grade inflammation, e.g. induced by oxidative stress or recurrent infections or allergic reactions. Interestingly, there are interactions between these two entities. Some of the cytokines and chemokines (e.g. TGFβ) not only play a role in inflammatory and remodelling processes, but in utero play a role as natural growth factors (12, 13).

Influencing factors act differently on lung function trajectories during these distinct time windows (4, 5, 8, 14-17) and are dependent on the nature of influencing factors (**Figure 2**). There are several theoretical situations that model how lung growth becomes disturbed. An early life injury can theoretically be limited in time (e.g. premature birth, severe early life lung injury), or continuous throughout childhood (e.g. air pollution). After a limited window of insult, three theoretical situations could follow: The lung could (**a**) recover its growth capacity after cessation of the insult, (**b**) catch-up lost growth, or (**c**) growth velocity becomes altered by the insult. If the insult to the lung is continuous (**d**) (e.g. air pollution, constant oxidative stress, chronic low-grade inflammation), lung growth velocity can be sustainably impaired, whereby it is unclear whether such toxic stimuli need to reach a certain threshold or whether they act cumulatively. In this review, we therefore discuss genetic, prenatal, perinatal and postnatal environmental factors separately.

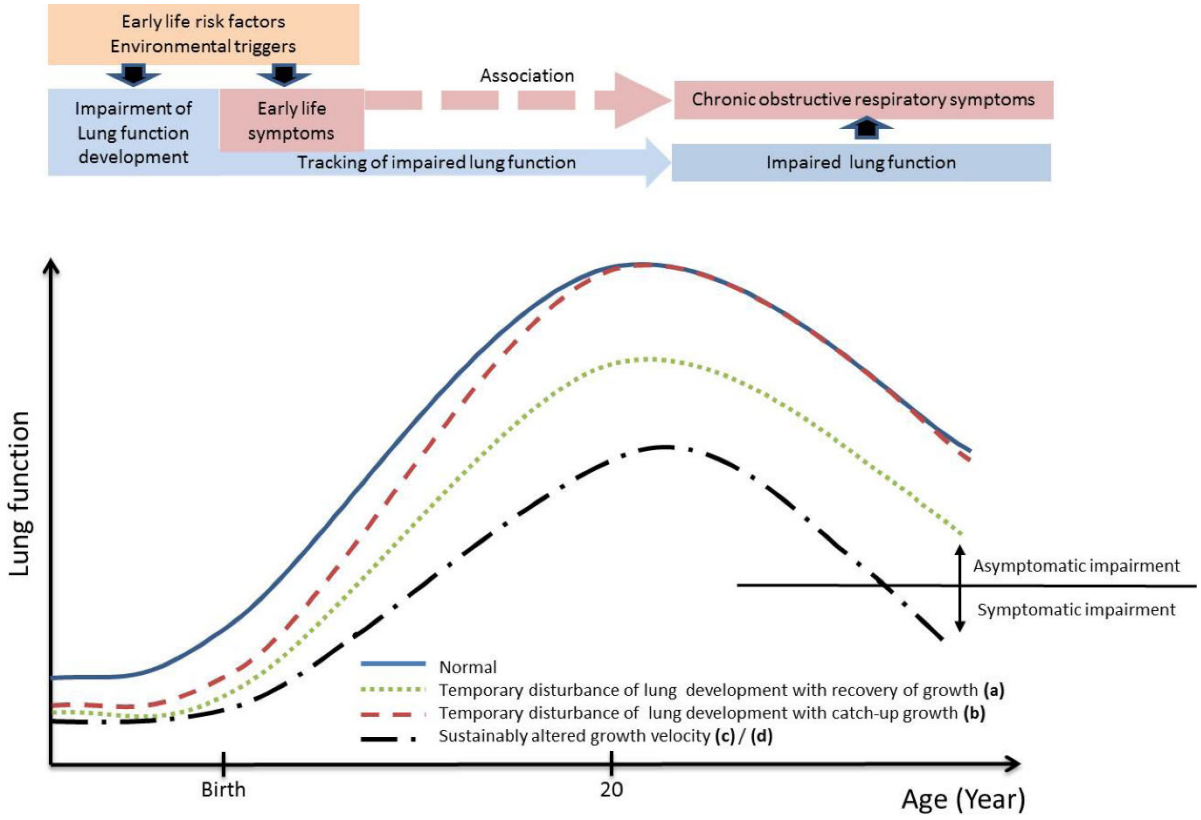

Figure 2. Schematic model of lung function trajectories throughout life. Temporary insult including antenatal injury, prematurity or early life severe lung injury may lead to lung function trajectories (**a**), (**b**) or (**c**). Continuous insults/effects including genetic deficits, gene environment susceptibility, chronic oxidative stress, air pollution, and environmental tobacco smoke can lead to impaired lung growth velocity (d**),** whereby it is unclear whether such toxic stimuli need to reach a certain threshold or whether they act cumulatively.

Hereditary and prenatal factors

Genetics

Genetic determinants of lung function have been found using targeted gene approaches as well as whole genome-wide association studies (GWASs) (18). Recent large-scale GWASs identified 97 genetic loci (19-23) in which variation was associated with lung function mainly

in adults. Some of these studies investigated the association of lung function with genetic variants in populations of children (19, 23). Interestingly, some of them also play a role in asthma and chronic obstructive pulmonary disease (COPD) (24-26). There was also overlap of some variants for lung function in adults and children, but the effect was lower in children. This lower effect in children could be an indication of the different effect size of the same locus on lung function in a different age group (from childhood to adulthood) which may be caused by gene-environment interaction. Researchers also focused on cross-sectional lung function measured at a single time-point, which may represent the combination of the peak lung function during early adulthood and subsequent age-related decline in lung function in the adult population (21). Indeed, genetic variants identified in cross-sectional GWASs of lung function showed no effect on the rate of lung function decline in adults (27, 28). GWASs assessment of the association between genetic variants and lung function trajectories in childhood is limited. McGeachie et al. (29) reported a significant association between a SNP located on chromosome 8 (rs4445257) and lung function trajectories in asthmatic children. The complex biological mechanism linked to this association, however, has not been extensively studied.

Genetic factors limiting fetal lung development may persist after birth. Further investigations of genetic determinants of lung function in early life, childhood and adulthood comparing children and those with airway disease (e.g. asthma) will help disentangle the biological mechanisms of fetal lung growth and later lung development, and related respiratory disease. Despite an increased number of studies on the genetic basis of lung function, genetic variants underlying lung function at birth or lung development trajectories from birth into adulthood are still unclear. The birth cohort studies exploring the association between genetics and lung function in infancy remain challenging and are often limited by sample size.

In experimental studies of the fetal lungs of mice, effects of genetic variants on lung development in the embryonic stage were shown (30). Therefore, the possibility that SNPs

could partly influence lung function and respiratory disease later in life through lung developmental changes exists.

Environmental factors

Prenatal exposure to environmental factors influencing lung development and growth might have deleterious long-term effects on lung health. In this section, we discuss the effects of environmental factors during pregnancy, such as air pollution, maternal smoking and nutritional factors.

Outdoor air pollution

Outdoor air pollutants, such as nitrogen dioxide (NO_2), particulate matter with a diameter smaller than 2.5µm or 10µm ($PM_{2.5}$ or PM_{10}), ozone (O_3), benzene and many others are known airway irritants causing inflammation. In epidemiological studies, pre- and postnatal exposure to these pollutants had a negative impact on lung function in childhood and adolescence (31-35). At school-age, several studies demonstrated that high levels of air pollution were not only associated with low lung function, but also with lower functional growth (35). Improving air quality in these cities reversed this impairment and decreasing air pollution levels either over time or by moving to a less polluted area decreased lung growth impairment (35, 36). Both findings suggest certain causality. At preschool age, fewer longitudinal studies are available. Latzin et al. (32) demonstrated that PM_{10} exposure during pregnancy is associated with altered lung function after birth. Examining the effect of air pollutants during different time windows, the most vulnerable phase seems to be during pregnancy and early infancy, during lung maturation and growth. During these time intervals, low to moderate levels of air pollution already impact upon lung function in childhood (31-33). However, the effect sizes of each of these pollutants are small. A recent exposomics study (37) showed no significant effect of toxic environmental substances on lung functional growth in preschool children, likely due to these

small effect sizes. While the effect sizes are small for the individual, for the population at large, even small effects can become a significant health burden (e.g. (38)). Nonetheless, exact mechanisms explaining the effects of air pollutants on lung growth and function are not known. Current evidence is consistent with lung functional growth trajectory type (a) (Figure 2), with today's infants exposed to air pollution potentially experiencing long-term effects of respiratory morbidity into old age (39).

Studies in more susceptible subgroups, such as asthmatics, preterm-born children or boys are needed, and further air pollution reduction measures are necessary to ameliorate the long-term negative effects on health.

Maternal smoking and environmental tobacco smoke

Exposure to environmental tobacco smoke (ETS) pre- and postnatally has been negatively correlated with lung function at school-age and adolescence (40-42). Maternal smoking during pregnancy in particular seems to have the strongest and most persistent effect on lung function up to adulthood with dose-dependent effects (43-46). Mechanisms related to tobacco toxicity are manifold; one interesting mechanism relates to the methylation of genes, important in the detoxification process of tobacco smoke components. Joubert et al. (47) identified DNA-sites in cord blood of 1062 newborns, which were methylated following maternal smoking during pregnancy. These genes (AHRR, CYP1A1) are known to impact the detoxification of tobacco smoke components. They were able to show a clear interaction between genetic background and environmental stimuli.

Additionally, it has been suggested by animal studies that maternal smoking during pregnancy modifies the epithelial-mesenchymal interaction of the developing alveolus and therefore increases the production of myofibroblasts in airways (48). Further experimental studies examining the effects of maternal smoking on offspring showed structural lung impairment,

hyperplasia of neuro-endocrine cells and diminished lung growth (49, 50), resulting in different structure and function of the lungs. Dai and colleagues (41) contributed to the hypothesis that maternal smoking during pregnancy might affect airway and alveolar growth and therefore lead to impaired lung function in adolescents. They showed within a prospective birth cohort of 620 children that mean FEV_1 growth was reduced by 154 ml between 12 and 18 years, when parents smoked during perinatal time windows, compared to children of non-smoking parents.

Literature on the effect of passive exposure to tobacco during childhood on lung functional growth is heterogeneous. Some evidence suggests a joint effect of parental and active smoking on lung function in young adults (44). Nonetheless, we must consider that prenatal ETS and active maternal smoking might lead to low birth weight and prematurity (51), which both independently influence lung function and susceptibility of the lungs (52).

Maternal nutrition and intrauterine growth retardation

Maternal nutrition and a healthy and balanced diet, eating patterns and supplementation with vitamins and their associations with the respiratory health of the offspring have been the subject of current research. Most studies examining the effects of the maternal diet on the lung function of the child found no associations between the two (53-56), whereas malnutrition during pregnancy has been associated with an increased prevalence of obstructive airway disease in adults (57). However, results from recent experimental studies in mice proved inconclusive on how a mother's high fat diet impacts the developing lungs of her child. Some studies described negative effects of a high fat diet, resulting in decreased pulmonary resistance, elastance, compliance and decreased lung-weight-to-body-weight ratios (58); while other studies showed increased lung growth with higher volumes related to bigger body length (59).

In examining supplementation with specific vitamins during pregnancy, no associations between vitamins B12, folate, vitamin D or E, or probiotics and respiratory health of the

offspring were seen (53-56). Nonetheless, vitamin A-supplementation in chronically undernourished Nepalese women increased FEV_1 by 46 ml and FVC by 46 ml in their children aged 11 years (60). Appropriate levels of supplements such as Vitamin A, D and E are instrumental in lung regeneration after chronic lung disease after premature birth (61, 62). There is no convincing evidence that higher doses of Vitamins A, D and E support catch-up growth in the lung of formerly premature infants. Diets with fish oil derived fatty acids had no effect on lung function in the offspring (63).

Further factors influencing lung function trajectories include intrauterine growth retardation (IUGR). Reasons for IUGR are various. They differ from genetic, placental, fetal, and maternal factors or a combination of any or all of these (64). Two maternal conditions previously mentioned are: maternal active smoking and malnutrition. Further, IUGR may have many adverse implications later on, one of which is premature birth, leading to impaired lung function in the offspring (see 2.2.1.) (14, 52) and therefore reduced peak lung function in early adulthood.

Perinatal factors

Prematurity, bronchopulmonary dysplasia and Cesarean Section

The negative impact of premature birth on later respiratory health is becoming better understood due to the increasing rates worldwide of adults who were born prematurely. Premature birth interrupts natural lung development and leads to incompletely developed alveoli, inefficient gas exchange and immature surfactant system (65). The resulting airways are smaller and less compliant. Long-term follow-up of preterm-born children strongly correlated with low lung function from early infancy into adulthood (6, 66-68). Prematurity itself leads to impaired lung growth and function, but so too do the consequences of premature birth and low birth weight

(68, 69). We will focus on these aspects in "*Catch-up weight gain, obesity, and breastfeeding*" section.

Another important aspect of premature birth is bronchopulmonary dysplasia (BPD, or chronic lung disease of infancy CLDI), which occurs in premature born infants, especially in those with respiratory distress requiring ventilation and oxygen supplementation, resulting in impaired alveolar, airway and vascular growth (66, 70). In summary, children with BPD showed even more lung function impairment in early infancy and later follow-ups than did preterm infants without BPD (67). It remains unclear, whether infants born prematurely have sustainably impaired growth (c) (71) (Figure 2) or limited growth deficit (a or b). Some novel functional evidence suggests the continuation of alveolarization until school-age (72).

Further perinatal impacts, such as delivery mode and its effects on lung function shortly after birth have been extensively published (73). However, studies on the effects of delivery mode on lung function show no evidence of long-term lung functional deficits.

Catch-up weight gain, obesity and breastfeeding

Rapid weight gain after prematurity or low birth weight has been shown in several studies to be an independent risk factor for adverse respiratory outcomes (14, 68). In a meta-analysis of 24938 children from 24 different cohorts, greater weight gain during infancy was significantly associated with higher FEV_1 but lower FEV_1/FVC-ratio and FEF_{75} in childhood (68). These findings corroborate associations found between obesity and decreased lung function (69, 74-76). The mechanisms behind these associations are as yet unclear, but genetic predisposition, prenatal exposures and immunomodulation as well as mechanical changes are discussed (76, 77). A recent dietary interventional study in obese adolescent patients showed a recovery from lung functional impairment during growth (78), indicating that obesity related lung function impairment has the potential to be reversed (trajectory model case c).

The effects of breastfeeding on respiratory symptoms, particularly in the first six months of life (e.g. (79)), are evident, however the effect of breastfeeding on subsequent lung function is discussed controversially. Longer or exclusive breastfeeding may have positive effects on lung function at school-age (80, 81). These effects are mainly seen in children of asthmatic mothers (81). The effect of breastfeeding on lung functional growth is not understood, but seems to be dependent on the underlying asthma disease process (81).

Postnatal factors

Lower respiratory tract infections

The effects of early viral infections of the respiratory tract, especially with human rhinovirus (HRV) and respiratory syncytial virus (RSV), on lung functional growth are subject to intense research. It is well known, that infants with severe bronchiolitis, especially when triggered by RSV or HRV, are at risk of asthma and impaired respiratory function later in childhood (5, 82-86) leading to decreased maximally attained lung function values in early adulthood. The mechanism behind the association of viral infections with impaired respiratory outcomes is not fully known. It is assumed that lung functional abnormalities are the result of virus-induced inflammatory processes of the airways. Hypotheses include a two-hit theory, where either viral infections raise susceptibility to a second and succeeding stimulus or are the result of a genetic or immune susceptibility or pre-existing lung function deficit (87-90). Evidence supporting the theory of genetic predisposition to viral infections are the identification of genetic variants, such as the gene CDHR3 on chromosome 7q22, which is known as the asthma susceptibility gene encoding for the RSV receptor (91, 92). Additionally, genetic variants of the 17q21 locus are associated with HRV-induced wheezing and infections in early childhood and later respiratory morbidity (93, 94). Assumptions supporting the hypothesis of viral infections raising the susceptibility for further stimuli include immune response modulation or direct airway damage

through the viral infection (90). Alternatively, it is possible, that pre-existing functional abnormalities predispose the lungs to more virally induced airway inflammation in infancy (95) or that some host factors are responsible for both airway development and response to viral infections. The hen and egg conundrum remain unresolved.

Microbiome

Environmental microbiota and the microbiome in the respiratory tract play an important role in the development of asthma. Current research is inconclusive as to whether colonization with certain bacteria lead to an inflammation response, and therefore to the development of asthma phenotypes, or if predisposed children or children with immunological deficits are prone to certain bacterial colonization and promote a niche for their growth (96, 97). Studies on the effects of the microbiome on lung functional growth and trajectories are lacking.

Studies promoting the notion of predisposition to bacterial colonization include Hales et al. publications (98-100). They could show that asthmatic infants and school-aged children had impaired humoral immunity against common bacteria leading to respiratory disease. In particular, these children had low specific IgG1 antibody production. Further, Bisgaard et al. (101) showed, that colonization with S. pneumoniae, H. influenza, M. catarrhalis, or more than one microorganism at a time in infants preceded asthma phenotypes at five years of age. Hilty et al. (102) obtained a different microbial diversity in bronchoalveolar lavages (BAL) and airway brushings in healthy or asthmatic children. BALs from asthmatic children showed more H. influenzae and other Proteobacteria. Due to the study design, disentangling the sequence of colonization and respiratory disease was impossible. No study has thus shown an asthma independent effect of microbiome on lung functional development.

Allergic sensitization

There is broad epidemiologic evidence of early sensitization and subsequent asthma in childhood and asthma related to impaired lung function later in life (5, 103-107). Interactions between environmental factors, such as the previously mentioned viral infections, pollutants and endotoxins and allergic disease have been widely shown (107, 108).

In the absence of the asthma disease process, the effect of allergic sensitization on lung function development alone is less evident. In one cohort of 1314 children, sensitization to perennial allergens in the first three years of life was associated with subsequent decreased lung function at school-age (107). This effect was augmented when exposure levels to allergens were high. Nonetheless, sensitization later in life, or sensitization to seasonal allergens, had only a weak-to-no effect on lung function. These findings suggest the significance of early sensitization. However, there is no clear evidence that early sensitization leads to respiratory disease and impaired lung function in adulthood.

Interventional studies examining the effects of allergen avoidance were able to show a significantly better sRaw in three-year-old children, but were unable to find any protective long-term effect on lung function (109). Additionally, studies investigating the impact of allergen immunotherapy on lung function showed no effects on FEV_1 and FVC, but small effects on MEF_{25-75} (110). Nonetheless, long-term observational studies investigating the effect of allergic sensitization on lung function in adulthood are missing.

Premorbid impairment of lung functional growth and subsequent respiratory disease and asthma

It is long known that impairment of lung function and respiratory morbidity are associated throughout life (3, 5, 9, 15, 86, 111). However, what is less known is whether premorbid lung function impairment is an independent risk factor for subsequent respiratory morbidity and

asthma. Initial evidence comes from birth cohort studies correlating infant lung function and subsequent wheezing disorders at preschool age (6, 106, 112, 113). Furthermore, a recent multi-center study suggests that the associations of gestational age, birth weight, and infant weight gain with childhood asthma are at least partly explained by adaptions in airway caliber (68). Similarly, there is strong evidence that comes from survivors of prematurity. Proietti et al. demonstrated that postnatal lung abnormalities were associated with respiratory morbidity in the first year of life (114). A recent meta-analysis showed that infants with chronic lung disease of prematurity showed impairment of lung function into early adulthood (115). Antenatal tobacco smoke exposure was associated with impaired infant lung function (116) and subsequent asthma (42). Similarly, impaired premorbid neonatal lung function enhanced the effect size of air pollution on respiratory symptoms in the first year of life. In summary, there is increasing evidence that premorbid impairments of lung functional growth mediated by a variety of early life risk factors are associated with subsequent respiratory morbidity and asthma, emphasizing the instrumental role of lung functional growth on asthma development.

Conclusion

Impairment of lung function in early life is one mechanism that explains some of the sustained effects of early life environmental risk factors on long term respiratory morbidity even in senescence. The analysis of the risks and mechanisms of early life factors influencing lung function trajectories are important to our understanding of the long-term effects of genetics and environmental stimuli on later respiratory morbidity and mortality. During pregnancy and the first year of life there is a phase of rapid lung growth, and the development and adaptation of the anti-oxidative, inflammatory and immune system are still ongoing. This results in an increased plasticity of the respiratory system, leading to increased vulnerability to environmental stimuli. Within this phase genetic and environmental factors can lead to impaired lung growth or secondarily through inflammation and consequent remodelling

processes to structural changes, resulting in impaired lung function in early adulthood. There is convincing evidence that perinatal factors such as prematurity and chronic lung disease in infancy result in impaired lung development, likely resulting in a form of chronic lung disease in adulthood. There is increasing evidence that premorbid impairments of lung functional growth may be an independent risk factor for asthma development.

Preventative therapeutic measures had a significant impact on the prevalence of long-term lung functional sequelae in this disease group. The effect of smoking during pregnancy on impairment of lung functional trajectories has been shown in several studies. Here, genetic predisposition and epigenetic phenomena modulate the effects. Preventative measures likely reduce the negative long-term effects on lung growth. The effect of environmental air pollution on lung function and lung functional growth has been shown at school-age, whereas evidence in infants and pre-schoolers is emerging. Again, preventative measures and the reduction of air pollution levels have been shown to be effective in reducing these effects. Nutrition, obesity, and catch-up growth show some effect, again preventable by dietary measures. Whereas consistent evidence of the impact of nutritional supplements (e.g. Vitamin A, D, E, fatty acids) on lung functional growth is outstanding. There is controversial evidence whether breastfeeding is associated with subsequent lung functional growth in healthy children and whether probiotics influence the microbiome.

Recurrent viral infections in early infancy are associated with long-term respiratory morbidity and subsequent impairment of lung function. Causality and reverse causation are however outstanding questions. Little is known about the dynamics of lung functional impairment. It is unclear whether environmental insult over a limited time period during early life leads to temporary impairment of lung development, or sustained growth velocity impairment. It has not yet been explored whether catch-up growth is spontaneously possible after cessation of the insult, or whether catch-up growth and lung regeneration can be induced in the future. The

heterogeneity of the evidence suggests that the latter option may depend on time, strength and type of the environmental insult as well as predisposing host factors.

In any case, even small effect size alterations in lung functional trajectory by exposure to environmental toxicity in today's infants, could lead to long-term respiratory morbidity in adults and the elderly. Given that the entire population is exposed, future research needs to look into the potentially significant future healthcare burden and explore preventative and therapeutic options to avoid such a development.

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4.2 Article II - Increased impact of air pollution on lung function in preterm vs. term infants: the BILD study

Fabienne Decrue, Olga Gorlanova, Yasmin Salem, Danielle Vienneau, Kees de Hoogh, Amanda Gisler, Jakob Usemann, Insa Korten, Uri Nahum, Pablo Sinues, Sven Schulzke, Oliver Fuchs, Philipp Latzin, Martin Röösli and Urs Frey on behalf of the BILD study group

American Journal of Respiratory and Critical Care Medicine (AJRCCM)

In Revision

Increased impact of air pollution on lung function in preterm vs. term infants: the BILD study

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Contributions UF, MR, PL conceived the study. FD, UF performed data analysis and manuscript writing. Measurements were recruited, recorded or analyzed by SS, FD, OG, JU, IK and YS. Statistical analyses were done by FD, OG and MR. DV and KdH modeled the air pollution data. PS provided biochemical expertise. All coauthors have critically reviewed the manuscript and have seen and approved the final manuscript.

Sources of support: This study was supported by the Swiss National Science Foundation grant 320030_163311.

Air pollution and postnatal lung function in preterm infants

Descriptor number: 14.3

Total word count: text 3460/3500

Today, approximately 10% of all infants are born prematurely, pre-existing vulnerability means that their lungs are potentially more susceptible to environmental air. This is the first study

showing that the effect size of low-to-moderate levels of air pollution exposure during pregnancy on postnatal lung function is significantly higher than in healthy term infants of similar postconceptional age of 44 weeks. The effect was best detectable in moderate to late preterm infants and these findings are highly relevant, since recent evidence indicates that early-life lung functional impairment and its lifespan developmental tracking may be an important early-life risk factor for chronic obstructive lung disease in adulthood.

This article has an online data supplement, which is accessible from this issue's table of contents online at www.atsjournals.org

Abstract

Rationale Infants born prematurely have an impaired capacity to deal with oxidative stress shortly after birth.

Objectives We hypothesize that the relative impact of pre- and postnatal exposure to air pollution on postnatal lung function is higher in preterm than in term infants.

Methods In the prospective Basel-Bern Infant Lung Development (BILD) cohort of 771 infants, 254 preterm and 517 term, we investigated the associations of pre- and postnatal air pollution levels for particulate matter with a diameter < 10 µm (PM_{10}) and nitrogen dioxide (NO_2) with postnatal lung function (tidal breathing flow volume loops) at 44 weeks postconceptional age and exhaled surrogate markers of inflammation and oxidative stress response (fraction of exhaled nitric oxide (FeNO)). Multilevel mixed-effects linear regression was used and adjusted for known confounders and study center.

Measurements and Main Results Significant negative associations of PM_{10} during the second trimester of pregnancy with lung function and FeNO were found in term and preterm infants. Importantly, we observed stronger associations in moderate-late preterm infants (32 – 37 weeks), with an increase of [184.9 (79.1 – 290.7) mL/min] minute ventilation per 10 µg/m^3 increase in PM_{10} ($p_{\text{prematurity} \times \text{PM10 interaction}} = 0.04$). Associations of air pollution and FeNO differed significantly between preterm and term infants ($p_{\text{prematurity} \times \text{PM10 interaction}} = 0.006$).

Conclusion Preterm infants showed significant higher susceptibility even to low-to-moderate air pollution exposure during pregnancy than term infants, leading to increased impairment of postnatal lung function. FeNO results further elucidate differences in inflammatory/oxidative stress response comparing preterms to terms.

Abstract word count: 248/250

Keywords: Particulate Matter; Premature Infants; Prenatal Injuries; Longitudinal Studies, COPD

Introduction

Adverse effects of high air pollution levels on respiratory health in children and adults have been shown (1-4), whereas low-to-moderate air pollution levels and their impact on lung function and respiratory symptoms in infants and children are a matter of ongoing discussion (5-8). Most recent studies indicate the necessity of further air pollution reductions, since clear associations between low-to-moderate air pollution and impaired lung function in preschool and school-aged children have been found (5-7). These studies further indicate the need for precise analysis and large prospective cohort studies to assess the impact on more vulnerable subgroups, such as asthmatics, preterm-born children or boys (9, 10). Children starting with impaired lung function are especially at risk for later respiratory morbidity and mortality (11, 12).

Survival rates of preterm infants are increasing throughout high-income countries (13) due to substantial progress in perinatal care. However, preterm infants are particularly known to have impaired capacity to deal with oxidative stress shortly after birth, which reflects in higher levels of inflammatory and oxidative stress markers, such as FeNO (14-17). Air pollution, a known oxidative stressor to the airways and lungs (17) and its effects on lung function in this vulnerable subgroup, should therefore be examined. We have previously shown that prenatal air pollution exposure affects lung function in healthy term born infants (18). We hypothesize that exposure to pre- and postnatal air pollution has a stronger effect on lung function in preterm infants due to increased vulnerability.

In a multivariable statistical approach after adjustment for known covariates of prematurity, we aimed to determine whether pre- and postnatal exposure to particulate matter with an aerodynamic diameter of < 10 μm (PM_{10}) and nitrogen dioxide (NO_2) affects lung function at 44 weeks of postconceptional age (PCA) in preterm more prominently than in term infants. We

additionally stratified the analysis in groups of preterm infants born prior to or after 32 weeks of gestational age (GA), knowing that in the earlier group lung functional abnormalities may be more affected by mechanical ventilation and chronic lung disease (CLD) of infancy (19). Some of the study participants (207 healthy term infants) from this study have been previously included in a study by Latzin et al. (18).

Methods

Study design

The prospective Basel-Bern Infant Lung Development (BILD) cohort (https://www.bild-cohort.ch/) comprises unselected neonates recruited since 1999 in the region of Bern and since 2012 in Basel, Switzerland (20). Prematurity was defined as GA at birth < 37 weeks (21). The ethics committees from Basel and Bern approved the study and written consent was obtained.

Air pollution exposure

Time-space hybrid models were used to calculate individual mean exposure by pollutant for different exposure windows: 1st, 2nd, 3rd trimester and postnatal. Weighted averages were calculated for children who changed their residential address or were hospitalized (mainly preterm infants in the 3$_{rd}$ trimester of pregnancy). The detailed NO_2 model is described in (22), and more simplified PM_{10} model in (5) with further detail in the online supplement.

Lung function outcomes

Pulmonary function was performed at 44 weeks of PCA using Exhalyzer D (EcoMedics, Duernten, Switzerland) according to current ERS/ATS guidelines (23) and procedures for analysis as described previously (18).

We investigated respiratory rate (RR), mean tidal volume (V_T) and minute ventilation (V_E). Ratio of time to peak tidal expiratory flow (PTEF) and expiratory time (t_{PTEF}/t_E) were used to describe TBFVL shapes. Further, fraction of exhaled nitric oxide (FeNO) was measured as a marker for airway inflammatory response and oxidative stress (17).

Covariates

After a stepwise backward regression and considering previous research (18, 19), we adjusted the regression model for the following covariates: sex, weight at lung function measurement, season and postconceptional age at lung function measurement, GA at birth, maternal smoking during pregnancy, days of supplementary oxygen, days of mechanical ventilation (defined as days of continuous positive airway pressure (cPAP) or intubation), maternal asthma (defined as self-reported or doctor-diagnosed). For FeNO analyses we additionally adjusted for V_E (18).

Statistical analysis

We performed a multilevel mixed-effects linear regression for term and preterm infants separately, adjusted for the above-mentioned covariates and corrected for clustering on center level (Basel vs. Bern). Inspection of the outcome variables suggested normal distribution.

Since we previously showed that lung functional abnormalities can be the result of prematurity and perinatal insults, particularly in extremely and very early preterm infants (19), we conducted a stratified analysis according to GA at birth into four clinically relevant groups: extremely (< 28 weeks of GA), very early (29 – 31 weeks of GA) or moderate to late (32 – 37 weeks of GA) preterm and term infants. We tested the presence of interaction within term and moderate to late preterm infants adding the interaction terms between exposure to air pollution and prematurity.

We performed sensitivity analyses in respect to CLD classification, sex as modifier (9, 10), education status of the mother as a confounder (due to many missing values) (9, 10) and within the FeNO analyses additionally adjusted for exposure to caffeine (24). We also checked differences of the main model with and without length at measurement date (25).

More detailed information on methods and power calculation are given in the online supplement.

Results

From 1999 to 2017, 1100 infants were recruited. We had complete data available for 890 (81%) children on pulmonary function testing at 44 weeks of PCA, modeled air pollution exposure and potential risk factors (**Figure E1**). Of these, data from 775 infants (87%) passed quality control of lung function measurements according to ERS/ATS guidelines (23), and after excluding outliers (see online supplement) in the moderate to late preterm infants with severe asphyxia a total of 771 infants were included for the main analyses. Anthropometric information and potential confounders are depicted in **Table 1**. Of the 771 infants, 517 (67%) were term and 254 (33%) were preterm infants. The preterm population consisted of 65 (26%) extremely, 90 (35%) very early and 99 (39%) moderate to late preterm infants. Overall, 60 infants (8%)

were children from actively smoking mothers, 85 (11%) had asthmatic mothers and 108 (14%) had a diagnosis of CLD. Comparison between participants lost to follow-up and included participants is given in **Table E1**. As most infants lost to follow-up or excluded were preterm infants, the excluded population differed significantly from the population included in the study. Main differences were in GA at birth, maternal smoking during pregnancy, diagnosis of maternal asthma and duration of oxygen supplementation.

Lung function data at 44 weeks of PCA, completed between 16th March 2000 and 12th January 2018 are shown in **Table 2**. Lung function values differed between subgroups with the highest values of V_E, V_T and FeNO in moderate to late (32 – 36 weeks of GA) preterm infants, when not adjusted for weight at lung function measurement. Distribution of duration of mechanical ventilation and of supplementary oxygen is shown for preterm infants in **Figure E2**.

The distribution of air pollution in the study region over the 18-year study period is depicted in **Figure 1**. Air pollution concentrations for each pollutant during the second trimester of pregnancy and postnatal time period are shown in **Table 3** and for all exposure windows in **Table E2**.

Association of air pollution with lung function at 44 weeks of postconceptional age (tidal breathing flow volume loops)

The association between prenatal and postnatal air pollution with lung function in early infancy is given in Table 4 and for all exposure windows in **Table E3**. Adverse associations of exposure to air pollution on lung function were highly dependent on the time window of exposure and most prominent when occurring during the second trimester of pregnancy. Moreover, when comparing preterm children, divided into three clinically used groups, to term born children, a clear differentiation was seen between moderate to late preterm (32 – 37 weeks of GA) and

term infants and within these two groups a significant interaction of prematurity and air pollution (p-value for interaction = 0.040). However, no association of air pollutants on lung function was seen in the subgroup of extremely (< 28 weeks of GA) and very early (29 – 31 weeks of GA) preterm infants (data not shown).

Within the term group V_E increased by [coefficient (β) (95% confidence intervals (CIs))] [75.27 (19.74–130.79) mL/min] per each 10 µg/m^3 increase in PM_{10} during the second trimester, whereas in all preterm infants, V_E increased by [88.62 (18.57–158.68) mL/min] and within the moderate to late preterm infants by [184.89 (79.12–290.66) mL/min] (**Figure 2**). Interactions of prematurity and PM_{10} during the second trimester of pregnancy for V_E within the term and moderate to late preterm infants were significant (p = 0.040). In the stratified analysis, similar associations were found between PM_{10} during the second trimester and V_T in the term, all preterm and moderate to late preterm infants with increases of [1.22 (0.18–2.26) mL], [1.33 (-0.18–2.85) mL] and [3.82 (1.57–6.08) mL], respectively.

The association of postnatal air pollution with pulmonary function is given in **Table 4**. In term infants, associations of postnatal PM_{10} exposure with RR and V_T were observed [2.12 (0.65–3.58) /min], and [-0.92 (-1.68– -0.17) mL], respectively. Further, V_T decreased by [-1.04 (-1.83– -0.25) mL] per increase of 10 µg/m^3 in NO_2. No significant associations between postnatal exposure to air pollutants and lung function were seen in the entire group of preterm infants, nor in the strata.

Fraction of exhaled nitric oxide (FeNO)

In the preterm group, FeNO increased significantly by [2.84 (0.84–4.84) ppb] and in the moderate to late preterm infants by [3.38 (-0.08–6.83) ppb] per each 10 µg/m^3 increase in PM_{10}

during the second trimester. However, in term infants, no clear associations between prenatal exposure to air pollution and FeNO were found.

Postnatal exposure to PM_{10} and NO_2 showed no association with FeNO either in term or preterm infants (**Table 4**).

Sensitivity analyses and results are described in the online supplement.

Discussion

Main findings

We showed significantly impaired lung function values with increasing air pollution exposure during the second trimester of pregnancy in term and preterm infants. However, in comparison to the age-matched term infants, the effect of air pollution on alterations of postnatal lung function in the preterm infants was significantly larger. The effect was best detectable in the subgroup of moderate to late preterm infants (32 – 37 weeks of GA). The stratified analysis was supported by complementary statistical analysis showing significant interaction of prematurity and PM_{10} exposure in the latter group. Statistical findings remained robust after extensive sensitivity analysis accounting for concomitant perinatal and biometric factors. This supports the suspected hypothesis of increased vulnerability of premature-born infants due to pre-existing conditions (26). In infants born < 32 weeks, this increased effect size was not detectable, likely due to the dominating influence of immaturity and perinatal treatment effects on lung function. Nevertheless, to the best of our knowledge this is the first study examining the effects of low-to-moderate air pollution levels during pregnancy and early infancy on infant lung function in preterm infants. Interestingly, across all infants, air pollution showed the strongest association with lung function impairment when occurring during the second trimester

of pregnancy. These findings are supported by the physiological development of the lung, where the second trimester is the most important and most susceptible phase (27).

Comparison with literature

Literature on the effects of outdoor air pollution on lung function in infancy is scarce. A study from the region of Grenoble (France) has shown associations between increasing prenatal NO_2 exposure levels and postnatal functional residual capacity (FRC) at 6 – 10 weeks of age (28) in healthy term infants. These results were not in line with the findings previously published from the BILD cohort by Latzin et al. (18), where no clear associations of prenatal air pollution exposure with FRC were shown. However, Latzin et al. (18) showed significant associations between prenatal PM_{10} exposure and increases in V_E at 44 weeks of GA in around 241 healthy term children (207 infants are part of the healthy control group in this study). These effects are qualitatively similar but around three-times higher than the effect sizes seen in the term population in this current extended study. We assume that due to the larger sample size of 771 infants, more precise modeling (22), and mainly decreasing air pollution levels over time. As a novel finding, effect sizes were significantly larger in preterm vs. term infants, particularly in moderate to late preterm infants. Interestingly, no associations of prenatal NO_2 exposure with infant lung function were seen in the current study. We presume, that this is due to the relative level of exposure. Mean NO_2 levels were on average only half of the World Health Organization annual guideline limits of 40 $\mu g/m^3$, whereas mean PM_{10} levels were around the WHO threshold levels of 20 $\mu g/m^3$ (29), with individual exposures above guideline thresholds. Colleagues from the GRAPHS trial, however, focused on the effects that indoor air pollution, in particular carbon monoxide (CO), might have on infant lung function in nearly 400 infants at 30-days of age (9). Within their cohort V_E and RR increased significantly with increasing levels of CO.

Air pollution also has an impact on biomarkers associated with airway inflammation and/or oxidative stress. In healthy term infants, Latzin et al. (18) found negative associations of air pollution on FeNO levels. In the current study, we found a positive association between PM_{10} and NO_2 levels during the second trimester, increasing FeNO values in preterm and moderate to late preterm infants and a no clear association in term infants. These findings reflect a different inflammatory context or oxidative stress response to pollutants in preterm infants in comparison to term infants. We hypothesize, that in preterm infants there is a persisting underlying process, which is consistent with the observations of Filippone et al. (30) or Teig et al. (31), who show that even in adolescent children after prematurity, oxidative stress response is different. Alternatively, one could speculate differences in underlying inflammatory response in accordance with other observations e.g. of Paunescu et al. (32) who show increased FeNO values after higher black carbon exposures only in children with persistent respiratory symptoms. On a cellular level, inflammation in preterm infants after birth could be related to persistent neutrophil activity and oxidative stress, which have both previously been identified as affecting FeNO levels (17, 33). Both promote the oxygenation of nitrogen oxide (NO) to soluble NO metabolites, which have been shown in increased concentrations in plasma and bronchoalveolar lavage (BAL) fluids during the first month of life (34, 35). However, after peaking during this time window, the neutrophil activity seems to decrease thereafter (33). It is unclear how long this process persists after term. We suggest that in future studies, along with FeNO, additional, well-established markers of oxidative stress such as 4-Hydroxy-2-nonenal (36) could potentially be measured non-invasively in exhaled breath of infants by modern mass spectrometric techniques (37). Such, metabolic analyses may allow for a more comprehensive characterization of the relationship between air pollution and its detrimental effects on the respiratory system.

We and others have shown that infant lung function is most severely altered in very immature infants with chronic lung disease of infancy (19, 38, 39). Furthermore, in our cohort, extreme and very early preterm infants did not show any clear associations of air pollution on lung function. Although we carefully adjusted for known confounders and covariates, and performed extensive sensitivity analysis, in the severely ill groups (GA < 32 weeks) the relatively small association between air pollution and lung function may have been undetectable. We assume, that the lack of association in these populations is due to many other dominant developmental and perinatal therapeutic factors which may outweigh the small effects that air pollution might have on overall lung mechanics (19, 40) and ventilatory needs. When discriminating between term and preterm infants born before and after 32 weeks of GA, we were able to show that moderate to late preterm infants showed an increased effect of air pollution on lung function in comparison to term infants.

Clinical relevance

Harmful effects of air pollution on the developing lung, especially during time windows of fast growth and when exposed to high air pollution levels have been explored widely (6, 41, 42). As a novelty and proof of principle, we demonstrated that groups of infants with pre-existing vulnerability, such as preterm infants, are more susceptible to detrimental effects of air pollution. Furthermore, the effect of air pollution may depend on the window of exposure during rapid lung development (27, 42), e.g. during the second trimester of pregnancy. The results additionally support the hypothesis of susceptibility of preterm infants to oxidative stress and therefore the necessity to protect them. The findings also have a highly relevant impact on population health. Recent evidence has shown that early-life lung function impairment may track through the lifespan, and poor lung functional trajectories from childhood to late senescence are important early-life risk factors for chronic respiratory disease in adults (3, 43-

45). Prevention in early-life is therefore one of the most important factors to avert development of chronic respiratory disease (e.g. COPD) and long-term negative health effects in adulthood, and subsequently senescence (3, 12, 43-47). Even though the effect size on an individual level might be small, the effect on a population basis (attributable risk) is of major concern (48). Today, approximately 10% of all infants are born prematurely (13). We best see pollution effects in the epidemiologically largest group of moderate to late preterm infants (32 – 37 weeks of GA) and, in some areas of the world, air pollution levels might be even higher (29).

Strengths and limitations

A challenge in this study is the comparison of the exposure modeling. The second trimester (a priori chosen exposure window) offered the best comparability and is known for its importance in respect to lung development (27, 42). In our cohort, mothers of preterm infants were often hospitalized on prenatal obstetrics wards several days before giving birth. Term infants left hospital after days, whereas preterm infants were typically hospitalized for weeks after birth. Thus, the calculated and weighted outdoor air pollution exposures for the perinatal (mainly third trimester and postnatal) time periods probably overestimate the true level of air pollution these children were exposed to inside the hospital. This may explain why we did not find any associations between air pollution exposure and lung function during those time periods in the preterm infants.

One clear strength of our study was the assessment of air pollution during pregnancy and after birth, enabling us to study different exposure windows. To our knowledge, this is the first study to assess low-to-moderate levels of pre- and postnatal air pollution and association with infant lung function in preterm infants. Further, we have included children between 1999 and 2017 in this study, which resulted in a fairly large sample size of 771 infants. Due to the prospective

design of the BILD cohort, we additionally had detailed data on potential confounders and effect modifiers, gathered from questionnaires and medical records.

However, the estimation model of air pollutant levels does not include satellite data, due to the early recruitment of participants (1999) when modeling techniques for satellite data were not yet available. Therefore, we used well-established and previously validated PM_{10} exposure values. Although $PM_{2.5}$ effects may biologically be more detrimental, there is a high collinearity between $PM_{2.5}$ and PM_{10} exposure.

Due to a limited number of high-risk children (e.g. children from asthmatic or smoking mothers), we could not perform additional subgroup analyses. Therefore, we cannot conclude on the impact of air pollution in these potentially susceptible subgroups, as reported by others (7, 41).

Children lost to follow-up, were significantly more often exposed to tobacco during pregnancy compared to those followed up. A lower rate of maternal smoking during pregnancy in those children investigated may have led to a selection of children with potentially better lung function.

Conclusion

In this study, we demonstrated that prenatal exposure to low-to-moderate air pollution levels as observed in Switzerland was associated with impaired postnatal lung function in term and preterm infants. Significantly increased effects were shown in the moderate to late preterm infants, suggestive of an amplified susceptibility to environmental stimuli such as air pollution. A broad body of literature provides evidence that impaired early-life lung function is associated with later respiratory morbidity in adulthood (e.g. COPD). This proof of principle study

highlights that some parts of the population, in our situation 10% of infants born prematurely, have differences in susceptibility to air pollution. This may impact future health and prevention policies in respect to our newborns.

Acknowledgments The authors thank the participants for participating in the study, the entire BILD cohort team (University of Basel Children's Hospital, Switzerland; Division of Respiratory Medicine, Department of Pediatrics, Inselspital, Bern University Hospital) and Fiona Beck (University of Basel Children's Hospital) for her critical reading of the manuscript.

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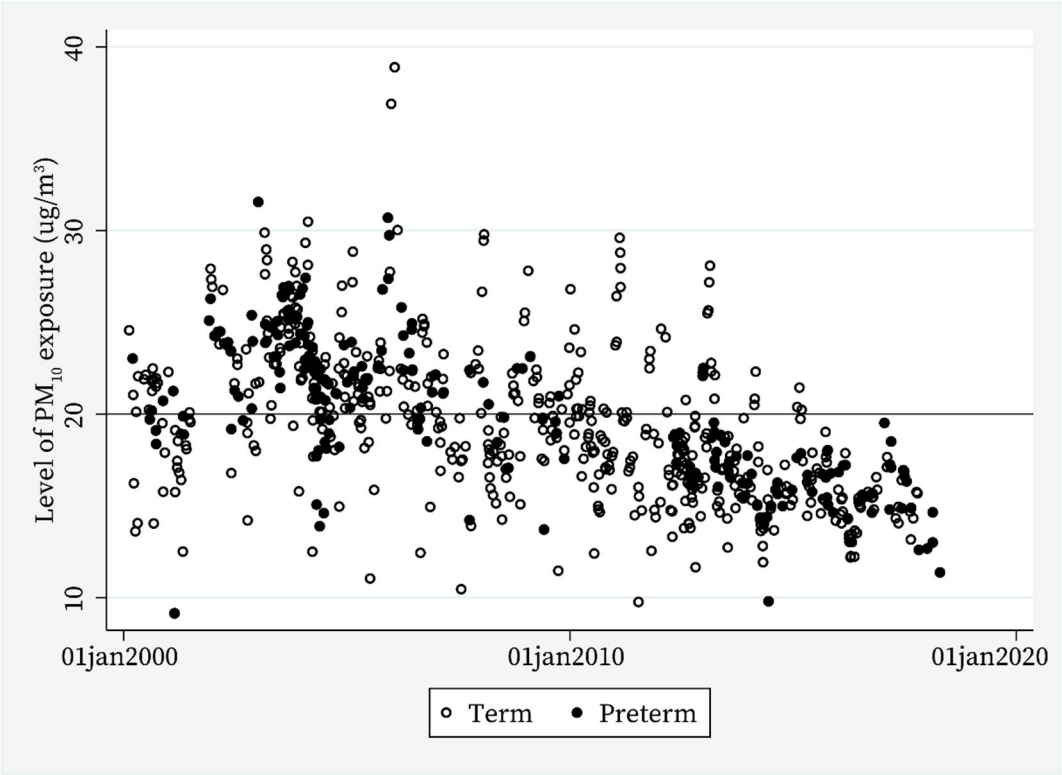

Figure 1 Decreasing air pollution levels over the 18 years of observation. Depicted are the individual mean levels of PM_{10} ($\mu g/m^3$) exposure for the whole observation period, consisting of pregnancy and postnatal time (defined time between birth and lung function measurement).

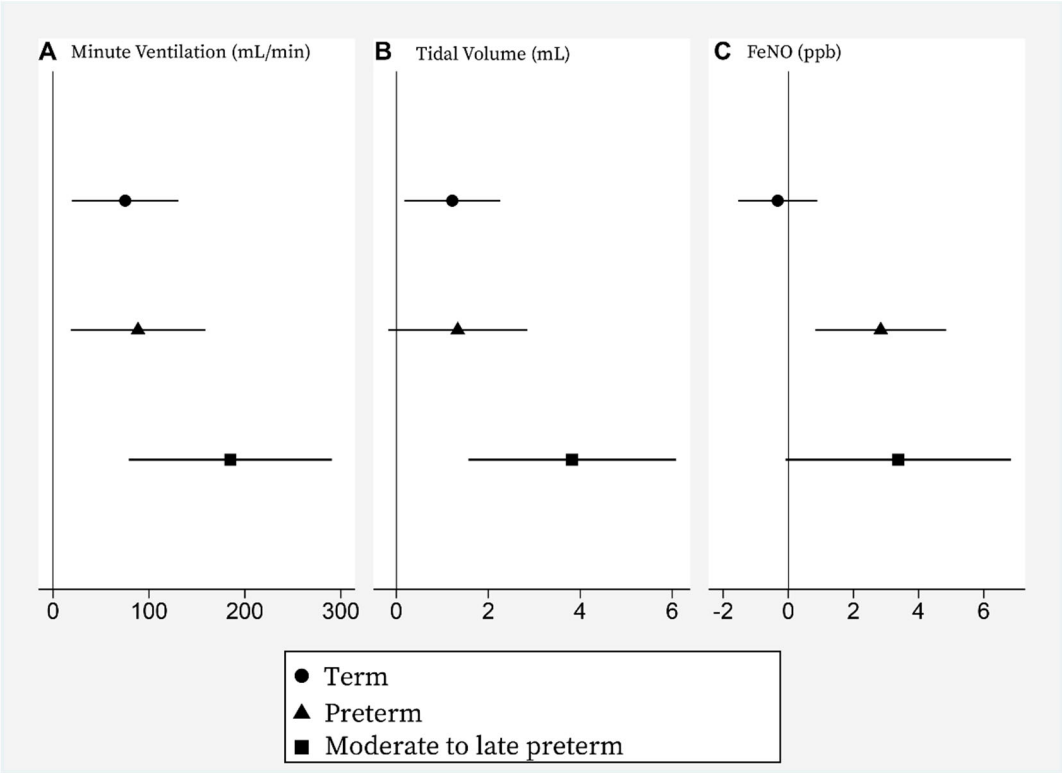

Figure 2 Adjusted effect of PM_{10} during the second trimester of pregnancy on lung function parameters in term, preterm and moderate to late preterm infants. Effect estimates of (**A**) minute ventilation (mL/min), (**B**) tidal volume (mL) and (**C**) FeNO (ppb) are presented as coefficient (β) and 95% confidence intervals (CIs) for each 10 $\mu g/m^3$ increase in PM_{10} during second trimester of pregnancy for term, preterm and moderate to late preterm infants. The model was adjusted for sex, weight at lung function measurement, season and postconceptional age at lung function measurement, gestational age at birth, maternal smoking during pregnancy, maternal asthma (defined as self-reported or doctor-diagnosed), days of supplementary oxygen, days of mechanical ventilation (defined as cPAP or intubation). Analyses for FeNO were additionally adjusted for minute ventilation (mL/min).

Table 1 Anthropometric data and considered risk factors

	Overall	**Term**	**Preterm**	**Moderate to late preterm (32 – 36 weeks)**
Study participants, n	771	517	254	99
Gestational age at birth, w	36.7 (4.8)	39.8 (3.3)	30.6 (3.2)	33.9 (1.4)
Postconceptional age at lung function measurement, w	44.8 (1.1)	44.8 (1.1)	44.7 (1.2)	44.5 (1.0)
Weight at lung function measurement, g	4327.8 (631.4)	4394.5 (548.1)	4192.0 (757.0)	4438.2 (711.0)
Length at lung function measurement, cm	54.2 (2.7)	54.7 (2.2)	53.2 (3.2)	54.5 (2.8)
Male sex	413 (54)	268 (52)	144 (57)	56 (57)
Maternal smoking during pregnancy	59 (8)	33 (6)	26 (10)	4 (4)
Maternal asthma *	85 (11.0)	65 (13)	20 (8)	9 (9)
Duration of oxygen supplementation, d	10.2 (30.7)	0	30.9 (47.1)	2.6 (5.8)
Duration of mechanical ventilation †, d	5.9 (15.1)	0	18.0 (21.7)	1.5 (2.7)

Values are mean (standard deviation) or number (percentage). * Asthma was defined as self-reported or doctor-diagnosed asthma. † mechanical ventilation was defined as cPAP or intubation

Table 2 Lung function data at 44 weeks of postconceptional age

	Overall	Term	Preterm	Moderate to late preterm (32 – 36 weeks)
Tidal breathing				
Minute ventilation, mL/min	1469.3 (323.0)	1451.4 (308.3)	1505.7 (348.7)	1580.3 (339.6)
Respiratory rate, /min	46.9 (11.6)	45.0 (10.6)	50.6 (12.7)	46.9 (9.5)
Tidal volume, mL	32.4 (7.0)	33.1 (5.9)	31.0 (8.6)	34.7 (8.2)
T_{PTEF}/T_E, %	34.0 (10.8)	36.6 (10.9)	28.8 (8.7)	31.9 (9.1)
FeNO, ppb *	14.1 (6.9)	13.7 (6.4)	15.0 (7.7)	17.6 (7.4)

Values are mean (standard deviation) or number (percentage). * information is missing for 71 children (26 term, 45 preterm infants)

Table 3 Distribution of estimated residential outdoor air pollutants

	Overall	Term	Preterm	Moderate to late preterm (32 – 36 weeks)
PM_{10} µg/m³				
PM_{10} 2nd trimester	19.9 (6.1–44.7)	19.9 (7.5–37.7)	20.0 (6.1–44.7)	18.5 (6.3–38.3)
Postnatal PM_{10} *	19.1 (6.8–54.7)	18.9 (6.8–54.7)	19.5 (10.0–43.9)	17.1 (10.0–43.9)
NO_2 µg/m³				
NO_2 2nd trimester	17.6 (5.3–52.8)	17.9 (5.5–40.7)	16.9 (5.3–52.8)	16.1 (5.3–52.8)
Postnatal NO_2 *	17.4 (4.8–42.3)	16.7 (5.3–41.8)	18.8 (4.8–42.3)	15.2 (4.8–42.3)

Values are mean (range). * postnatal was defined as time between birth and lung function measurement.

Table 4 Associations of pre- and postnatal air pollution exposure with lung function at 44 weeks of postconceptional age									
Minute ventilation, mL/min	**Term n = 517**			**Preterm n = 254**			**Moderate to late preterm (32 – 36 weeks) n = 99**		
	Coef	95% CIs	p-value	Coef	95% CIs	p-value	Coef	95% CIs	p-value
PM_{10} 2nd trimester	75.27	(19.74,130.79)	0.008	88.62	(18.57,158.68)	0.013	184.89	(79.12,290.66)	0.001
PM_{10} postnatal *	33.27	(-7.24,73.77)	0.107	0.83	(-75.43,77.08)	0.983	20.73	(-106.33,147.80)	0.749
NO_2 2nd trimester	-2.45	(-43.79,38.88)	0.907	-12.74	(-71.52,46.04)	0.671	45.42	(-32.35,123.20)	0.252
NO_2 postnatal *	7.27	(-35.18,49.73)	0.737	-44.16	(-107.92,19.60)	0.175	-8.38	(-97.70,80.95)	0.854
Tidal volume, mL									
PM_{10} 2nd trimester	1.22	(0.18,2.26)	0.022	1.33	(-0.18,2.85)	0.084	3.82	(1.57,6.08)	0.001
PM_{10} postnatal *	-0.92	(-1.68,-0.17)	0.016	0.08	(-1.61,1.78)	0.924	-0.22	(-2.92,2.48)	0.873
NO_2 2nd trimester	-0.58	(-1.36,0.19)	0.139	-0.42	(-1.73,0.88)	0.527	-0.02	(-1.69,1.64)	0.980

NO_2 postnatal *	-1.04	(-1.83,-0.25)	0.010	-0.58	(-2.00,0.84)	0.422	-1.25	(-3.13,0.64)	0.194
FeNO, ppb									
PM_{10} 2nd trimester	-0.32	(-1.54,0.89)	0.601	2.84	(0.84,4.84)	0.005	3.38	(-0.08,6.83)	0.056
PM_{10} postnatal *	-0.13	(-1.04,0.77)	0.771	0.84	(-1.52,3.21)	0.485	1.67	(-2.10,5.43)	0.385
NO_2 2nd trimester	-0.55	(-1.44,0.34)	0.226	1.46	(-0.15,3.07)	0.075	0.53	(-1.39,2.45)	0.591
NO_2 postnatal *	-0.47	(-1.39,0.44)	0.312	0.43	(-1.43,2.29)	0.651	0.00	(-2.23,2.23)	0.999

Estimates derived from mixed modelling for the association per 10 µg/m^3 increase of PM_{10} and NO_2 with lung function measurements at 44 weeks of postconceptional age. Multivariable model adjusted for sex, weight at lung function measurement, season and postconceptional age at lung function measurement, gestational age at birth, maternal smoking during pregnancy, maternal asthma (defined as self-reported or doctor-diagnosed), days of supplementary oxygen, days of mechanical ventilation (defined as cPAP or intubation). Analyses for FeNO were additionally adjusted for minute ventilation (mL/min). Results are presented as coefficient (Coef) and 95% confidence intervals (95% CIs) * postnatal was defined as time between birth and lung function measurement.

Online supplement

Methods

Study design

The prospective and ongoing Basel-Bern Infant Lung Development (BILD) birth cohort (https://www.bild-cohort.ch/) comprises unselected neonates recruited antenatally since 1999 in the region of Bern and since 2012 in the region of Basel, Switzerland (E1). Potential risk factors were assessed by interviews using standardized questionnaires (E1). Children underwent lung function measurement at 44 weeks of PCA. Prematurity was defined following International Statistical Classification of Diseases and Related Health Problems (ICD 10) as GA at birth < 37 weeks (E2). The ethics committees of the regions of Bern and of Basel approved the study and written consent was obtained.

Air pollution exposure

Air pollution data included daily mean levels of PM_{10}, NO_2 for the period from September 1999 to October 2017. Background air pollution was measured at the monitoring station of Payerne (part of the Swiss National Air Pollution Monitoring Network). We estimated NO_2 exposure using a time-space hybrid model, in order to capture seasonal air pollution variations during the entire study period and spatial variation in different study areas. This model was based on high-quality information on land use, population density, traffic, road network, dispersion models, meteorological data and air quality from the fixed measurement station (i.e. Payerne), trained with 28,849 NO_2 biweekly and monthly passive sampler measurements. Observations were collected consecutively over more than ten years at 146 locations, for external validation of the model (E3). We estimated PM_{10} exposure using a simplified spatial-temporal model in which

the temporal variation from Payerne was superimposed on the annual dispersion model from Pollumap (E4) which provided the spatial contrast in exposures. Thus, in this model, the averaged PM_{10} per time period (e.g. 1st trimester), based on daily measurements at the central monitoring site (i.e., Payerne), were corrected for each address using the ratio between the annual dispersion model value at the home or hospital address (i.e., Pollumap from METEOTEST, 200×200m resolution, 1998- 2015 for PM_{10} (E4)) and the annual mean from Payerne.

From these data we calculated the mean exposure by pollutant for each subject for different exposure windows: 1st trimester, 2nd trimester, 3rd trimester and postnatal. For families who changed their residential address during the study period or for preterm infants hospitalized, we calculated an average exposure estimate weighted by the time spent at each residence or hospital. Addresses were geocoded using a reference file from the Swiss Federal Statistical Office (Neuchâtel).

Lung function outcomes

Pulmonary function was performed at 44 weeks of postconceptional age (PCA) using Exhalyzer D (EcoMedics, Duernten, Switzerland) according to current ERS/ATS guidelines (E5). For analysis we used the first 100 regular breaths during non-REM (non-rapid eye movement) sleep from the total recorded breathing. We excluded sighs and ten breaths before and after a sigh. Simultaneous to tidal breathing recording, the fraction of exhaled nitric oxide (FeNO) was measured online with a chemiluminescence analyzer during the third quartile of expiration and averaged over the 100 breaths used for analysis. Following ERS/ATS guidelines for infant lung function testing, mean tidal flows, volume and flow-volume loop were calculated.

The following parameters were investigated: respiratory rate (RR), mean tidal volume (V_T), minute ventilation (V_E). Ratio of time to peak tidal expiratory flow (PTEF) and expiratory time ($t_{PTEF/tE}$) were used to describe TBFVL shapes. Online FeNO measurements were performed with a rapid-response chemiluminescence analyser (CLD 77 AM; EcoMedics AG, Duernten, Switzerland) concurrently with the TBFVL recording. We used air free of nitric oxide (NO) for respiration to prevent contamination of FeNO with ambient NO. The third quartile of each expiration was used to calculate mean FeNO over the 100 breaths recorded and was adjusted for V_E, as previously described (E6).

Covariates

Potential confounders were assessed by interviews using standardized questionnaires (E1) including sociodemographic factors, pre- and postnatal smoke exposure and, parental atopic disease. To confirm prenatal smoke exposure, the cotinine level in the infant's first urine was used.

After a stepwise backward selection and considering previous research (E7, E8), we adjusted the regression model for the following covariates: sex, weight at lung function measurement, season and postconceptional age at lung function measurement, GA at birth, maternal smoking during pregnancy, days of supplementary oxygen, days of mechanical ventilation (defined as days of continuous positive airway pressure (cPAP) or intubation) and, maternal asthma (defined as self-reported or doctor diagnosed). For FeNO analyses we additionally adjusted for V_E, as previously described (E7, E9).

Statistical analysis

We performed a multilevel mixed-effects linear regression for term and preterm infants separately, adjusted for the above-mentioned covariates and corrected for clustering on center level (Basel vs. Bern). Inspection of the outcome variables suggested normal distribution.

Since we previously showed that lung functional abnormalities can also be the result of prematurity and perinatal insults, particularly in extreme and very early premature born infants (E8), we also performed analysis in subgroups of preterm infants. We therefore stratified the preterm group according to their gestational age (GA) at birth into three clinically relevant groups; extreme (< 28 weeks of GA), very early (29 – 31 weeks of GA) and moderate to late (32 – 37 weeks of GA) preterm. We tested the presence of interaction within term and moderate to late preterm infants adding the interaction term between exposure to air pollution and prematurity.

We performed sensitivity analyses in respect to CLD classification, education status of the mother, a known potential confounder for lung function of the offspring. We did not include this risk factor in the main analysis as we had several missing values (around 70 children had no information on education status of the mother). Further, we investigated the modifying effect of sex on the association between air pollution and lung function (E10, E11) in the main model and therefore stratified for it and used interaction terms. Within the FeNO analyses we added exposure to caffeine as a confounder (E9, E12) to the main model. We also checked differences of the main model with and without length at measurement date (E6).

Pearson's correlation was used to assess correlation between different exposure windows of air pollution.

Effect estimates are presented as coefficient (β) and 95% confidence intervals (CIs) per 10 μg/m^3 increase in each pollutant. Data were analyzed using Stata® (Stata Statistical Software: release 16. STATA Cooperation; College Station, TX).

Power analysis

We calculated that a sample of 61 subjects would have a power of >80%, at a significance level of 0.05 to detect changes of 24.9 mL/min in V_E per 1 ug/m^3 increase in PM_{10} (E7).

Results

Sensitivity analyses

Using CLD classification as an additional confounder to the main model resulted in an increase in V_E [197.6 (92.06–303.19) mL/min] in moderate to late and [86.60 (16.86–156.35) mL/min] in preterm infants per 10 μg/m^3 increase in PM_{10} during the second trimester.

When stratifying the main model by sex and using interaction terms of sex and air pollution, no clear differences between boys and girls were seen, nor did the effect sizes change substantially to the earlier reported findings. Interaction terms were not significant, and were therefore not included in the main model.

In the preterm group, FeNO increased by [8.95 (3.27–14.63) ppb] per 10 μg/m^3 increase in PM_{10} and by [5.05 (-0.50–10.60) ppb] per 10 μg/m^3 increase in NO_2 during the second trimester when exposure to caffeine citrate was included in the main model. However, information on exposure to caffeine citrate was only available for 47 preterm infants, all infants were off drugs for > 6 weeks.

As we had many missing data on education status of the mother (70 missing), we included it as a confounder in the sensitivity analysis. Education status of the mother, categorized into low (less than four years of apprenticeship), middle (four years of apprenticeship and above) and high (tertiary education), as an additional confounder in the main model, did not have any substantial changes in effect sizes nor significance level (V_E in term infants increased by [75.27 (19.74–130.79) ppb] per 10 $\mu g/m^3$ increase in PM_{10} during the second trimester).

Including length as a confounder in the main model, did not change the effect sizes, significance levels or interaction terms (V_E in term infants increased by [77.05 (21.30–132.79) ppb] per 10 $\mu g/m^3$ increase in PM_{10} during the second trimester). However, correlation between weight and length at measurement date was high with 0.76 (Pearson correlation).

Correlation between different exposure windows for air pollutants was low-to-moderate for different exposure windows of PM_{10} (**Table E4**).

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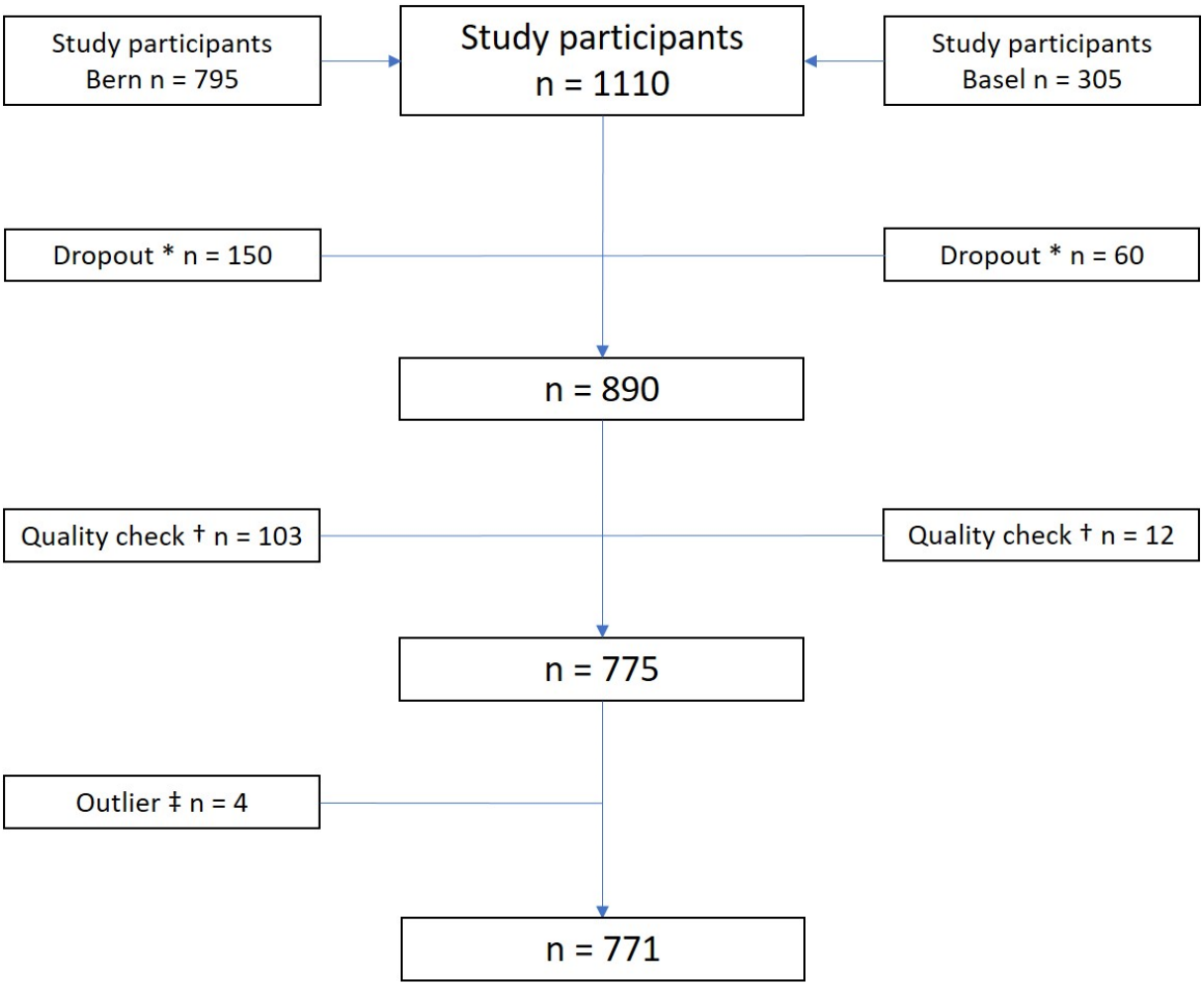

Figure E1 Flow chart of the study population. *Dropout due to missing lung function measurement at 44 weeks of postconceptional age, missing air pollution exposure values, missing information on important risk factors and dropout of the child from the study.

† Quality check included lung function quality or gestational age at lung function measurement < 42 or > 48 weeks of postconceptional age. ‡ Outliers included moderate to late preterm infants, which had severe asphyxia and therefore needed mechanical ventilation > 20days and/or supplementary oxygen > 80days.

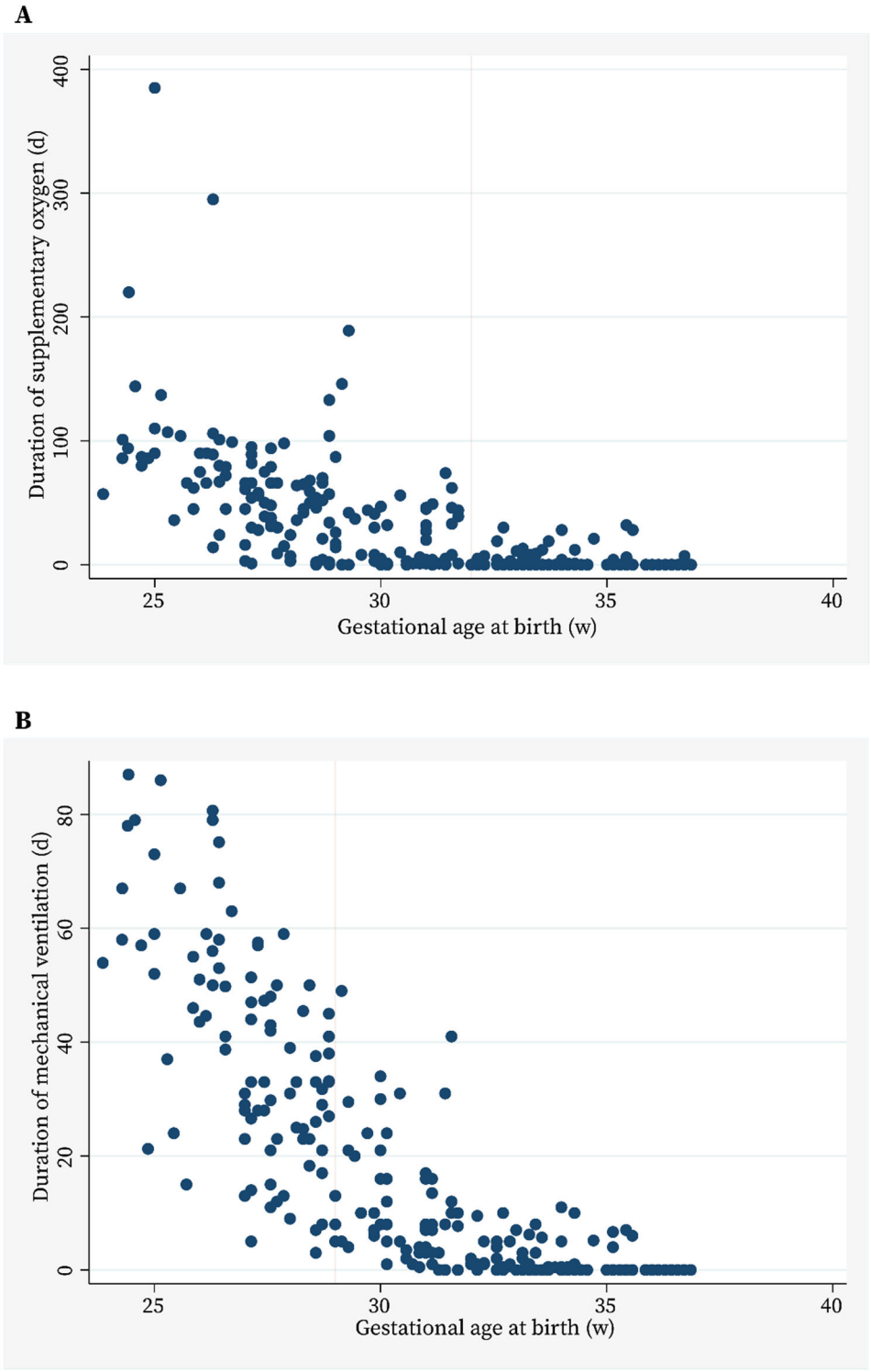

Figure E2 Duration of mechanical ventilation (defined as cPAP or intubation) (**A**) and of supplementary oxygen (**B**) according to gestational age at birth shown for preterm infants. Vertical lines represent the three groups of preterm infants (extremely, very early and moderate to late).

Table E1 Comparison between participants lost to follow-up / excluded vs. included participants

	Included	**Lost to follow-up / excluded**	**p-value**
Study participants, n	771	329	
Gestational age at birth, weeks	36.7 (4.8)	34.4 (5.6)	<0.001
Weight at study date, g	4327.8 (631.4)	4179.6 (835.1)	0.003
Length at study date, cm	54.2 (2.7)	53.7 (4.0)	0.027
Male sex	413 (54)	190 (58)	0.126
Maternal smoking during pregnancy *	59 (8)	29 (11)	0.102
Maternal asthma †	85 (11)	12 (5)	0.028
Duration of oxygen supplementation, d ‡	10.2 (30.7)	52.3 (80.4)	<0.001

Values are mean (standard deviation) or number (percentage). * information is missing for 74 children lost to follow-up. † Asthma was defined as self-reported or doctor-diagnosed asthma, missing information for 100 children lost to follow-up. ‡ information is missing for 144 children lost to follow-up.

Table E2 Distribution of estimated residential outdoor air pollutants over all time windows

	Overall	Term	Preterm	Moderate to late preterm (32 – 36 weeks)
PM_{10} µg/m^3				
PM_{10} 1st trimester	20.2 (6.3–44.6)	20.1 (9.0–44.6)	20.2 (6.3–41.8)	18.5 (6.3–36.1)
PM_{10} 2nd trimester	19.9 (6.1–44.7)	19.9 (7.5–37.7)	20.0 (6.1–44.7)	18.5 (6.3–38.3)
PM_{10} 3rd trimester	19.5 (6.6–62.7)	19.8 (8.2–40.4)	18.7 (6.6–62.7)	17.1 (6.6–36.6)
Postnatal PM_{10} *	19.1 (6.8–54.7)	18.9 (6.8–54.7)	19.5 (10.0–43.9)	17.1 (10.0–43.9)
NO_2 µg/m^3				
NO_2 1st trimester	17.6 (5.1–46.4)	17.7 (6.5–46.4)	17.5 (5.1 - 45.4)	16.6 (5.1–45.4)
NO_2 2nd trimester	17.6 (5.3–52.8)	17.9 (5.5–40.7)	16.9 (5.3–52.8)	16.1 (5.3–52.8)
NO_2 3rd trimester	16.9 (4.9–49.4)	17.3 (5.4–41.5)	15.9 (4.9–49.4)	14.6 (4.9–49.4)
Postnatal NO_2 *	17.4 (4.8–42.3)	16.7 (5.3–41.8)	18.8 (4.8–42.3)	15.2 (4.8–42.3)

Values are mean (range). * postnatal was defined as time between birth and lung function measurement.

Table E3 Associations of pre- and postnatal air pollution exposure (for all time windows) with lung function at 44 weeks of postconceptional age

	Term n = 517			**Preterm n = 254**			**Moderate to late preterm (32 – 36 weeks) n = 99**		
Minute ventilation, mL/min	Coef	95% CIs	p-value	Coef	95% CIs	p-value	Coef	95% CIs	p-value
PM_{10} 1st trimester	22.43	(-21.39,66.24)	0.316	6.51	(-53.84,66.86)	0.833	160.66	(51.74,269.59)	0.004
PM_{10} 2nd trimester	75.27	(19.74,130.79)	0.008	88.62	(18.57,158.68)	0.013	162.62	(59.37,265.87)	0.002

PM_{10} 3rd trimester	13.11	(-32.32,58.53)	0.572	-35.94	(-86.18,14.31)	0.161	-86.4	(-185.16,12.36)	0.086
PM_{10} postnatal *	33.27	(-7.24,73.77)	0.107	0.83	(-75.43,77.08)	0.983	20.73	(-106.33,147.80)	0.749
NO_2 1st trimester	3.97	(-31.57,39.52)	0.827	11.74	(-40.52,63.99)	0.66	75.25	(0.29,150.22)	0.049
NO_2 2nd trimester	-2.45	(-43.79,38.88)	0.907	-12.74	(-71.52,46.04)	0.671	45.42	(-32.35,123.20)	0.252
NO_2 3rd trimester	-3.53	(-40.37,33.32)	0.851	-36.93	(-91.16,17.30)	0.182	-4.44	(-78.71,69.82)	0.907
NO_2 postnatal *	7.27	(-35.18,49.73)	0.737	-44.16	(-107.92,19.60)	0.175	-15.47	(-103.51,72.57)	0.731
Tidal volume, mL									
PM_{10} 1st trimester	0.52	(-0.30,1.34)	0.215	-0.1	(-1.44,1.24)	0.888	1.37	(-0.87,3.61)	0.23
PM_{10} 2nd trimester	1.22	(0.18,2.26)	0.022	1.33	(-0.18,2.85)	0.084	3.82	(1.57,6.08)	0.001
PM_{10} 3rd trimester	-0.96	(-1.80,-0.12)	0.025	-0.1	(-1.22,1.02)	0.856	-2.55	(-4.62,-0.48)	0.016
PM_{10} postnatal *	-0.92	(-1.68,-0.17)	0.016	0.08	(-1.61,1.78)	0.924	-0.22	(-2.92,2.48)	0.873
NO_2 1st trimester	0.09	(-0.57,0.76)	0.787	-0.17	(-1.33,0.99)	0.772	0.45	(-1.18,2.07)	0.589

NO_2 2nd trimester	-0.58	(-1.36,0.19)	0.139	-0.42	(-1.73,0.88)	0.527	-0.02	(-1.69,1.64)	0.98
NO_2 3rd trimester	-1.1	(-1.78,-0.41)	0.002	-0.59	(-1.79,0.62)	0.339	-0.87	(-2.44,0.70)	0.277
NO_2 postnatal *	-1.04	(-1.83,-0.25)	0.01	-0.58	(-2.00,0.84)	0.422	-1.25	(-3.13,0.64)	0.194
FeNO, ppb									
PM_{10} 1st trimester	0.06	(-0.89,1.00)	0.909	-0.45	(-2.22,1.32)	0.622	0.46	(-2.42,3.33)	0.756
PM_{10} 2nd trimester	-0.32	(-1.54,0.89)	0.601	2.84	(0.84,4.84)	0.005	3.38	(-0.08,6.83)	0.056
PM_{10} 3rd trimester	-0.71	(-1.72,0.29)	0.162	1.19	(-0.26,2.64)	0.109	2.39	(-0.16,4.94)	0.066
PM_{10} postnatal *	-0.13	(-1.04,0.77)	0.771	0.84	(-1.52,3.21)	0.485	1.67	(-2.10,5.43)	0.385
NO_2 1st trimester	-0.22	(-0.98,0.55)	0.576	0.63	(-0.87,2.12)	0.412	0.48	(-1.51,2.47)	0.637
NO_2 2nd trimester	-0.55	(-1.44,0.34)	0.226	1.46	(-0.15,3.07)	0.075	0.53	(-1.39,2.45)	0.591
NO_2 3rd trimester	-0.43	(-1.23,0.37)	0.288	0.87	(-0.64,2.38)	0.26	0.21	(-1.59,2.00)	0.822
NO_2 postnatal *	-0.47	(-1.39,0.44)	0.312	0.43	(-1.43,2.29)	0.651	0.00	(-2.23,2.23)	0.999

Respiratory rate, /min									
PM_{10} 1st trimester	-0.46	(-2.03,1.11)	0.567	0.43	(-2.12,2.97)	0.741	2.00	(-1.33,5.32)	0.239
PM_{10} 2nd trimester	-0.27	(-2.19,1.66)	0.787	0.29	(-2.60,3.19)	0.843	0.66	(-2.87,4.18)	0.715
PM_{10} 3rd trimester	1.75	(0.11,3.38)	0.036	-1.42	(-3.53,0.69)	0.186	1.17	(-1.98,4.33)	0.467
PM_{10} postnatal *	2.12	(0.65,3.58)	0.005	-0.37	(-3.59,2.84)	0.820	1.93	(-2.06,5.92)	0.343
NO_2 1st trimester	-0.34	(-1.63,0.95)	0.609	0.77	(-1.43,2.97)	0.494	1.79	(-0.59,4.18)	0.141
NO_2 2nd trimester	0.47	(-1.03,1.96)	0.539	0.17	(-2.31,2.65)	0.893	1.77	(-0.67,4.22)	0.156
NO_2 3rd trimester	1.36	(0.03,2.70)	0.045	-0.75	(-3.04,1.53)	0.517	1.5	(-0.83,3.82)	0.206
NO_2 postnatal *	1.51	(-0.03,3.04)	0.054	-0.85	(-3.54,1.85)	0.539	2.07	(-0.72,4.86)	0.145
t_{PTEF}/t_E									
PM_{10} 1st trimester	-0.91	(-2.51,0.70)	0.267	0.7	(-1.00,2.39)	0.422	1.88	(-1.21,4.98)	0.233

PM_{10} 2nd trimester	0.27	(-1.74,2.28)	0.794	-0.66	(-2.59,1.27)	0.506	-0.49	(-3.71,2.74)	0.767
PM_{10} 3rd trimester	0.55	(-1.12,2.21)	0.519	-0.73	(-2.17,0.71)	0.323	-0.43	(-3.37,2.51)	0.774
PM_{10} postnatal *	0.04	(-1.46,1.54)	0.959	-1.88	(-4.02,0.25)	0.084	-2.68	(-6.45,1.09)	0.164
NO_2 1st trimester	0.50	(-0.82,1.83)	0.454	1.24	(-0.23,2.70)	0.098	1.78	(-0.43,3.99)	0.114
NO_2 2nd trimester	1.07	(-0.46,2.61)	0.17	0.82	(-0.83,2.47)	0.331	1.25	(-1.02,3.51)	0.281
NO_2 3rd trimester	1.14	(-0.23,2.51)	0.102	0.19	(-1.37,1.75)	0.813	0.36	(-1.80,2.53)	0.743
NO_2 postnatal *	1.19	(-0.39,2.76)	0.139	0.06	(-1.74,1.87)	0.944	0.39	(-2.29,3.08)	0.774

Estimates derived from mixed modelling for the association per 10 $\mu g/m^3$ increase of PM_{10} and NO_2 with lung function measurements at 44 weeks of postconceptional age. Multivariable model adjusted for sex, weight at lung function measurement, gestational age at birth, season at lung function measurement, maternal smoking during pregnancy, maternal asthma (defined as self-reported or doctor-diagnosed), days of supplementary oxygen, days of mechanical ventilation (defined as cPAP or intubation). Analyses for FeNO were additionally adjusted for minute ventilation (mL/min). * postnatal was defined as time between birth and lung function measurement.

PM_{10}	1st trimester	2nd trimester	3rd trimester	Postnatal
1st trimester	1.0000			
2nd trimester	0 .2051	1.0000		
3rd trimester	0.0025	0.2114	1.0000	
Postnatal *	0.1518	-0.0019	0.2672	1.0000
NO_2				
1st trimester	1.0000			
2nd trimester	0.7154	1.0000		
3rd trimester	0.4502	0.7662	1.0000	
Postnatal *	-0.1508	-0.3361	0.1935	1.0000

Table E4 Pearson's correlation of air pollutants (PM_{10} and NO_2) among different time windows. * postnatal was defined as time between birth and lung function measurement.

4.3 Article III - Exposure to moderate air pollution and associations with lung function at school-age: A birth cohort study

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Environment International

https://doi.org/10.1016/j.envint.2018.12.019

Accepted 10 December 2018

Exposure to moderate air pollution and associations with lung function at school-age: a birth cohort study

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Word count: Abstract 362, text 4568

Number of tables: 4

Number of figures: 2

Number of references: 39

Number of tables and figures in online supplement: 10

Highlights

- Low-level early life NO_2 exposure associates with impaired school-age lung function
- These effects occurred at annual NO_2 < 20 $\mu g/m^3$, thus below recommended WHO threshold
- These dose dependent effects were mainly found for NO_2 exposure in infancy
- Thus, infancy seems to be a susceptible window for air pollution effects

Abstract

Background: Adverse effects of higher air pollution levels before and after birth on subsequent lung function are often reported in the literature. We assessed whether low-to-moderate levels of air pollution during preschool-age impact upon lung function at school-age.

Methods: In a prospective birth cohort of 304 healthy term-born infants, 232 (79%) completed lung function at follow-up at six years. Using spatial-temporal models, levels of individual air pollution (nitrogen dioxide (NO_2) and ozone (O_3), particulate matter with a diameter < 10 µm (PM_{10})) were estimated for the time windows pregnancy, first up to the sixth year of life separately, and birth until follow-up at the age six. Time window means were compared to World Health Organization (WHO) guideline limits. Associations of exposure windows with spirometry and body plethysmography indices were analyzed using regression models, adjusting for potential confounders. For subgroup analysis, air pollution exposure was categorized into quartiles (four groups of 52 children).

Results: Mean NO_2 level from birth until follow-up was [mean (range)] [11.8 (4.9 to 35.9 µg/m^3)], which is almost 4-times lower than the WHO suggested limit of 40 µg/m^3. In the whole population, increased air pollution levels from birth until follow-up were associated with reduced lung function at six years. In the subgroup analysis, the 52 children exposed to NO_2 levels from the highest quartile during pregnancy, the first and second year of life and from birth until follow-up, had a significant decrease in forced expiratory volume in one second (FEV_1). Per interquartile range increase of NO_2, FEV_1 decreased by [z-score change (95% confidence interval)] [-1.07 (-1.67 to -0.47)], [-1.02 (-1.66 to -0.39)], [-0.51 (-0.86 to -0.17)] and [-0.80 (-1.33 to -0.27)], respectively. Air pollution exposure during pregnancy and childhood resulted in a non-significant decrease in lung volume at six years, as assessed by functional residual capacity measured by body plethysmography (FRC_{pleth}).

Conclusion: Our results suggest that exposure to higher NO_2 levels, which are still much lower than WHO guideline limits, especially during the sensitive period of early lung development, may be associated with reduced lung function at school-age. These findings support the concept of age and dose-dependent pollution effects on lung function in healthy school-aged children and underline the importance of pollution reduction measures.

Keywords: air pollution; infant; cohort; prospective; lung function; school-age

Introduction

Adverse effects of air pollution on lung function in newborns (1), during childhood (2-7) and adolescence (8) have been reported. Different exposure windows during lung development have been investigated in order to identify a critical time frame. While some studies reported small-scale associations between higher air pollution exposure during pregnancy and reduced lung function at school-age (2-6), other studies reported that exposure during infancy, but not pregnancy, was associated with reduced lung function at school-age (7, 8). These conflicting findings suggest that different exposure windows during lung development may differently affect lung function. Effects may be dose and age dependent and prospective studies with continuous exposure assessment, measuring from pregnancy onward, might help to identify sensitive exposure windows.

Few studies continuously assessed air pollution before and after birth and studied its association with lung function at a mean age of 4.5 (5) and 6-11 years (6, 9). Importantly, pollution levels in these studies were high. While there is a clear association between higher air pollution and subsequent reduced lung function (5, 6), adverse effects of moderate air pollution in adults are recognized (10); however, in primarily healthy children are less understood. Studying the latter is particularly important given recent attempts to decrease air pollution in some countries (e.g. Switzerland) (11), which may result in less severe adverse effects on lung development.

Our research group previously measured lung volume in 5-week-old unsedated infants using multiple breath washout (1), a method feasible for measuring functional residual capacity (FRC) in newborns (12). We found a negative association between nitrogen dioxide (NO_2) during pregnancy and FRC in newborns (1), suggesting deleterious effects of air pollution on functional lung volumes in early infancy. However, little is known about critical exposure windows during early childhood and their impact on lung volume during childhood, and

particularly effects of low-level air pollution exposure below the World Health Organization (WHO) guideline limits (NO_2 < 40 μg/m^3) (13).

The aim of this study was, to determine within a prospective birth cohort of unselected infants, if low-to-moderate pollution levels were associated with changes in airway obstruction and lung volume at six years of age. For a detailed exposure assessment, we assessed individual air pollution levels from pregnancy until school-age, enabling us to assess the dose-dependent effect of different exposure windows, but particularly in very early life during suspected windows of susceptibility on subsequent lung function (5, 7).

Methods

Study design and subjects

The prospective Basel-Bern Infant Lung Development (BILD) birth cohort study (https://www.bild-cohort.ch/) comprises a group of unselected, healthy neonates recruited antenatally since 2002 in the region of Bern, Switzerland (14). Exclusion criteria for the study were preterm delivery (< 37 weeks) and significant perinatal disease, including respiratory distress and later diagnosis of chronic respiratory disease. Potential risk factors were assessed by interviews using standardized questionnaires. Children underwent a lung function measurement at follow-up at six years. The Ethics Committee of the Region of Bern approved the study and written consent was obtained at enrolment.

Air pollution exposure during pregnancy and childhood

Air pollution data included daily mean levels of nitrogen dioxide (NO_2), ozone (O_3), and particulate matter with an aerodynamic diameter of <10 μm (PM_{10}) for the period February 2002 to July 2016. Background air pollution was measured at the monitoring station of Payerne (part of the Swiss National Air Pollution Monitoring Network). We estimated NO_2 exposure using a time-space hybrid model, in order to capture seasonal air pollution variations during the entire study period and spatial variation in different study areas. This model was based on high quality information on land use, population density, traffic, road network, dispersion models, meteorological data, and air quality from the fixed measurement station (i.e. Payerne), trained with 28,849 NO_2 bi-weekly and monthly passive sampler measurements in the region of Bern. Observations were collected consecutively over more than ten years at 146 locations, for external validation of the model (15). We estimated PM_{10} and O_3 exposure using a simplified spatial-temporal model in which the temporal variation from Payerne was superimposed on the annual dispersion model from Pollumap (16) which provided the spatial contrast in exposures.

Thus, in this model, the averaged PM_{10} per time period (e.g. pregnancy, first year of life), based on daily measurements at the central monitoring site (i.e. Payerne), was corrected for each address using the ratio between the annual dispersion model value at the home address (i.e. Pollumap from METEOTEST, 200×200m resolution, 1998-2015 for PM_{10} and O_3 (16)) and the annual mean from Payerne.

From these data we calculated the mean exposure by pollutant for each subject for different exposure windows: pregnancy, annual levels from birth until end of follow-up, as well as short-term exposure, defined as 14 days average before lung function testing at school-age. For families who changed their residential address during the study period, we calculated an average exposure estimate weighted by the time spent at each residence. Addresses were geocoded using a reference file from the Swiss Federal Statistical Office (Neuchâtel).

To assess the degree of air pollution, we compared mean levels of PM_{10}, NO_2, and O_3 for all study participants to the annual mean WHO guidelines for each air pollutant (13, 17). To assess dose-dependent effects, we parted air pollution exposure into quartiles of the range of each pollutant.

Lung function at six years

Spirometry was performed using the MasterLab setup (Jaeger, Wurzburg, Germany) according to current ERS/ATS guidelines [18]. The following lung function parameters were investigated: FEV_1, FVC, FEV_1/FVC ratio and mid expiratory flow at 25-75% of FVC (MEF25-75%). A reproducible test was defined as FVC and FEV_1 agreeing within 100 ml or 10% between the best two blows (18). Spirometry data were expressed as z-scores using the Global Lung Function Initiative (GLI) 2012 prediction equations (19). Body plethysmography measurements were done to assess the functional residual capacity (FRC_{pleth}), which is the volume of air present in the lungs at the end of passive expiration (18). The mean of three high

quality measurements (coefficient of variation (CV) <25% between measurements) was calculated.

Potential confounders

At baseline, exposure to potential pre-and postnatal risk factors for lung function deficits was assessed by interviews using standardized questionnaires. We assessed environmental tobacco smoke (ETS), an important risk factor for decreased lung function at school-age (20-22). ETS was defined as passive smoke exposure at work or home during pregnancy, and parental smoking at any time during childhood. Maternal atopic disease was defined as self-reported, doctor-diagnosed asthma, hay fever, or eczema. Parental education was categorized into low (less than four years of apprenticeship), middle (four years of apprenticeship and above) and high (tertiary education). At follow-up, we assessed the asthma diagnosis of the child, defined by wheezing over the past 12 months and doctor diagnosed asthma, or wheezing and use of asthma medication (glucocorticoid or beta mimetic), and short-term air pollution exposure (14 days average before lung function testing).

Statistics

First, we performed univariable linear regression analysis to investigate the association of pulmonary function measures (in z-scores) with mean exposure levels of each pollutant from each exposure window. Second, we adjusted the models for maternal atopy, ETS, asthma of the child and short-term air pollution exposure. Sensitivity analysis was performed using lung function values in ml adjusted for the above-mentioned confounders and additionally adjusted for sex, age and height at lung function. We also performed the analyses not considering the child´s asthma status as a confounder. For subgroup analysis, we divided the population into four equally sized groups of 52 participants (quartiles). Quartiles were defined by levels of each air pollutant (for NO_2, O_3 and PM_{10}) for each time window separately. Changes in lung function

parameters are presented as coefficient (β) values and 95% confidence intervals (CIs) per interquartile range (IQR) for each pollutant. Data were analyzed using Stata® (Stata Statistical Software: release 15. STATA Cooperation; College Station, TX).

Power analysis

Using Quanto (23), we calculated that a sample of 200 subjects would have a power of more than 80%, at a significance level of 0.05 to detect changes of more than 15 ml in FEV_1 per 1 $\mu g/m^3$ increase of PM_{10}.

Results

Between 2002 and 2010, 304 children were recruited, of which 241 (79%) eligible study participants completed lung function testing at six years of age. From these, we had complete data on modelled air pollution exposure and lung function measurements at school-age for 232 (96%), of which spirometry was available for 208 (90%) and body plethysmography for 212 (91%) (**Figure S1**). **Table 1** shows the anthropometric data and potential risk factors. Of the 232 children, 44 (19%) were exposed to ETS during the study period, 17 (7%) had asthma at six years, and 89 (38%) had atopic mothers.

There were differences between children followed-up and those lost to follow-up. Participants followed-up were less often exposed to tobacco smoke during pregnancy (5% vs 11%, p=0.042), and born from parents with higher education levels (p=0.001) (**table S1**).

Table 2 shows spirometry and body plethysmography data at school-age, completed between the 4th of August 2008 and the 20th of May 2016. For each of the outcome parameters, the summary statistics were: FEV_1 (mean z-score; SD) (0.02; 0.87), FVC (-0.19; 0.93), and

FEV_1/FVC (0.45; 0.9). The FRC_{pleth} was (mean ml; SD) (1105; 256). The (mean [range]) CV of body plethysmography was (6.7% [0.7% to 21.6%]).

The distribution of air pollution during the study period is given in **table 3**.

The distribution of air pollution for each quartile (n=52) is given in **table S2**. The WHO guideline limits for NO_2 (annual mean 40 µg/m^3) (13) were not exceeded in our study during any exposure window. However, annual mean PM_{10} levels of the study participants surpassed the WHO annual guideline limit of 20 µg/m^3 during pregnancy and the first year of life by 9% [mean (range)] [21.8 µg/m^3 (12.5–28.6 µg/m^3)], and 5% [21.0 µg/m^3 (10.8–26.8 µg/m^3)], respectively. Air pollution in Switzerland decreased during the study period. Mean PM_{10} in 2002 was 21.2 µg/m^3, and 12.8 µg/m^3 in 2016. Mean NO_2 in 2002 was 14.7 µg/m^3, and 11.3 µg/m^3 in 2016. Annual O_3 levels were not comparable to the WHO guideline limits as these were only defined as 8 hour means (8 hour mean 100 µg/m^3), rather than annual means as performed in this study. The temporal decrease of NO_2 for each quartile is shown in **figure 1**.

The highest exposed group was exposed to NO_2 levels of [mean (range)] [26.1 µg/m^3 (22.0 µg/m^3 to 37.2 µg/m^3)] during pregnancy, and during sixth year of life to NO_2 levels of [18.3 µg/m^3 (14.4 µg/m^3 to 26.6 µg/m^3)]. The lowest exposed group was exposed to NO_2 levels of [12.3 µg/m^3 (10.4 to 14.1 µg/m^3)] during pregnancy, and during sixth year of life to NO_2 levels of [8.4 µg/m^3 (7.0 µg/m3 to 9.6 µg/m^3)], respectively (**table S2**).

Association of different air pollution exposure windows with lung function at six years

Spirometry

Air pollution exposure during pregnancy was associated with reduced FEV_1 at six years (**table S3**). In the adjusted regression model, per IQR increase in NO_2 during pregnancy (8.0 µg/m^3)

and from birth until follow-up (5.2 µg/m^3), FEV_1 decreased by [z-score change (95% confidence interval)] [-0.16 (-0.35 to 0.04)] and significantly by [-0.24 (-0.45 to -0.02)], respectively. Per IQR increase in O_3 during pregnancy (14.6 µg/m^3) and from birth until follow-up (5.9 µg/m^3), FEV_1 decreased by [-0.11 (-0.29 to 0.06), and significantly by [-0.24 (-0.45 to -0.02)], respectively. Per IQR increase in PM_{10} during pregnancy (4.4 µg/m^3) and from birth until follow-up (3.3 µg/m^3), FEV_1 decreased by [-0.14 (-0.30 to 0.14)] and by [-0.09 (-0.26 to 0.08)], respectively. Results reported per ml change in FEV_1 are given in table 4. Similar associations were found for the other time windows (second, third, fourth and fifth year of life) and FEV_1 at six years (data not shown).

Dose and age dependent effects

Adverse effects of air pollution exposure on lung function depended upon the exposure level. Regression analysis of the quartiles showed significant negative associations of NO_2 levels with FEV_1 in the highest exposed group. In this subgroup, per IQR increase in NO_2 during pregnancy (8.0 µg/m^3), during the first year of life (7.2 µg/m^3), during the second year of life (5.7 µg/m^3) and from birth until follow-up (5.2 µg/m^3), FEV_1 decreased highly significant (p=0.001) by [-1.07 (-1.67 to -0.47)], by [-1.02 (-1.66 to -0.39)], by [-0.51 (-0.86 to -0.17)] and by [-0.80 (-1.33 to -0.27)] respectively.

There was no significant association of NO_2 and FEV_1 at six years in the subgroup with the lowest exposure levels. In this subgroup (n=52), per IQR increase in NO_2 during pregnancy (8.0 µg/m^3), during the first year of life (7.2 µg/m^3), during the second year of life (5.7 µg/m^3) and from birth until follow-up (5.2 µg/m^3), FEV_1 decreased non-significantly by [-0.21 (-2.05 to 1.63)], [-0.15 (-1.97 to 1.67)], [-0.88 (-2.37 to 0.60)] and [-0.36 (-1.97 to 1.24)], respectively (**table S4**). Within the other quartiles, also non-significant effects of NO_2 levels on FEV_1 were observed (**figure 2**, **table S4**). O_3 and PM_{10} exposure during investigated time windows was

associated with a non-significant FEV_1 decrease for all investigated exposure windows (**table S5, S6).**

Sensitivity analysis

In a sensitivity analysis, we did not adjust for the child´s asthma status at six years, which resulted in similar but weaker associations between air pollution and FEV_1 at six years. For example, in the entire study sample, in the adjusted regression models not adjusted for asthma, per IQR increase in NO_2 during pregnancy and from birth until follow-up, FEV_1 decreased by [z-score change (95% confidence interval)] [-0.14 (-0.34 to 0.07)] and by [-0.21 (-0.44 to -0.01)], respectively (**table S7**).

Adverse effects of NO_2 exposure in the highest exposure group and spirometry at six years was also very similar when we did not consider the child´s asthma status as a confounder. In this subgroup, per IQR increase in NO_2 during pregnancy, during the first year of life, during the second year of life and from birth until follow-up, FEV_1 decreased by [-1.07 (-1.66 to -0.46)], by [-1.01 (-1.64 to -0.38)], by [-0.51 (-0.85 to -0.17)] and by [-0.79 (-1.31 to -0.27)], respectively (**table S8**).

Body plethysmography

The association of exposure to air pollution during pregnancy and childhood with lung volume assessed at six years is given in **table S9** in the online supplement. Air pollution exposure during pregnancy and childhood resulted in a non-significant decrease in lung volume, as assessed by FRC_{pleth}.

Discussion

Main findings

To our knowledge, this is the first study to examine moderate air pollution exposure before and after birth and the association with airway obstruction and lung volume at school-age. We defined several exposure windows (pregnancy, first year of life, sixth year of life, and birth until follow-up at six years) to examine the dose-dependent impact of air pollution from different exposure time points on lung function at school-age.

When analyzing the entire study population, NO_2 exposure during pregnancy and the first year of life was weakly associated with a decrease in FEV_1 at school-age. In order to investigate dose-dependent exposure groups, NO_2 exposure was categorized into quartiles. Children in the highest exposure group (mean annual NO_2 exposure above 20 $\mu g/m^3$) had a significant decrease in FEV_1 at school-age. This effect was observed for exposure during pregnancy and early childhood, indicating that early lung development is especially sensitive to air pollution exposure. Children exposed to lower NO_2 levels (mean annual NO_2 exposure below 20 $\mu g/m^3$) had no decrease in lung function at school-age. Together, these findings support the hypothesis of dose-dependent effects of air pollutants on lung function, especially observed in young infants exposed to high levels of NO_2.

Comparison with literature

There are several studies demonstrating the effects of higher air pollution exposure and reduced lung function during childhood and adolescence, underlying the clinical relevance of air pollution on lung growth (4, 5, 24) and later respiratory morbidity in the elderly (25-28). In regard to studies with lower air pollution exposure levels, a previous population-based cohort in Sweden calculated air pollution exposure from birth until follow-up at eight years and measured lung function at follow-up in almost 2000 children (7). In that study, different

exposure windows were calculated and mean levels of PM_{10} were lower than in our study. Per 7 $\mu g/m^3$ increase in PM_{10}, which was equal to the difference between the 5th and 95th percentile, the authors reported a decrease of -59.3 ml in FEV_1 in the whole study population, which was more pronounced in boys and susceptible subgroups (e.g. children sensitized to common allergens). In our population, PM_{10} exposure during the first year of life was not significantly associated with reduced FEV_1. However, an increase of 7.7 $\mu g/m^3$ PM_{10}, assessed from birth until six years of age, which was equal to the difference between the 5th and 95th percentile, resulted in a -24.0 ml decrease in FEV_1.

These differences may be explained by several mechanisms. Our population of six-year-olds has lower mean FEV_1 values compared to the eight-year-olds in the study by Schultz et al. (7) (1277 ml vs 1780 ml), and hence, adverse effects of air pollution may result in a lower absolute reduction in FEV_1 due to lower lung volumes. When expressing the reduction in FEV_1 in percent change, per 7 $\mu g/m^3$ PM_{10} increase, Schultz et al. measured a FEV_1 decrease of 3.3 %, while we observed a FEV_1 decrease of 1.9 %. Reasons underlying these differences may be related to different statistical models and adjustments, a different assessment of air pollution exposure, as well as exposure to different risk factors (e.g. environmental and genetic). Results from a large cohort study in the area of Paris support our findings. Bougas et al. reported associations of pre-and postnatal exposure to NO_x (NO and NO_2) and lung function parameters at 8-9 years of age (2), with more pronounced effects observed in vulnerable subgroups (e.g. children with early lower respiratory tract infections or allergic sensitization). Of note, exposure levels of NO_2 measured from birth until lung function in that study were approximately four times higher (median NO_2 65.4 $\mu g/m^3$) than exposure levels in our study (median NO_2 14.2 $\mu g/m^3$).

A large cohort study in the US investigated the association of air quality regulations in the 1990s, and lung function in 600 eight-year-old children. The authors found no significant

associations between air pollution exposure and lung function, except for $PM_{2.5}$ exposure one year before lung functional testing, and reduced FEV_1 values (29). This weak association supports our findings, where we adjusted rigorously for potential confounders (e.g. short-term air pollution exposure, asthma status of the child), and found only weak associations within the overall population. As the observed decrease in FEV_1 in our study reached significance level mainly for children in the highest NO_2 exposure group, we conclude that current air pollution exposure in Switzerland is associated with only small reductions in lung function at school-age. However, the small reductions in lung function as reported in this and previous studies (4, 5, 7) should encourage further reduction in ambient air pollution levels to protect susceptible children during vulnerable time windows of lung development.

Previous studies measured pollutants at one to three time points, and found associations with reduced lung function (4, 5, 7, 30). Such an approach may have its limitations, however, since air pollution exposure reflects only a snap shot, and mean individual exposure levels during longer time periods may differ. This may have led to an overestimation of air pollution effects, especially when air pollution decreases over time and the pollutants were measured at an early time point and these estimates were used for the whole study period (7, 31). Furthermore, in order to not report spurious associations, studies should attempt to approximate exposure levels as precisely as possible, taking into account spatial and temporal variation, as factors such as proximity to major roads and short-term air pollutant exposure, are known to impact upon individual exposure levels (15, 32).

We observed a relevant decline in air pollutant levels over the 14-year study period (**figure 1**).

NO_2 within our overall population decreased nearly 30% (from 20.6 $\mu g/m^3$ to 14.4 $\mu g/m^3$) between 2002 and 2010. While in 2002 30% of all infants were exposed to NO_2 levels >20 $\mu g/m^3$, in 2010 only 16% remained exposed to these levels. This finding is encouraging, since recent attempts to reduce air pollutant exposure, at least in Switzerland, were effective (11).

Gauderman et al. investigated the effect of a decrease in air pollution levels on lung development in eleven-year-old children up to 15-year-old adolescents (31), but not at earlier ages. For an increase of 2 $\mu g/m^3$ in $PM_{2.5}$, they observed a decrease of -21.8 ml in FVC. Nevertheless, the study by Gauderman et al. did not track longitudinal lung function on an individual level, or at a young age (31). The study is, however, important as it was the first to report improved lung function due to reduced air pollution in a large cohort, and underlines the necessity to measure pollutant levels continuously in prospective studies. Assessing more precise exposure estimates could help to better evaluate the effects of improved air quality on children's health, especially when air pollution decreases over time.

Clinical and physiological relevance

Air pollution has detrimental effects on lung development, especially in areas with higher exposure levels (5, 24, 33, 34). Depending on the assessment of the pollutant, time period of interest, and study areas, the reported adverse effects differ (7, 29, 31, 35). Although the absolute effect of some pollutants may seem small, a decline in lung function may be most significant for those starting with impaired lung function early in life (e.g. preterm infants) or susceptible subjects (e.g. cystic fibrosis patients) (36-38).

Preschool NO_2 exposure was associated with impaired lung function at six years of age, whether or not the multivariable model was adjusted for the presence of asthma status of the child. We speculate that this may imply that air pollution has a direct impact on lung functional growth, and it is not only the effect of airway obstruction as a consequence of asthma related airway inflammation.

We observed an association between higher air pollution levels and reduced FEV_1, but not with FRC_{pleth} at six years of age. This finding may be due to different breathing maneuvers done during lung function testing. For spirometry (to assess FEV_1) the child is breathing at the

mechanical limits of the respiratory system (flow limitation). This typically results in lower variability of forced flow lung function parameters. During body plethysmography, the child is breathing close to FRC. At FRC, the respiratory system exhibits more adaptive variability of the airway resistance and thus small deleterious effects of air pollutants might not be detectable. These physiological differences may explain why the small impact of air pollution on lung function may only be visible when assessed at the extreme, which is during forced breathing maneuvers, but not during regular FRC breathing. Nevertheless, data are consistent with the impact of low-level air pollution on airway obstruction (7) but not lung restriction.

Further, air pollution is just one parameter resulting in reduced lung function, and other factors, such as toxins (e.g. ETS), as well as physiological decline over time, should be considered. Hence, even though low-level air pollution may have only small adverse effects, children exposed to moderate-to-high air pollution levels during susceptible time windows showed a significant decrease in lung function (2, 5, 24, 39), which might lead to later respiratory morbidity in the elderly (25-28). Therefore, further attempts should be made to reduce air pollution levels in order to minimize detrimental effects.

Strengths and limitations

One strength of our study was the assessment of air pollution during pregnancy and after birth, enabling us to study different exposure windows. To our knowledge, this is the first study to assess low-to-moderate levels of pre- and postnatal air pollution and association with airway obstruction and lung volume. We estimated NO_2 continuously using models based on bi-weekly measurements over the whole study period and validated it with independent data (15). Lung function was performed at one center by trained staff reducing potential inter-center differences. Especially in young children, precision of measurements largely depends on the child's ability to perform the breathing maneuvers, and only high-quality measurements were included.

Children lost to follow-up, had significantly lower educated parents and were more often exposed to ETS compared to those followed-up. A lower rate of ETS exposure in those children investigated may have led to a selection of children with potentially better lung function at school-age.

Due to a limited sample size, we could only perform subgroup analyses by dividing participants into quartiles depending on their exposure level, but could not perform additional subgroup analyses. Therefore, we cannot conclude on the impact of air pollution in susceptible subgroups (e.g. children with asthma), as reported by others (7, 29). Our analyses were adjusted for known confounders impacting upon the association between air pollution and reduced lung function, but the possibility of residual confounding remains.

When looking at the whole study population, we observed small effects of higher NO_2 exposure during the entire study period (pregnancy until six years) with reduced FEV_1 values at school-age. This effect became even more obvious in the highest exposed group, where NO2 exposure >20 $\mu g/m^3$ during pregnancy the 1st, and the 2nd year of life were associated with severely reduced lung function at school-age. This indicates that NO_2 exposure >20 $\mu g/m^3$ may be a critical exposure level. We are not able conclude on critical exposure levels of NO_2 during later childhood (5th and 6th year of life), since exposure levels during these time points were lower (<20 $\mu g/m^3$) due the natural decline of air pollution during the study period.

Conclusion and implications

In this study, we found associations between moderate air pollution exposure assessed before birth and during early childhood and lung function at school-age on a population level in healthy children. Stronger associations between air pollution and reduced FEV_1 values were observed in a subgroup exposed to the highest pollution levels above 20 $\mu g/m^3$ NO_2. It is remarkable that in infants these dose-dependent effects are unexpectedly observed even at NO_2 values below

the WHO guideline limits of 40 µg/m^3 (13). Thus, functional growth impairment may already be present, if infants are exposed to low-level air pollution during pregnancy and the first two years of life, time windows of rapid lung growth and potentially vulnerable lung development. In order to protect the vulnerable infant population and to develop health policies, further studies are needed to particularly investigate the relative impact of exposure during young age and low-level air pollution. Not only age, but also other preexisting risk factors (e.g. male sex, allergic sensitization (7)), may affect vulnerability to air pollution in small infants, requiring large multicenter birth cohort air pollution studies with a wide range of accurately measured air pollution exposures. There is sufficient evidence in the literature (25-28), that impaired early life lung functional growth is associated with later respiratory morbidity in the elderly. Thus, impairment of lung functional growth at even low concentrations will affect a large proportion of the normal healthy population and will potentially have a significant impact on public health in the future.

Acknowledgments

Funding This work was supported by the Swiss National Science Foundation grant 320030_163311 and a research fellowship of the UKBB issued to J. Usemann. The authors thank all the study participants for participating in the study, the entire BILD cohort team (University of Basel Children's Hospital, Switzerland; Division of Respiratory Medicine, Department of Pediatrics, Inselspital, Bern University Hospital, University of Bern, Switzerland), Karine Hugentobler (University of Basel Children's Hospital) for her critical reading of the manuscript, METEOTEST for providing Pollumap and its GIS information.

Competing interest The authors have no conflicts of interest to declare.

Contributions UF conceived the study. Measurements were recorded by JU, OF, IK, EP, AS, and PL. EP, DV and MR developed the NO2 air pollution model. DV derived the PM10 and O3 exposure estimates. Statistical analyses were performed by FD, JU, IK and OG. FD and JU drafted the manuscript; all authors have seen and approved the final manuscript.

Patient consent Obtained.

Ethics approval The Ethics Committee of the Region of Bern approved the study

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Table 1 Anthropometric data and potential risk factors of the study participants at six years

Study participants, n	232
Age at follow-up, years	6.1 (0.2)
Length at follow-up, m	1.17 (0.05)
Weight at follow-up, kg	22.2 (3.3)
Male sex, n (%)	114 (49)
Asthma of the child at follow-up [a], n (%)	17 (7)
Maternal atopy [b], n (%)	89 (38)
Maternal smoking during pregnancy, n (%)	11 (5)
ETS [c], n (%)	44 (19)
Parental education [d]	
low, n (%)	43 (19)
middle, n (%)	97 (42)
high, n (%)	92 (40)

Values are mean (SD) or number (percentage). [a] Asthma was defined by wheezing over the past 12 months and doctor diagnosed asthma, or wheezing and use of asthma medication (glucocorticoid or beta mimetic); [b] defined as self-reported, doctor-diagnosed asthma, hay fever or eczema; [c] defined as passive smoke at work or at home during pregnancy, as well as parental smoking any time during follow-up; [d] categorized into low (less than four years of apprenticeship), middle (four years of apprenticeship and above) and high (tertiary education).

Table 2 Lung function data at school-age at six years

Summary of measures	Mean (SD)	Range
Spirometry [a]		
FEV_1 (ml)	1277 (191)	797 – 1785
FEV_1 (z-scores)	0.02 (0.87)	-2.25 – 2.41
FVC (ml)	1381 (235)	816 – 2251
FVC (z-scores)	-0.19 (0.93)	-2.70 – 2.49
FEV1/FVC	0.93 (0.05)	0.7 – 1.0
FEV1/FVC (z-scores)	0.45 (0.93)	-2.46 – 2.15
$MEF_{25-75\%}$ (ml/sec)	1676 (388)	606 – 2664
$MEF_{25-75\%}$ (z-scores)	-0.04 (0.90)	-2.96 – 1.94
Body plethysmography [b]		
FRC_{pleth} (ml)	1105 (256)	579 – 3320

Values are means (SD) and range. Lung function measurements are given in ml and as z-scores according to Quanjer et al. (19). FEV_1, forced expiratory volume in one second; FVC, forced vital capacity; $MEF_{25-75\%}$, forced expiratory flow from 25-75% of exhalation. FRC_{pleth}, function residual capacity measured by body plethysmography; [a] Data available for n=208; FVC and FEV_1 agreeing within 100 ml between the best two blows. [b] Data available for n=212.

Table 3 Distribution of estimated residential outdoor air pollutants

NO_2 µg·m^{-3}	Mean	(SD)	Range	IQR
Entire pregnancy	18.5	(5.5)	10.4 – 37.2	8.0
1st year of life	17.6	(5.1)	9.4 – 34.4	7.2
6th year of life	12.8	(4.0)	7.0 – 26.6	4.9
From birth until follow-up	15.0	(4.3)	8.5 – 30.2	5.3
Exposure at follow-up [a]	11.8	(4.7)	4.9 – 35.9	5.9
O_3 µg·m^{-3}				
Entire pregnancy	87.3	(10.5)	68.6 – 114.9	14.5
1st year of life	87.0	(5.6)	76.1 – 99.4	8.7
6th year of life	83.8	(4.7)	65.0 – 96.1	6.0
From birth until follow-up	85.0	(4.1)	74.4 – 95.3	6.0
Exposure at follow-up [a]	88.3	(30.3)	10.1 – 163.4	50.6
PM_{10} µg·m^{-3}				
Entire pregnancy	21.8	(3.3)	12.5 – 28.6	4.4
1st year of life	21.0	(2.8)	10.8 – 26.8	3.1
6th year of life	17.9	(2.6)	9.8 – 25.8	3.1
From birth until follow-up	19.4	(2.4)	10.6 – 23.6	3.2
Exposure at follow-up [a]	17.5	(7.9)	5.4 – 52.5	7.9

Values are means (SD), range and IQR. [a] 14 days average before lung function testing during follow-up at six years; data available for n=232.

Table 4 Association of air pollution during different exposure windows with spirometry (in ml) at six years

		Univariable association			Multivariable association [a]		
		FEV_1 (ml)					
Exposure		Coef	95% CI	p-value	Coef	95% CI	p-value
NO_2	pregnancy	12.0	(-25.6, 49.7)	0.530	-25.2	(-57.4, 7.1)	0.125
	1st year of life	-9.1	(-28.8, 47.1)	0.636	-25.6	(-59.6, 8.4)	0.140
	6th year of life	-1.0	(-32.4, 30.4)	0.949	-40.5	(-76.4, -4.7)	0.027
	birth until follow-up	1.4	(-30.8, 33.6)	0.933	-37.7	(-72.3, -3.1)	0.033
	short-term exposure	18.7	(-11.8, 49.2)	0.229	6.7	(-16.2, 29.6)	0.565
O_3	pregnancy	-25.1	(-60.8, 10.7)	0.168	-20.4	(-48.5, 7.7)	0.154
	1st year of life	-0.8	(-39.9, 38.3)	0.968	-16.5	(-45.0, 11.9)	0.253
	6th year of life	27.2	(-6.6, 61.0)	0.114	4.6	(-20.7, 29.9)	0.719
	birth until follow-up	4.3	(-33.7, 42.3)	0.823	-21.2	(-49.1, 6.8)	0.138
	short-term exposure	-35.9	(-79.4, 7.7)	0.106	-40.7	(-72.9, -8.5)	0.013
PM_{10}	pregnancy	-4.1	(-39.0, 30.8)	0.817	-23.5	(-49.6, 2.6)	0.078
	1st year of life	8.7	(-20.7, 38.2)	0.559	0.16	(-22.4, 22.7)	0.989
	6th year of life	0.6	(-30.9, 32.0)	0.973	-13.6	(-38.4, 11.1)	0.279
	birth until follow-up	2.1	(-33.3, 37.6)	0.906	-13.5	(-41.0, 14.0)	0.333
	short-term exposure	27.1	(1.3, 52.8)	0.039	5.8	(-13.9, 25.4)	0.565

Univariable and multivariable linear regression models for the association per IQR increase of NO_2, O_3, and PM_{10} with lung functional measurements in milliliters at six years.

[a] Multivariable model adjusted for sex, child's age, height, child's diagnosis of asthma, maternal atopy, environmental tobacco exposure and short-term exposure to the examined air pollutant. Coef, Coefficient; FEV_1, forced expiratory volume in one second; NO_2, nitrogen dioxide; O_3, ozone; PM_{10}, particulate matter with an aerodynamic diameter of <10 µm; data available for n=208.

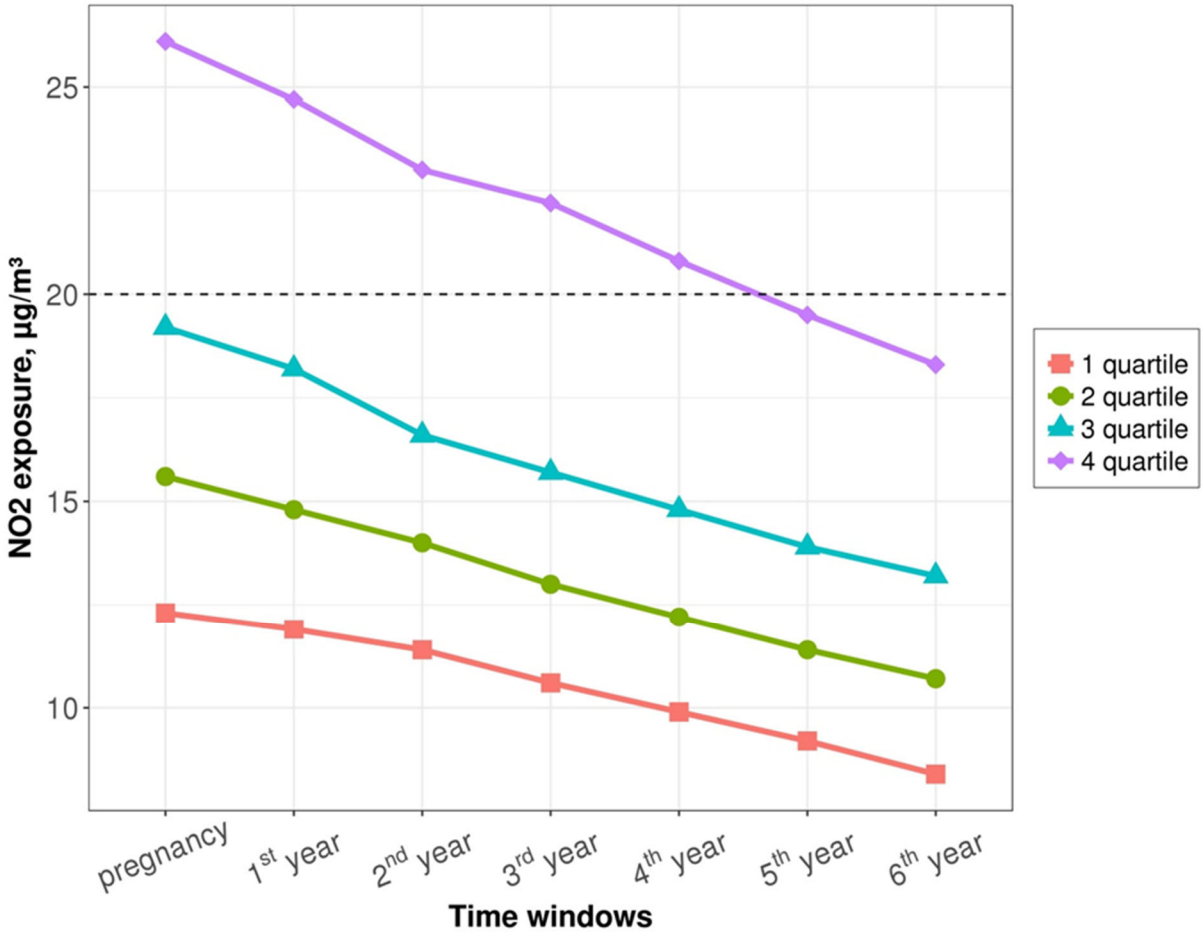

Figure 1 Temporal development of mean NO_2 levels (µg/m³) for each quartile. Temporal development over all investigated time windows (pregnancy, 1st year, 2nd year, 3rd year, 4th year, 5th year, 6th year). The population was divided into quartiles by individual NO_2 levels **(µg/m³)**; data available for n=208, per quartile n=52.

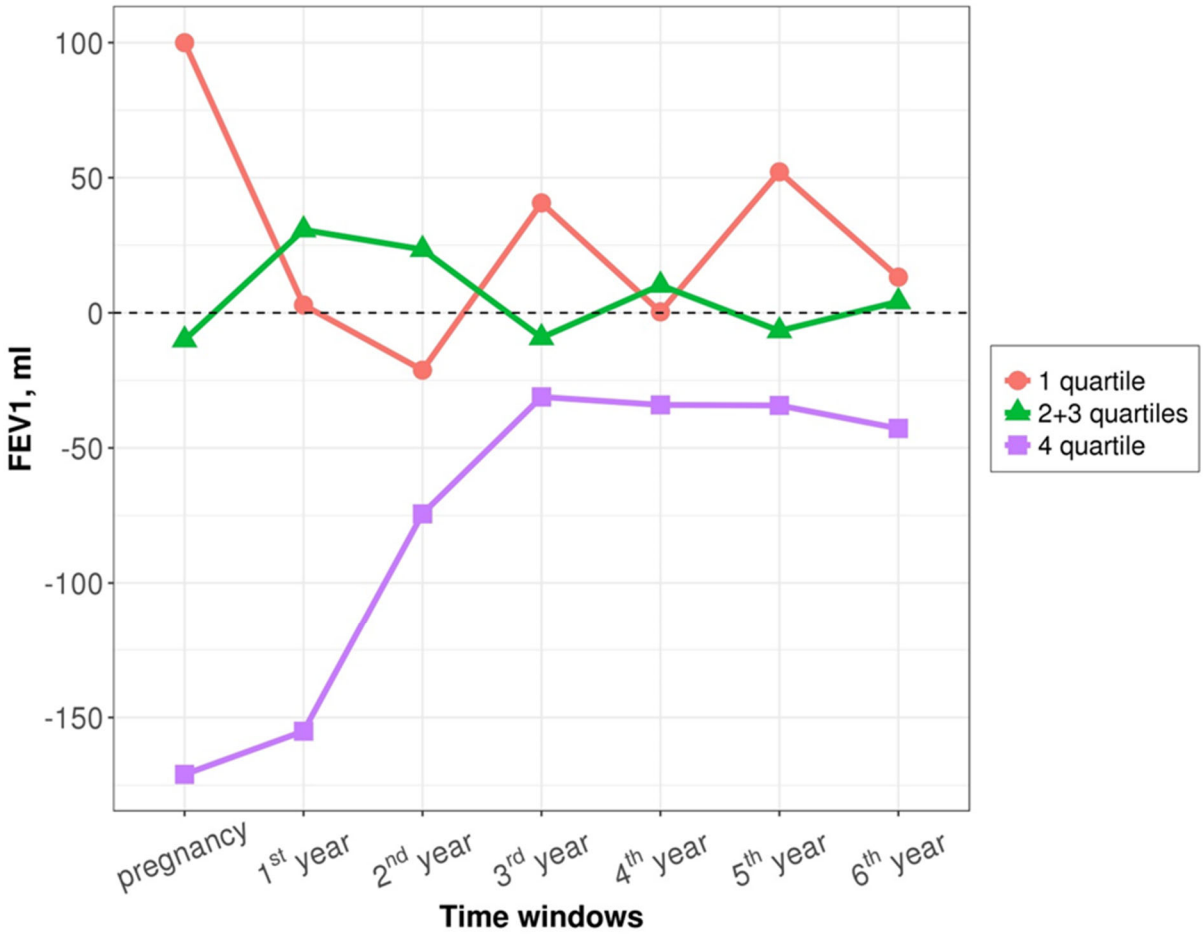

Figure 2 Effect of NO_2 levels ($\mu g/m^3$) on FEV_1 (ml) at six years. The population was divided into quartiles by individual NO_2 levels (per quartile n=52). This resulted in low exposed (1st quartile), mid exposed (2nd and 3rd quartile), and highest exposed (4th quartile) subgroups. NO_2 ($\mu g/m^3$) during different time windows (pregnancy, 1st year, 2nd year, 3rd year, 4th year, 5th year, 6th year) and its effects on FEV_1 (ml) at six years was calculated for 1st quartile, 2nd and 3rd quartile combined, and 4th quartile. Data available for n=208.

Online supplement

Table S1 Anthropometric data and potential risk factors of the study participants compared to children lost to follow-up

	Followed-up	Lost to follow-up	p-value
Number of children	241	63	
Male sex, n (%)	118 (56)	35 (49)	0.351
Length at birth, cm	49.5 (1.9)	49.5 (2.4)	0.419
Weight at birth, g	3359 (419)	3418 (508)	0.170
Maternal atopy [a], n (%)	92 (38)	22 (37)	0.829
Maternal smoking during pregnancy, n (%)	11 (5)	7 (11)	0.042
ETS [b], n (%)	45 (19)	11 (17)	0.825
Parental education [c]			
low, n (%)	46 (19)	14 (23)	0.001
middle, n (%)	101 (42)	10 (17)	
high, n (%)	94 (39)	36 (60)	

Values are mean (standard deviation) or number (percentage). [a] defined as self-reported, doctor-diagnosed asthma, hay fever or eczema; [b] defined as passive smoke at work or at home during pregnancy, as well as parental smoking any time during follow-up; [c] categorized into low (less than four years of apprenticeship), middle (four years of apprenticeship and above) and high (tertiary education).

Table S2 Distribution of estimated residential NO_2 exposure levels for each quartile

NO_2 µg·m^{-3}	Mean	(SD)	Range	IQR
pregnancy				
1. Quartile	12.3	1.1	10.4 – 14.1	
2. Quartile	15.6	1.0	14.2 – 17.4	8.0
3. Quartile	19.2	1.1	17.4 – 21.9	
4. Quartile	26.1	3.1	22.0 – 37.2	
1st year of life				
1. Quartile	11.9	1.0	9.4 – 13.4	
2. Quartile	14.8	0.9	13.4 – 16.3	7.2
3. Quartile	18.2	1.2	16.3 – 20.7	
4. Quartile	24.7	2.9	20.7 – 34.4	
2nd year of life				
1. Quartile	11.4	1.0	9.0 – 12.8	
2. Quartile	14.0	0.8	12.8 – 15.3	5.7
3. Quartile	16.6	1.0	15.3 – 18.4	
4. Quartile	23.0	3.4	18.6 – 33.6	
3rd year of life				
1. Quartile	10.6	0.9	8.8 – 11.8	
2. Quartile	13.0	0.7	11.9 – 14.1	5.7
3. Quartile	15.7	0.9	14.1 – 17.5	
4. Quartile	22.2	3.3	17.6 – 30.4	
4th year of life				
1. Quartile	9.9	0.8	8.3 – 11.2	
2. Quartile	12.2	0.7	11.2 – 13.3	4.9
3. Quartile	14.8	0.8	13.4 – 16.0	
4. Quartile	20.8	3.5	16.2 – 29.4	
5th year of life				
1. Quartile	9.2	0.8	7.6 – 10.3	
2. Quartile	11.4	0.7	10.4 – 12.6	4.8
3. Quartile	13.9	0.8	12.6 – 15.2	
4. Quartile	19.5	3.5	15.2 – 28.4	
6th year of life				
1. Quartile	8.4	0.7	7.0 – 9.6	4.9
2. Quartile	10.7	0.7	9.6 – 11.9	
3. Quartile	13.2	0.7	12.0 – 14.4	
4. Quartile	18.3	3.2	14.4 – 26.6	
Birth until follow-up				
1. Quartile	10.4	0.9	8.5 – 11.6	5.2
2. Quartile	12.8	0.8	11.6 – 14.2	
3. Quartile	15.4	0.8	14.2 – 16.8	
4. Quartile	21.0	3.1	16.9 – 30.2	

Values are means (SD), range and IQR; data available for n=208, per quartile n=52.

Table S3 Association of air pollution during different exposure windows with spirometry (in z-scores) at six years

		Univariable association			Multivariable association [a]		
		FEV_1 (z-scores)					
Exposure		Coef	95% CI	p-value	Coef	95% CI	p-value
NO_2	pregnancy	-0.09	(-0.26, 0.09)	0.325	-0.16	(-0.35, 0.04)	0.126
	1st year of life	-0.08	(-0.26, 0.09)	0.338	-0.16	(-0.37, 0.05)	0.127
	6th year of life	-0.07	(-0.22, 0.07)	0.307	-0.25	(-0.47,-0.02)	0.031
	birth until follow-up	-0.08	(-0.23, 0.06)	0.274	-0.24	(-0.45,-0.02)	0.033
	short-term exposure	0.02	(-0.12, 0.16)	0.740	0.03	(-0.11, 0.17)	0.662
O_3	pregnancy	-0.04	(-0.20, 0.12)	0.633	-0.11	(-0.29, 0.06)	0.203
	1st year of life	-0.09	(-0.26, 0.09)	0.334	-0.11	(-0.28, 0.07)	0.242
	6th year of life	-0.00	(-0.16, 0.15)	0.953	0.02	(-0.13, 0.18)	0.782
	birth until follow-up	-0.13	(-0.31, 0.04)	0.124	-0.24	(-0.45,-0.02)	0.033
	short-term exposure	-0.22	(-0.42,-0.03)	0.027	-0.25	(-0.45,-0.05)	0.014
PM_{10}	pregnancy	-0.12	(-0.28, 0.04)	0.135	-0.14	(-0.30, 0.14)	0.695
	1st year of life	0.01	(-0.13, 0.14)	0.924	-0.01	(-0.15, 0.13)	0.899
	6th year of life	-0.06	(-0.20, 0.08)	0.404	-0.09	(-0.24, 0.06)	0.251
	birth until follow-up	-0.06	(-0.22, 0.10)	0.433	-0.09	(-0.26, 0.08)	0.292
	short-term exposure	0.03	(-0.09, 0.15)	0.594	0.02	(-0.10, 0.14)	0.695
		FEV_1/FVC (z-scores)					
NO_2	pregnancy	0.03	(-0.15, 0.22)	0.739	-0.02	(-0.23, 0.19)	0.849
	1st year of life	0.03	(-0.16, 0.21)	0.773	-0.05	(-0.27, 0.17)	0.631
	6th year of life	-0.01	(-0.16, 0.15)	0.947	-0.14	(-0.37, 0.10)	0.242
	birth until follow-up	0.00	(-0.15, 0.16)	0.967	-0.12	(-0.35, 0.11)	0.303
	short-term exposure	0.08	(-0.07, 0.22)	0.321	0.06	(-0.09, 0.21)	0.414
O_3	pregnancy	-0.14	(-0.31, 0.04)	0.121	-0.16	(-0.34, 0.03)	0.092
	1st year of life	0.03	(-0.17, 0.22)	0.793	0.03	(-0.16, 0.22)	0.738
	6th year of life	0.11	(-0.05, 0.28)	0.177	0.13	(-0.03,0.29)	0.117
	birth until follow-up	-0.00	(-0.19, 0.18)	0.974	0.02	(-0.17, 0.20)	0.869
	short-term exposure	-0.06	(-0.27, 0.16)	0.598	-0.04	(-0.25, 0.17)	0.713
PM_{10}	pregnancy	-0.07	(-024, 0.10)	0.441	-0.07	(-0.24, 0.10)	0.413
	1st year of life	-0.05	(-0.19, 0.10)	0.536	-0.05	(-0.20, 0.09)	0.481
	6th year of life	-0.04	(-0.19, 0.11)	0.603	-0.03	(-0.19, 0.13)	0.722
	birth until follow-up	-0.05	(-0.22, 0.12)	0.570	-0.06	(-0.24, 0.12)	0.523
	short-term exposure	-0.01	(-0.14, 0.11)	0.817	0.00	(-0.12, 0.13)	0.950

Univariable and multivariable linear regression models for the association per IQR increase of NO_2, O_3, and PM_{10} with lung function measurements in z-scores according to Quanjer et al. (19) at six years. [a] Multivariable model adjusted for child's diagnosis of asthma, maternal atopy, environmental tobacco exposure and short-term exposure to the examined air pollutant. Coef, Coefficient; FVC, forced vital capacity; FEV_1, forced expiratory volume in one second; NO_2, nitrogen dioxide; O_3, ozone; PM_{10}, particulate matter with an aerodynamic diameter of <10 µm; data available for n=208.

Table S4 Association of quartiles of NO_2 exposure during different exposure windows with spirometry at six years

	Univariable association			Multivariable association [a]		
	FEV_1 (z-scores)					
Exposure window	Coef	95% CI	p-value	Coef	95% CI	p-value
Pregnancy						
1. quartile	-0.41	(-2.16,1.33)	0.635	-0.23	(-2.06, 1.59)	0.797
2. quartile	0.19	(-1.97,2.35)	0.858	0.31	(-1.80, 2.42)	0.768
3. quartile	-0.14	(-1.83,1.55)	0.871	-0.05	(-1.83, 1.73)	0.955
4. quartile	-0.84	(-1.40,-0.28)	0.004	-1.06	(-1.66, 0.46)	0.001
1st year of life						
1. quartile	-0.13	(-1.79,1.54)	0.877	-0.38	(-2.13, 1.37)	0.665
2. quartile	1.36	(-1.05,3.77)	0.263	1.44	(-0.99, 3.86)	0.239
3. quartile	-0.24	(-1.65,1.18)	0.737	-0.45	(-1.89, 1.00)	0.538
4. quartile	-0.72	(-1.28,-0.17)	0.012	-1.01	(-1.64, 0.38)	0.002
2nd year of life						
1. quartile	-0.70	(-2.09,0.69)	0.314	-0.91	(-2.35, 0.54)	0.214
2. quartile	-0.33	(-2.44,1.79)	0.758	-0.34	(-2.49, 1.81)	0.752
3. quartile	-0.52	(-1.92,0.88)	0.459	-0.56	(-2.07, 0.96)	0.462
4. quartile	-0.34	(-0.68,-0.01)	0.043	-0.51	(-0.85, 0.17)	0.004
3rd year of life						
1. quartile	0.17	(-11.26,1.59)	0.816	0.12	(-1.59, 1.35)	0.867
2. quartile	1.29	(-1.15,3.72)	0.294	1.32	(-1.17, 3.81)	0.293
3. quartile	-0.28	(-1.36,1.92)	0.731	0.41	(-1.36, 2.18)	0.640
4. quartile	-0.03	(-0.39,0.33)	0.866	-0.12	(-0.54, 0.29)	0.866
4th year of life						
1. quartile	-0.39	(-2.02,1.24)	0.632	-0.66	(-2.37, 1.05)	0.442
2. quartile	1.28	(-0.66,3.21)	0.191	1.22	(-0.82, 3.25)	0.191
3. quartile	-0.81	(-2.39,0.78)	0.312	-0.80	(-2.46, 0.85)	0.335
4. quartile	-0.07	(-0.35,0.20)	0.598	-0.16	(-0.48, 0.16)	0.315
5th year of life						
1. quartile	0.37	(-1.07,1.80)	0.609	0.16	(-1.34, 1.66)	0.831
2. quartile	0.91	(-0.92, 2.75)	0.321	0.98	(-0.94, 2.90)	0.309
3. quartile	0.11	(-1.59,1.80)	0.899	0.12	(-1.65, 1.90)	0.891
4. quartile	-0.06	(-0.35,0.24)	0.707	-0.14	(-0.48, 0.19)	0.384
6th year of life						
1. quartile	-0.30	(-1.99,1.39)	0.724	-0.39	(-2.16, 1.38)	0.663
2. quartile	1.38	(-0.52,3.27)	0.152	1.23	(-0.77, 3.23)	0.222
3. quartile	-0.35	(-2.14,1.45)	0.698	-0.40	(-2.34, 1.54)	0.681
4. quartile	-0.10	(-0.41,0.22)	0.540	-0.11	(-0.45, 0.23)	0.512
Birth until follow-up						
1. quartile	-0.03	(-1.53,1.47)	0.970	-0.38	(-1.95, 1.20)	0.631
2. quartile	1.18	(-0.91,3.28)	0.262	1.49	(-0.62, 3.59)	0.162
3. quartile	0.17	(-1.53,1.86)	0.844	0.30	(-1.52,2.12)	0.740
4. quartile	-0.70	(-1.17,-0.22)	0.005	-0.79	(-1.31,-0.27)	0.004

Univariable and multivariable linear regression models for the association per IQR increase of NO_2 with lung functional measurements in z-scores according to Quanjer et al. (19) at six years. [a] Multivariable model adjusted for child's diagnosis of asthma, maternal atopy, environmental tobacco exposure and short-term exposure to the examined air pollutant. Coef, Coefficient; FEV_1, forced expiratory volume in one second; NO_2, nitrogen dioxide; data available for n=208, per quartile n=52.

Table S5 Association of quartiles of O_3 exposure during different exposure windows with spirometry at six years

	Univariable association			Multivariable association [a]		
	FEV_1 (z-scores)					
Exposure window	Coef	95% CI	p-value	Coef	95% CI	p-value
Pregnancy						
1. quartile	0.07	(-1.22, 1.37)	0.909	-0.02	(-1.32, 1.28)	0.974
2. quartile	1.99	(-0.05, 4.04)	0.056	1.99	(-0.16, 4.15)	0.069
3. quartile	1.63	(-0.33, 2.93)	0.015	1.68	(-0.32, 3.04)	0.017
4. quartile	-0.71	(-1.32,-0.11)	0.022	-0.72	(-1.39, 0.05)	0.035
1st year of life						
1. quartile	0.40	(-0.87, 1.67)	0.531	1.05	(-0.23, 2.33)	0.107
2. quartile	-1.78	(-4.13, 0.57)	0.134	-1.82	(-4.16, 0.53)	0.125
3. quartile	1.64	(0.36, 2.93)	0.013	1.66	(0.33, 2.99)	0.015
4. quartile	0.08	(-0.83, 0.98)	0.865	0.07	(-0.95, 1.08)	0.895
2nd year of life						
1. quartile	-0.35	(-1.30, 0.60)	0.459	-0.16	(-1.15, 0.84)	0.756
2. quartile	-0.19	(-2.74, 2.37)	0.885	-0.37	(-2.98, 2.24)	0.777
3. quartile	0.40	(-0.78, 1.58)	0.504	0.65	(-0.58, 1.88)	0.290
4. quartile	-0.35	(-1.10, 0.41)	0.358	-0.35	(-1.15, 0.45)	0.384
3rd year of life						
1. quartile	0.48	(-0.36, 1.31)	0.260	0.49	(-0.38, 1.36)	0.266
2. quartile	-0.85	(-2.60, 0.89)	0.331	-0.90	(-2.89, 1.09)	0.366
3. quartile	-1.20	(-2.89, 0.48)	0.157	-0.79	(-2.38, 0.81)	0.326
4. quartile	0.17	(-0.50, 0.85)	0.605	0.14	(-0.55, 0.83)	0.680
4th year of life						
1. quartile	0.16	(-0.62, 0.95)	0.674	0.23	(-0.62, 1.08)	0.589
2. quartile	-1.14	(-2.89, 0.60)	0.195	-0.82	(-2.49, 0.85)	0.329
3. quartile	0.31	(-1.33, 1.95)	0.705	0.33	(-1.45, 2.11)	0.709
4. quartile	-0.16	(-0.83, 0.51)	0.631	-0.06	(-0.73, 0.61)	0.862
5th year of life						
1. quartile	0.22	(-0.32, 0.77)	0.417	0.23	(-0.35, 0.80)	0.430
2. quartile	-0.34	(-2.06, 1.38)	0.692	-0.38	(-2.15, 1.39)	0.669
3. quartile	-0.57	(-2.28, 1.14)	0.505	-1.00	(-2.73, 0.73)	0.250
4. quartile	0.02	(-0.57, 0.61)	0.952	0.09	(-0.51, 0.69)	0.762
6th year of life						
1. quartile	0.11	(-0.34, 0.56)	0.631	0.07	(-0.41, 0.55)	0.781
2. quartile	-0.45	(-2.35, 1.45)	0.636	-0.55	(-2.52, 1.42)	0.579
3. quartile	-0.41	(-2.07, 1.26)	0.626	-0.59	(-2.32, 1.13)	0.492
4. quartile	0.05	(-0.60, 0.70)	0.869	0.05	(-0.63, 0.72)	0.893
Birth until follow-up						
1. quartile	0.13	(-0.75, 1.01)	0.773	0.05	(-0.96, 1.06)	0.928
2. quartile	1.67	(-0.70, 4.04)	0.163	1.78	(-0.56, 4.12)	0.133
3. quartile	0.03	(-1.30, 1.35)	0.970	-0.41	(-1.91, 1.08)	0.578
4. quartile	-0.11	(-0.74, 0.53)	0.733	-0.09	(-0.80, 0.60)	0.788

Univariable and multivariable linear regression models for the association per IQR increase of O_3 with lung functional measurements in z-scores according to Quanjer et al. (19) at six years. [a] Multivariable model adjusted for child's diagnosis of asthma, maternal atopy, environmental tobacco exposure and short-term exposure to the examined air pollutant. Coef, Coefficient; FEV_1, forced expiratory volume in one second; O_3, Ozone; data available for n=208, per quartile n=52.

Table S6 Association of quartiles of PM_{10} exposure during different exposure windows with spirometry at six years

	Univariable association			Multivariable association [a]		
	FEV_1 (z-scores)					
Exposure window	Coef	95% CI	p-value	Coef	95% CI	p-value
Pregnancy						
1. quartile	0.09	(-0.46, 0.63)	0.746	0.17	(-0.42, 0.76)	0.567
2. quartile	0.72	(-1.17, 2.62)	0.446	0.43	(-1.55, 2.41)	0.664
3. quartile	-0.04	(-1.70, 1.63)	0.964	0.29	(-1.55, 2.13)	0.750
4. quartile	-0.54	(-1.52, 0.45)	0.279	-0.49	(-1.51, 0.54)	0.343
1st year of life						
1. quartile	0.03	(-0.32, 0.38)	0.867	0.02	(-0.35, 0.40)	0.898
2. quartile	0.87	(-0.80, 2.55)	0.302	0.75	(-0.86, 2.36)	0.355
3. quartile	0.83	(-1.15, 2.81)	0.403	0.95	(-1.10, 3.00)	0.355
4. quartile	-0.03	(-0.69, 0.64)	0.937	-0.02	(-0.76, 0.73)	0.966
2nd year of life						
1. quartile	-0.03	(-0.44, 0.38)	0.889	-0.02	(-0.47, 0.42)	0.922
2. quartile	1.50	(-0.26, 3.27)	0.094	1.18	(-0.76, 3.11)	0.226
3. quartile	-0.05	(-1.54, 1.44)	0.944	-0.37	(-1.98, 1.25)	0.647
4. quartile	-0.72	(-1.33,-0.11)	0.022	-0.70	(-1.33,-0.07)	0.031
3rd year of life						
1. quartile	0.15	(-0.23, 0.53)	0.420	0.10	(-0.33, 0.53)	0.629
2. quartile	-1.23	(-2.92, 0.45)	0.147	-2.26	(-3.83,-0.69)	0.006
3. quartile	-0.89	(-3.11, 1.33)	0.424	-0.98	(-3.32, 1.37)	0.407
4. quartile	-0.03	(-0.54, 0.48)	0.900	-0.06	(-0.58,0.47)	0.823
4th year of life						
1. quartile	0.10	(-0.24, 0.43)	0.569	0.10	(-0.27, 0.47)	0.580
2. quartile	-0.04	(-1.85, 1.78)	0.967	-0.25	(-2.06, 1.56)	0.782
3. quartile	0.51	(-1.25, 2.26)	0.565	0.53	(-1.24, 2.30)	0.546
4. quartile	-0.00	(-0.43, 0.42)	0.982	-0.13	(-0.59, 0.32)	0.557
5th year of life						
1. quartile	0.38	(-0.03, 0.78)	0.065	0.38	(-0.10, 0.87)	0.116
2. quartile	-0.45	(-2.45, 1.14)	0.571	-0.64	(-2.28, 1.00)	0.437
3. quartile	0.54	(-1.56,2.65)	0.606	0.49	(-1.67, 2.64)	0.653
4. quartile	0.11	(-0.90, 1.11)	0.834	-0.07	(-1.11, 0.97)	0.893
6th year of life						
1. quartile	0.21	(-0.20, 0.62)	0.305	0.33	(-0.11, 0.76)	0.136
2. quartile	-0.75	(-2.18, 0.69)	0.301	-0.81	(-2.25, 0.62)	0.259
3. quartile	0.04	(-1.91, 1.99)	0.968	0.28	(-1.81, 2.38)	0.786
4. quartile	-0.35	(-1.06, 0.36)	0.325	-0.43	(-1.19, 0.34)	0.275
Birth until follow-up						
1. quartile	0.21	(-0.15, 0.58)	0.250	0.17	(-0.23, 0.58)	0.397
2. quartile	-0.05	(-1.85, 1.75)	0.956	-0.18	(-2.09, 1.72)	0.846
3. quartile	0.13	(-1.91, 2.16)	0.902	0.05	(-2.14, 2.25)	0.961
4. quartile	-0.99	(-2.16, 0.18)	0.096	-0.97	(-2.19, 0.26)	0.119

Univariable and multivariable linear regression models for the association per IQR increase of PM_{10} with lung functional measurements in z-scores according to Quanjer et al. (19) at six years. [a] Multivariable model adjusted for child's diagnosis of asthma, maternal atopy, environmental tobacco exposure and short-term exposure to the examined air pollutant. Coef, Coefficient; FEV_1, forced expiratory volume in one second; PM_{10}, particulate matter <10 µm; data available for n=208, per quartile n=52.

Table S7 Association of air pollution during different exposure windows with spirometry (in z-scores) at six years with/without adjustment for asthma of the child

		Multivariable WITHOUT asthma [a]			Multivariable WITH asthma [b]		
		FEV_1 (z-scores)					
Exposure		Coef	95% CI	p-value	Coef	95% CI	p-value
NO_2	pregnancy	-0.14	(-0.34, 0.07)	0.188	-0.16	(-0.35, 0.04)	0.126
	1st year of life	-0.15	(-0.37, 0.06)	0.167	-0.16	(-0.37, 0.05)	0.127
	6th year of life	-0.22	(-0.45, 0.01)	0.065	-0.25	(-0.47,-0.02)	0.031
	birth until follow-up	-0.21	(-0.44, 0.01)	0.059	-0.24	(-0.45,-0.02)	0.033
	short-term exposure	0.02	(-0.12, 0.17)	0.756	0.03	(-0.11, 0.17)	0.662
O_3	pregnancy	-0.11	(-0.29, 0.08)	0.251	-0.11	(-0.29, 0.06)	0.203
	1st year of life	-0.09	(-0.28, 0.09)	0.317	-0.11	(-0.28, 0.07)	0.242
	6th year of life	-0.05	(-0.12, 0.21)	0.583	0.02	(-0.13, 0.18)	0.782
	birth until follow-up	-0.13	(-0.31, 0.05)	0.147	-0.24	(-0.45,-0.02)	0.033
	short-term exposure	-0.20	(-0.40,-0.01)	0.062	-0.25	(-0.45,-0.05)	0.014
PM_{10}	pregnancy	-0.13	(-0.30, 0.04)	0.124	-0.14	(-0.30, 0.14)	0.695
	1st year of life	-0.01	(-0.16, 0.13)	0.866	-0.01	(-0.15, 0.13)	0.899
	6th year of life	-0.08	(-0.24, 0.08)	0.314	-0.09	(-0.24, 0.06)	0.251
	birth until follow-up	-0.09	(-0.27, 0.09)	0.312	-0.09	(-0.26, 0.08)	0.292
	short-term exposure	0.01	(-0.11, 0.14)	0.826	0.02	(-0.10, 0.14)	0.695
		FEV_1/FVC (z-scores)					
NO_2	pregnancy	-0.03	(-0.25, 0.18)	0.768	-0.02	(-0.23, 0.19)	0.849
	1st year of life	-0.05	(-0.28, 0.18)	0.657	-0.05	(-0.27, 0.17)	0.631
	6th year of life	-0.15	(-0.39, 0.09)	0.209	-0.14	(-0.37, 0.10)	0.242
	birth until follow-up	-0.12	(-0.36, 0.11)	0.289	-0.12	(-0.35, 0.11)	0.303
	short-term exposure	0.09	(-0.06, 0.24)	0.216	0.06	(-0.09, 0.21)	0.414
O_3	pregnancy	-0.15	(-0.34, 0.04)	0.123	-0.16	(-0.34, 0.03)	0.092
	1st year of life	0.05	(-0.14, 0.24)	0.617	0.03	(-0.16, 0.22)	0.738
	6th year of life	0.13	(-0.04, 0.30)	0.120	0.13	(-0.03,0.29)	0.117
	birth until follow-up	0.04	(-0.16, 0.22)	0.719	0.02	(-0.17, 0.20)	0.869
	short-term exposure	-0.07	(-0.28, 0.15)	0.545	-0.04	(-0.25, 0.17)	0.713
PM_{10}	pregnancy	-0.05	(-0.22, 0.13)	0.591	-0.07	(-0.24, 0.10)	0.413
	1st year of life	-0.04	(-0.19, 0.11)	0.610	-0.05	(-0.20, 0.09)	0.481
	6th year of life	-0.01	(-0.18, 0.15)	0.873	-0.03	(-0.19, 0.13)	0.722
	birth until follow-up	-0.04	(-0.22, 0.15)	0.699	-0.06	(-0.24, 0.12)	0.523
	short-term exposure	0.02	(-0.11, 0.15)	0.809	0.00	(-0.12, 0.13)	0.950

Multivariable linear regression models for the association per IQR increase of NO_2, O_3, and PM_{10} with lung function measurements in z-scores according to Quanjer et al. (19) at six years. [a] Multivariable model adjusted for maternal atopy, environmental tobacco exposure and short-term exposure to the examined air pollutant. [b] Multivariable model adjusted for child's diagnosis of asthma, maternal atopy, environmental tobacco exposure and short-term exposure to the examined air pollutant. Coef, Coefficient; FVC, forced vital capacity; FEV_1, forced expiratory volume in one second; NO_2, nitrogen dioxide; O_3, ozone; PM_{10}, particulate matter with an aerodynamic diameter of <10 µm; data available for n=208.

Table S8 Association of quartiles of NO_2 exposure during different exposure windows with spirometry at six years with/without adjustment for asthma of the child

	Multivariable WITHOUT asthma [a]			Multivariable WITH asthma [b]		
	FEV_1 (z-scores)					
Exposure window	Coef	95% CI	p-value	Coef	95% CI	p-value
Pregnancy						
1. quartile	-0.23	(-2.06, 1.59)	0.797	-0.21	(-2.05,1.63)	0.820
2. quartile	0.31	(-1.80, 2.42)	0.768	0.27	(-1.82,2.36)	0.794
3. quartile	-0.05	(-1.83, 1.73)	0.955	0.18	(-1.69,2.05)	0.848
4. quartile	-1.06	(-1.66, 0.46)	0.001	-1.07	(-1.67,-0.47)	0.001
1st year of life						
1. quartile	-0.38	(-2.13, 1.37)	0.665	-0.15	(-1.97,1.67)	0.867
2. quartile	1.44	(-0.99, 3.86)	0.239	1.37	(-1.03,3.77)	0.256
3. quartile	-0.45	(-1.89, 1.00)	0.538	-0.42	(-1.84,1.00)	0.557
4. quartile	-1.01	(-1.64, 0.38)	0.002	-1.02	(-1.66,-0.39)	0.002
2nd year of life						
1. quartile	-0.91	(-2.35, 0.54)	0.214	-0.88	(-2.37,0.60)	0.237
2. quartile	-0.34	(-2.49, 1.81)	0.752	-0.12	(-2.23,1.99)	0.911
3. quartile	-0.56	(-2.07, 0.96)	0.462	-0.58	(-2.09,0.93)	0.445
4. quartile	-0.51	(-0.85, 0.17)	0.004	-0.51	(-0.86,-0.17)	0.040
3rd year of life						
1. quartile	0.12	(-1.59, 1.35)	0.867	0.00	(-1.54,1.54)	0.998
2. quartile	1.32	(-1.17, 3.81)	0.293	1.49	(-0.94,3.92)	0.223
3. quartile	0.41	(-1.36, 2.18)	0.640	-0.75	(-1.23,2.37)	0.524
4. quartile	-0.12	(-0.54, 0.29)	0.866	-0.17	(-0.56,0.27)	0.495
4th year of life						
1. quartile	-0.66	(-2.37, 1.05)	0.442	-0.61	(-2.38,1.16)	0.490
2. quartile	1.22	(-0.82, 3.25)	0.191	1.18	(-0.83,3.18)	0.244
3. quartile	-0.80	(-2.46, 0.85)	0.335	-0.75	(-2.42,0.91)	0.366
4. quartile	-0.16	(-0.48, 0.16)	0.315	-0.17	(-0.49,0.16)	0.311
5th year of life						
1. quartile	0.16	(-1.34, 1.66)	0.831	0.14	(-1.39,1.66)	0.858
2. quartile	0.98	(-0.94, 2.90)	0.309	1.22	(-0.64,3.08)	0.193
3. quartile	0.12	(-1.65, 1.90)	0.891	0.13	(-1.67,1.92)	0.887
4. quartile	-0.14	(-0.48, 0.19)	0.384	-0.17	(-0.50,0.15)	0.293
6th year of life						
1. quartile	-0.39	(-2.16, 1.38)	0.663	-0.39	(-2.18,1.40)	0.665
2. quartile	1.23	(-0.77, 3.23)	0.222	1.36	(-0.52,3.24)	0.152
3. quartile	-0.40	(-2.34, 1.54)	0.681	-0.42	(-2.39,1.55)	0.668
4. quartile	-0.11	(-0.45, 0.23)	0.512	-0.19	(-0.54,0.16)	0.276
Birth until follow-up						
1. quartile	-0.38	(-1.95, 1.20)	0.631	-0.36	(-1.97,1.24)	0.650
2. quartile	1.49	(-0.62, 3.59)	0.162	1.27	(-0.79,3.34)	0.220
3. quartile	0.30	(-1.52,2.12)	0.740	0.46	(-1.37,2.28)	0.618
4. quartile	-0.79	(-1.31,-0.27)	0.004	-0.80	(-1.33,-0.27)	0.004

Multivariable linear regression models for the association per IQR increase of NO_2 with lung functional measurements in z-scores according to Quanjer et al. (19) at six years.

[a] Multivariable model adjusted for maternal atopy, environmental tobacco exposure and short-term exposure to the examined air pollutant. [b] Multivariable model adjusted for child's diagnosis of asthma, maternal atopy, environmental tobacco exposure and short-term exposure

to the examined air pollutant. Coef, Coefficient; FEV_1, forced expiratory volume in one second; NO_2, nitrogen dioxide; data available for n=208, per quartile n=52.

4.4 Article IV - Combination of exhaled metabolites analysis with parallel lung function and FeNO measurements in infants

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Submitted to Analytical Chemistry

Combination of exhaled metabolites analysis with parallel lung function and FeNO measurements in infants

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Why appropriate for Analytical Chemistry:

Secondary electrospray ionization high-resolution mass spectrometry (SESI-HRMS) analysis of exhaled metabolites in adults is becoming an increasingly prominent technique to address clinical questions. The technology has been deployed in the recent years in clinical settings, boosting its relevance in translational research. However, as it stands today, the technique is limited to subjects which can actively provide real-time exhalations into the device. In this manuscript, we describe the development of a novel method to extend the technology to neonates in a clinical setting and potentially other non-cooperative patients (e.g. elderly). This allows for the first time combining established clinical tests such as lung function with non-invasive breath metabolomics. This method will be incorporated into a battery of other -omic platforms and clinical investigations in the context of an epidemiological study; Basel-Bern Infant Lung Development (BILD) cohort (https://www.bild-cohort.ch/)

Abstract

Breath analysis by secondary electrospray ionization-high resolution mass spectrometry (SESI-HRMS) offers the possibility to measure comprehensive metabolic profiles. The technology is currently being deployed in several clinical settings. However, patients are required to exhale directly into the device located in a dedicated room. Consequently, clinical implementation in patients incapable of performing necessary exhalation maneuvers (e.g. infants) or immobile (e.g. too weak, elderly or in intensive care), remains a challenge. The aim of this study was to develop a method to extend such breath analysis capabilities to this sub-population of patients by collecting breath samples remotely (off-line) and promptly (within 10 min) transfer them to SESI-HRMS for chemical analysis. We initially assessed the method in adults by comparing breath mass spectra collected off-line with Nalophan® bags against spectra of breath samples collected in real-time. In total, 13 adults provided 176 pairs of on-line and off-line measurements. Lin's Concordance Correlation Coefficient (CCC) was used to estimate the agreement between off-line and on-line analyses. 1,249 mass spectral features (55% of total detected) exhibited Lin's CCC > 0.6. Subsequently, the method was successfully deployed to analyze breath samples from infants (n = 16), obtaining as a result SESI-HRMS breath profiles. To demonstrate the clinical feasibility of the method, we measured in parallel other clinical variables: i) lung function, which characterizes the breathing patterns and ii) nitric oxide, which a surrogate marker of airway inflammation. As a showcase, we focused our analysis on the exhaled oxidative stress marker 4-hydroxynonenal and its association with nitric oxide and minute ventilation.

Introduction

Breath analysis by mass spectrometry (MS) as a non-invasive technique to capture metabolic profiles of patients, has been subject to research over the last decades (1, 2). Its non-invasive nature makes it very attractive for clinical diagnosis and therapeutic monitoring (3). Within the plethora of available MS-based methods, broadly speaking, there exist two main strategies: real-time and off-line techniques. The former covers several ionization variants, including proton transfer reaction-MS (PTR-MS) (4), selected ion flow tube-MS (SIFT-MS) (5) and secondary electrospray ionization-high resolution mass spectrometry (SESI-HRMS). Real-time breath analysis entails several advantages. Mostly, it is fast (allowing for large screening) and, because there is no sample manipulation, it is less prone to alterations (i.e. analytes undergoing further chemical transformations during analysis (6)). All three MS-based real-time breath analysis platforms have been deployed in clinical settings (7, 8). However, there are cases in which, even if the MS is located in the hospital, real-time breath analysis of patients is not feasible. This includes for example compromised patients that cannot approach the MS facility or non-cooperative patients like infants or elderly. In such cases, hybrid approaches whereby samples are collected in a recipient and analyzed shortly afterwards by real-time mass spectrometry are the only available option (9, 10). Much work has been done in the field to determine the impact of collection of volatile organic compounds in bags for subsequent mass spectrometric analysis (11, 12). However, this has never been determined for SESI-HRMS, whereby hundreds of mass spectral features are typically detected. The aim of this study was to perform such assessment and showcasing the feasibility of the developed method to analyze exhaled metabolites in infants in a real-life, but controlled clinical setting.

Experimental Section

Comparison between real-time and off-line exhaled metabolic profiles in adults

Study participants

In total 13 healthy adult participants (mean age 33.8 years, range 24-43 years, 62% female) included in the study were part of a study with the aim to standardize procedures for breath analysis by SESI-HRMS. Adults provided one to two pairs of on-line and off-line measurements per day with at least 4 hours of rest in between, at least one hour of fasting and abstaining from chewing gum or brushing their teeth prior to the measurement. This resulted in 190 pairs of on-line and off-line measurements, from which 14 pairs had to be excluded due to technical issues or non-adherence to study protocol. Anthropometric data for adults is presented in **Table S1**. The local ethics committees (Ethics Committee for Northwest and Central Switzerland) approved the study (EKBB-Nr. 360/11 and ID 2018-01324), and written informed consent was obtained.

Breath analysis

On-line measurements in adults were performed following the same procedure as previously described 13 (**Figure 1**). Briefly, the breath interface (Exhalion, FIT, Spain) was used to control flow rate and volume of exhalation maneuvers (via exhaled CO_2), maximizing the reproducibility across individuals (14). For each on-line measurement, six consecutive exhalations were acquired. Participants were asked to inhale through their nose and then exhale through a disposable bacterial/viral filter. Exhalion interface was coupled to a SESI ion source (SuperSESI, FIT, Spain) and a HRMS (Q-Exactive Plus, Thermo Fisher Scientific, Germany), as previously described (15, 16). The Q Exactive Plus MS was operated via Q Exactive Tune software in positive polarity full scan mode, over the mass-to-charge range of 70-1000 with a

resolution of 140,000, 4 microscans, automatic gain control target 106 and maximum injection time of 500 ms.

Off-line breath measurements were subsequently provided within 15 min after real-time breath measurements (**Figure 1**). The off-line device consisted of a one-way valve as a mouthpiece (Hudson RCI®), a Nalophan® bag (Nalophan® NA, 20 µm ± 5 µm thick, Kalle) of approximately 2 L volume and 700 cm^2 surface and a tube (Rotilabo® PTFE, 6 mm, 8 mm, length 90 mm, Carl Roth®) connected to a valve (VHK2-08F-08F, SMC Switzerland) at the end of the bag. After off-line collection of breath, the samples were infused into the ion source within 10 mins after collection. The ion source featured a low-pressure mass flow controller that ensured same flow conditions through the ion source for the on-line and the off-line measurements.

Quality control

In the morning before breath measurements, the performance of the SESI-HRMS was checked by measuring a gas standard including 8 components (Dalian Special Gases, Dalian, China; Table S2) diluted to 2 ppb (dilutor Model 2010, Sabio Environmental, Round Rock, US). For all measurements, it was confirmed that the signal intensity of protonated α-Terpinene (m/z 137) was greater than 107 a.u. In addition, each new measurement was compared against historic data obtained for the same gas standard and confirmed there were no significant deviations from previous data points.

Data analysis

Data pre-processing and further statistical analyses were performed using MATLAB (version 2020b, MathWorks Inc., USA). MS raw data was accessed via inhouse C# console apps based on Thermo Fisher Scientific's RawFileReader (version 5.0.0.38). After binning, the time traces for each mass spectra feature were extracted. The area under the curve during the exhalation

windows for each of the features detected was computed and normalized to the time window. The replicate exhalations within each on-line experiment were averaged. A similar procedure was used to calculate the signal intensities for the off-line analysis. As a result, for the adults a data matrix of 352 samples (176 real-time and 176 off-line) × 2,284 features was obtained. The data were 5^{th}-root transformed to approach normal distributions. Lin's concordance correlation coefficient 17 (Lin's CCC) was used to estimate the agreement of the off-line measurements against the real-time technique in adults.

Deployment of off-line method in infants, along with lung function and fractional exhaled nitric oxide (FeNO) in infants

Study participants

From the prospective and ongoing Basel-Bern Infant Lung Development (BILD) cohort (https://www.bild-cohort.ch/), unselected term and preterm infants were recruited to participate in this study (18). Prematurity was defined as gestational age at birth < 37 weeks (19). 16 (11 term and 5 preterm) infants (mean age 47 days, range 29-95 days, 50% female), provided a total of 25 measurements, of which 9 were replicate measurements. Anthropometric data for infants are given in **Table S3**.

Breath collection

Off-line breath collection from infants was performed in unsedated sleep at 44 weeks of postconceptional age. The sampling device consisted of the following elements: medical compressed air (~21 % oxygen, ~78 % nitrogen), manometer, bypass flow, infant mask (face mask size 1, GaeleMed Corporation), t-piece (Hudson RCI®), one-way valve and a Nalophan® bag of approximately 500 mL with 450 cm^2 surface (**Figure 1**). Medical compressed air was administered with a flow rate of 0.3 L/min, helping infants to overcome the resistance of the device (20). Excess air leaked through the bypass flow to the room. Infant face mask had a dead

volume of 25 mL and t-pieces 30 mL. Infants' oxygen saturation and pulse were continuously monitored during breath collection with a pulse oximeter (Masimo Signal Extraction Technology, Masimo). Off-line breath collection was performed until bags were completely filled. If infants were still asleep after the first bag was filled, a second bag was used to gather information on short-term repeatability.

Lung function and FeNO measurements

Tidal breathing flow volume loops were captured using Exhalyzer D (EcoMedics, Duernten, Switzerland) and FeNO measurement recorded by a chemiluminescence analyzer (CLD 77 AM; EcoMedics AG, Duernten, Switzerland) following ERS/ATS guidelines (21). For analysis, we used the first 20-30 regular breaths during non-REM (non-rapid eye movement) sleep from the total recorded breathing as previously described (22) and more into detail in supplementary information.

Data analysis

The data matrix collected for the infants consisted of 25 samples × 2,284 features, whereby nine of the samples were short-term replicates. This initial matrix was visualized using t-distributed stochastic neighbor embedding (t-SNE) (23). After computing the mean of the two short term replicates, the data matrix therefore consisted of 16 samples (unique individuals) × 2,284 features. Pearson’s correlation coefficient between minute ventilation, FeNO and exhaled metabolites was computed.

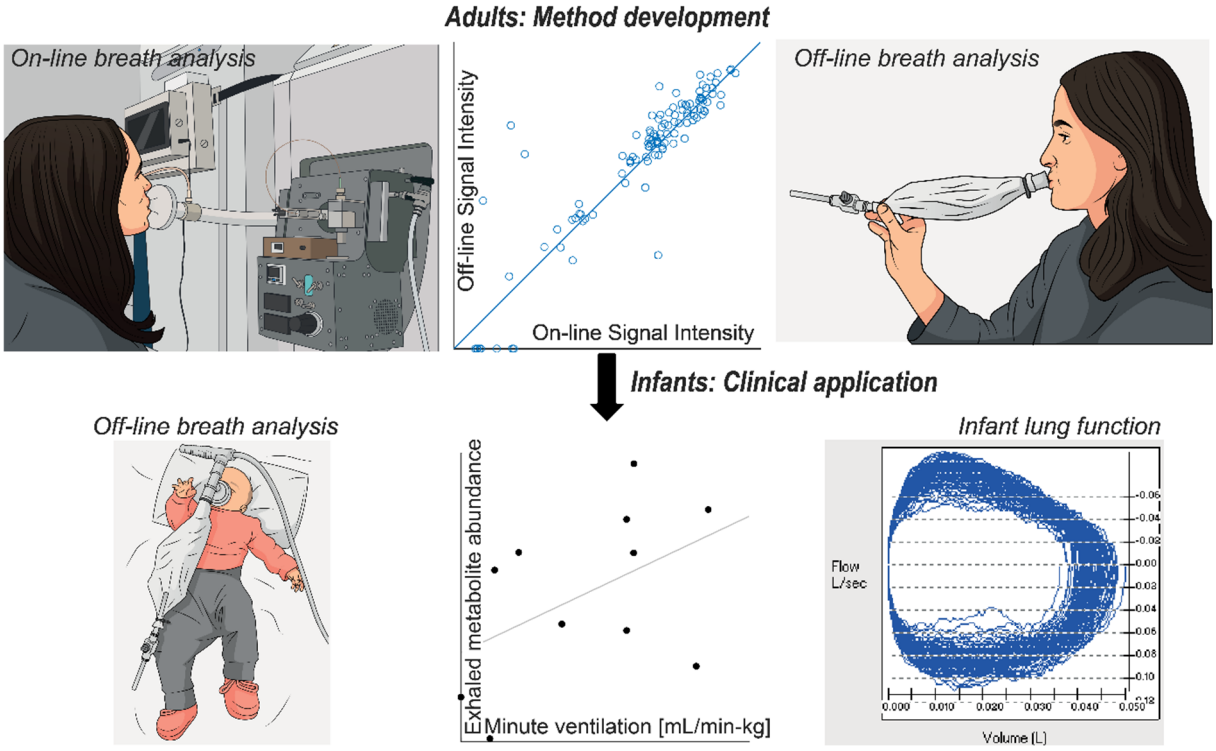

Figure 1. Experimental set-up for adults' and infants' breath analysis. Initially, adults provided on-line and off-line breath samples for comparison of the newly developed off-line technique against the preexisting on-line technique. Once validated, we deployed the method to measure infants during unsedated sleep. Additionally, infant lung function and FeNO measurements were performed in parallel and correlated to exhaled target metabolites.

Results and Discussion

Comparison between real-time and off-line exhaled metabolic profiles in adults

During the first phase of this study, we aimed to develop and assess the performance of the off-line collection method as compared to the real-time approach. The assessment of the agreement between on-line and off-line methods was performed by computing the Lin's CCC for each mass spectral feature (17). Lin's CCC ranges from -1 to +1 (i.e. agreement between two

methods). The histogram of the distribution of Lin's CCC for the 2,284 measured features is shown in **Figure 2a**. Out of 2,284 features, 845 showed a Lin's CCC > 0.8 additionally 404 features had Lin's CCC between 0.6 and 0.8. Hence, 55% of the features exhibited a Lin's CCC > 0.6. Similar moderate agreement between real-time and off-line collection methods have been observed for SIFT-MS (24). **Figure 2b** shows the distribution of the location shift for the portion of features with a Lin's CCC > 0.6. It shows a tendency towards negative values, indicating that the signals tend to be reduced in the off-line measurements, suggesting a general trend of adsorption and losses of the metabolites onto the surface of Nalophan® bag. Positive values probably correspond to impurities released by Nalophan® bags, some of which have been previously characterized (25).

We further investigated whether there was a trend for the heavier species ―generally less volatile― to exhibit a stronger tendency to be lost in the off-line collection process. **Figures S1-S3** show the location shift as a function of m/z. No apparent trend was observed. Further insights on the Lin's CCC analysis are shown in **Figures S4-S6**. We then investigated whether some chemical families of exhaled metabolites exhibited a systematic greater or lower Lin's CCC. To do so, we concentrated in a series of aldehydes previously identified in exhaled breath by SESI-HRMS (26). **Figure 2c** displays Lin's CCC vs chain length for three homologous series of aldehydes: 2-alkenals, 4-hydroxy-2,6-alkadienals and 4-hydroxy-2-alkenals. It shows that there is no clear dependency of the quality of the bag measurement (i.e. Lin's CCC) on the chain length, but it rather suggests that the functional groups in the molecular structure of the metabolites dictate how well the signals are preserved in the bag. For example, the overall Lin's CCC for the 2-alkenals family is lower than for other two series of aldehydes containing a hydroxyl group, suggesting that the former family has a greater affinity for Nalophan® (i.e. "sticky" nature). Some outliers are particularly evident. For example, 4-hydroxy-2-dodecenal's Lin's CCC falls well below the overall trend of the family. This may be rationalized by the

presence of several isomeric species —hence with different affinities for Nalophan®— which are simultaneously detected. This is one limitation of SESI-HRMS technique, as the lack of chromatography implies that isomers cannot be resolved. Within the latter family of aldehydes, we paid particular attention to 4-hydroxynonenal (4-HNE). 4-HNE is a major product of n-6 fatty acid oxidation. It has been shown to be involved in a great number of pathologies such as metabolic diseases, neurodegenerative diseases and cancers (27). We have recently found evidence that preterm infants are subject to increased oxidative stress (under review), hence we hypothesize that 4-HNE may be altered in this preterm population, which accounts to around 10 % of births worldwide (28).

Figure 2d shows the on-line vs off-line signal intensity for all pairs of measurements for 4-HNE. Lin's CCC for this metabolite was 0.61, which is in the lower end of the cut-off we considered in this study. Another common representation for method comparison is so-called Bland-Altman plots (**Figure 2e**). It shows that the mean difference between the two pairs of measurement (i.e., online minus offline) is below zero, indicating that 4-HNE tends to be retained in the bags, although the effect is not dramatic. It is also noticeable that outliers tend to appear at the weakest signal intensities (mean of two measurements < 13 a.u.), whereas for stronger signal intensities the information is more faithfully preserved. Overall, although far from perfect (i.e. Lin's CCC = 1), the collection of breath samples in such Nalophan® bags allows for the analysis of 4-HNE with a reasonable degree of confidence. Please note that this is within the timeframe of ten minutes, which is approximately the time it requires to transport the sample within the hospital facilities. Longer storing times at room temperature may probably compromise the quality of the measurement.

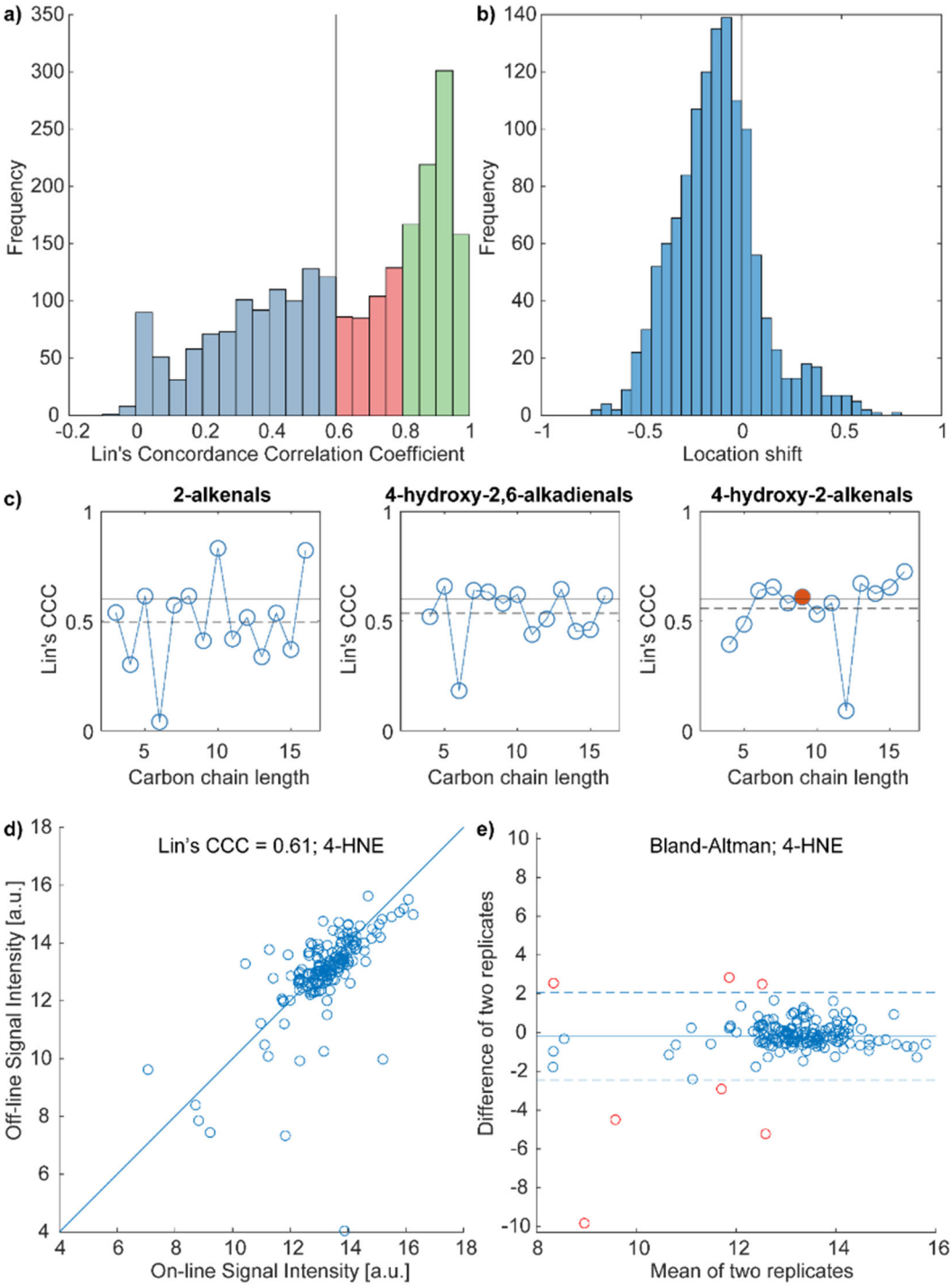

Figure 2 a) Distribution of Lin's CCC for the 2,284 measured features. **b**) Distribution of the location shift for the features with a Lin's CCC > 0.6 **c**) Lin's CCC vs. chain length for three homologous series of aldehydes: 2-alkenals, 4-hydroxy-2,6-alkadienals and 4-hydroxy-2-alkenals (red = 4-HNE; solid lines Lin's CCC = 0.6; dashed lines mean Lin's CCC for the group) **d**) On-line vs off-line signal intensity for all pairs of measurements for 4-HNE **e**) Bland-

Altman plot shows the mean difference of 4-HNE between on-line and off-line measurement pairs.

Deployment of off-line method in infants

Repeatability

Once the off-line technique in adults was validated against the gold standard (i.e., real-time analysis), we extended the method to the analysis of exhaled metabolites in infants, while in parallel lung function tests and FeNO measurements were carried out. Based on the validation results, we further considered only the mass spectral features with a Lin's CCC > 0.6. In order to summarize the information contained in the high-dimensional space of the breath mass spectra, we performed t-SNE analysis (23). **Figure 3a** shows the resulting scatter plot (last two numbers of patient ID shown). The plot shows that, for those infants where two replicate samples could be collected, the measurements cluster together. This further reassures the quality of the measurement. The plot suggests four main clusters of patients. We did verify whether this could be influenced by a batch effect, but this was not the case. For example, patients BILD4386 and BILD4387 were twins measured on the same day, yet they tend to occupy different spaces in the t-SNE domain. Taking together, this provides an initial indication that the method can provide a true metabolic read-out from the exhaled breath of infants.

Exhaled metabolites vs lung function

Subsequently, we further explored the data to evaluate whether the breath metabolites may be associated with the lung function parameters. For the first time, we were capable of collecting parallel breath specimens from infants who were subjected to routine clinical examinations of the respiratory system (n = 11). **Figure 1** shows an example of a flow-volume loop for one of

the participants. **Figure 3b** shows distribution of the minute ventilation per kg body weight. The data falls within previously measured values in a large cohort (histogram of data from Fuchs et al. (22) shown for reference), suggesting that these patients are a representative sample of a typical infant population. To better understand the associations between breathing mechanics and the exhaled metabolites, we then computed Pearson's correlation coefficients. **Figure 3b** shows the histogram of the distribution of correlation coefficients between minute ventilation and all mass spectral features with Lin's CCC > 0.6. The distribution is centered around zero, suggesting an independence of the vast majority of the exhaled metabolites from the breathing pattern (for reference, random associations resulting from a Monte Carlo simulation are overlaid). **Figure 3b** shows a specific example on the (in)dependence between one such metabolite (i.e. 4-HNE) and minute ventilation. Despite our rather limited sample size, we found no correlation between 4-HNE and minute ventilation. This is somehow to be expected as the concentration of metabolites within the sampling bag will be "averaged" independently of the breathing patterns.

Exhaled metabolites vs inflammation marker FeNO

The FeNO values observed in our small cohort were also within the expected range according to previous studies, albeit leaning towards highest values of the spectrum (**Figure 3c**). In contrast to minute ventilation, we found a rather clear trend towards negative correlations between FeNO and exhaled metabolites, including 4-HNE (**Figure 3c**). The reasons why negative associations may exist between exhaled 4-HNE and FeNO are at this point unclear and drawing any conclusion from this limited dataset would be premature. However, FeNO has been associated with a wide range of pathophysiological processes (mainly inflammation of the airways), whereas 4-HNE is regarded as an oxidative stress marker. Hence it is reasonable to think that they will provide complementary information.

Our goal in the coming years is to identify significant associations between markers of oxidative stress such as 4-HNE and clinical characteristics using the methodology presented in this manuscript. For this reason, we then took advantage of this pilot study to estimate the number of patients that we will require to enroll in a follow-up study. The results of the calculation are shown in **Figure 3d** (see supporting information for details). Assuming a significance level of 5%, with 90% power, the goal of detecting an absolute correlation of at least r = 0.22 (like HNE vs FeNO), will require a sample size of n = 210 participants.

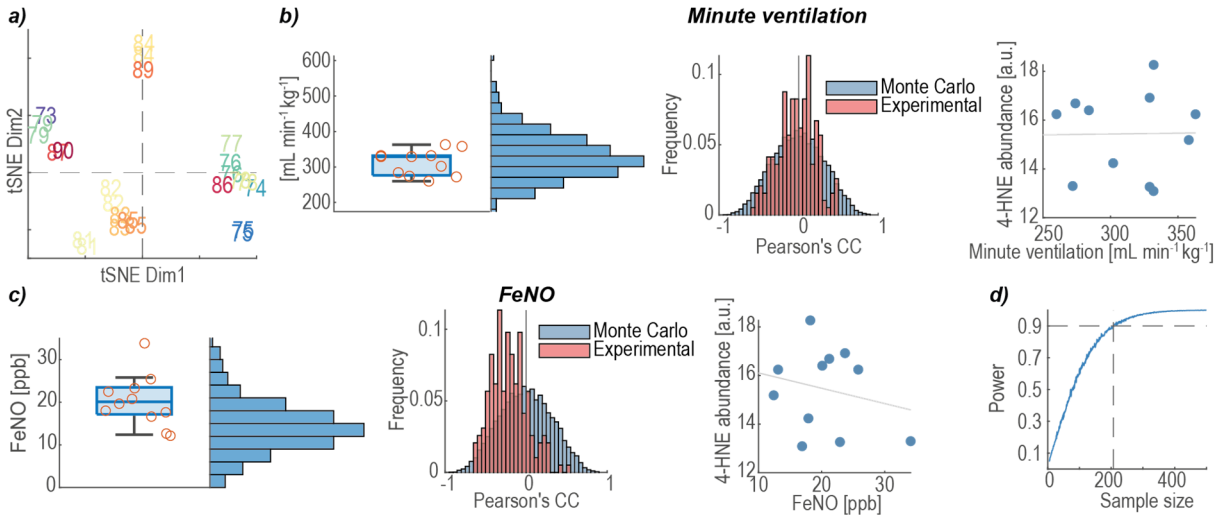

Figure 3 a) Results from t-SNE analysis for the breath mass spectra detected for infants (each color represents an individual); patients' number are represented in the scatter plot, note how replicate measurements cluster together. **b**) Distribution of minute ventilation/kg body weight of infants enrolled in this study, data falls within previously measured values in a large cohort (histogram of data from Fuchs et al. (22) shown for reference). Histogram of experimental correlation coefficients and of Monte Carlo simulation of the expected distribution of correlations for minute ventilation/kg with the MS data, with a relationship for 4-HNE vs minute ventilation/kg (r = 0.01; p = 0.97) **c**) Distribution of measured FeNO of infants enrolled in this study and histogram of normative data on FeNO in a birth cohort of infants. Histogram

of experimental correlation coefficients and of Monte Carlo simulation of the expected distribution of correlations for FeNO with the MS data, with a relationship for 4-HNE vs FeNO ($r = 0.22$; $p = 0.51$) **d**) Estimation of number of patients needed in a follow-up study.

Clinical relevance

Adding a metabolic dimension to lung function and clinical tests via exhaled breath analysis holds promise towards a more personalized approach. Extending this possibility to non-cooperative patients (e.g. children or cognitive disabled patients) and immobile patients due to the nature of their disease or treatment (e.g. infectious disease or treatment in intensive care unit) opens new possibilities for therapeutic drug monitoring and diagnosis of disease, especially in pediatric medicine.

Conclusion

We have developed an off-line collection and subsequent high-resolution mass spectrometric analysis method for breath in a clinical setting. The characterization of the agreement of the off-line and real-time analysis revealed over 1,000 mass spectral features of moderate to high quality. Among these metabolites there were relevant ones such as 4-HNE, which could be measured for the first time in infants, along with lung function characteristics and FeNO as a clinical outcome.

Acknowledgments

The authors thank all the study participants for participating in the study, Fiona Beck (University of Basel Children's Hospital) for her critical reading of the manuscript and Mélina Richard for her graphical support and research coordination. Isabel Gonzalez Novoa is gratefully acknowledged for her support during the lung function tests. The authors further thank Oliver Fuchs and Philipp Latzin for sharing normative lung function data. This work is also part of the Zurich Exhalomics project under the umbrella of the University of Medicine Zurich/Hochschulmedizin Zürich. This study was funded by the Fondation Botnar, Switzerland (Professorship to Pablo Sinues) and the Swiss National Science Foundation (grant No. 320030_173168).

Conflict of interests

PS is co-founder of Deep Breath Initiative A.G. (Switzerland), which develops breath-based diagnostic tools. KDS is consultant for Deep Breath Initiative A.G. (Switzerland)

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Online supplement

Lung function measurements

Pulmonary function was performed at 44 weeks of postconceptional age using Exhalyzer D (EcoMedics, Duernten, Switzerland) according to current ERS/ATS guidelines (1). For analysis we used the first 20-30 regular breaths during non-rapid eye movement (non-REM) sleep from the total recorded breathing. We excluded sighs and 10 breaths before and after a sigh. Simultaneous to tidal breathing recording, the fraction of exhaled nitric oxide (FeNO) was measured online with a chemiluminescence analyzer during the third quartile of expiration and averaged over the 20-30 breaths used for analysis (2). Following ERS/ATS guidelines for infant lung function testing, mean tidal flows, volume and flow-volume loop were calculated. We investigated respiratory rate (RR), mean tidal volume (V_T) and minute ventilation (V_E). Ratio of time to peak tidal expiratory flow (PTEF) and expiratory time ($t_{PTEF/tE}$) were used to describe TBFVL shapes.

Sample size calculation

The required sample size for a future study (**Figure 3d**) was determined based on Monte Carlo simulations generating 10,000 samples of size 5 to 500 to test the correlation between the two variables: 4-hydroxynonenal (4-HNE) and other clinical variables (e.g. minute ventilation). For a given sample size, we generated Monte Carlo simulations to determine an approximate cutoff value for a test of the correlation. We then generated samples under the alternative hypothesis, and estimated the power of the test, being:

Null hypothesis: exhaled 4-HNE and lung function variables are uncorrelated.

Alternative hypothesis: exhaled 4-HNE and lung function variables are correlated with an absolute value of r at least as high as 0.22 (4-HNE vs FeNO).

Table S1 Anthropometric data from adult participants

ID	Age (year)	Gender	Number of measurements
DOPAEx2_BS07	36	Female	56
DOPAEx2_BS09	24	Male	50
DOPAEx2_BS10	28	Female	54
DOPAEx2_BS11	36	Male	20
DOPAEx2_BS12	36	Male	22
DOPAEx2_BS13	28	Female	22
DOPAEx2_BS14	32	Female	20
DOPAEx2_BS15	34	Female	22
DOPAEx2_BS16	30	Female	20
DOPAEx2_BS17	36	Male	22
DOPAEx2_BS18	43	Female	20
DOPAEx2_BS19	38	Female	22
DOPAEx2_BS20	38	Male	22

Table S2 Anthropometric data from infant participants

ID	Number of measurements	Gender	Birth	Age (days)	Respiratory rate (breaths/minute)	Mean tidal volume (mL)	Mean tidal volume/kg (mL/kg)	Minute ventilation (mL/minute)	Minute ventilation/kg (mL/minute-kg)	FeNO [ppb]
BILD_4373	1	Female	Term	32						
BILD_4374	1	Male	Term	33						
BILD_4375	2	Female	Term	34	52	37	6.9	1933	358	12.4
BILD_4376	2	Male	Term	29	37	33	6.9	1248	260	25.8
BILD_4377	1	Male	Term	47	33	39	9	1299	302	17.9
BILD_4378	2	Male	Term	35	40	42	9.1	1670	363	13.1
BILD_4379	2	Female	Term	34						
BILD_4381	2	Female	Term	35	41	32	8	1306	329	23.7
BILD_4382	2	Female	Term	35						
BILD_4383	2	Male	Preterm	78	52	33	6	1729	332	18.2
BILD_4384	2	Male	Term	33						
BILD_4385	2	Female	Preterm	41	29	36	9	1046	274	21.2
BILD_4386	1	Male	Preterm	75	39	38	8.5	1479	329	22.9
BILD_4387	1	Male	Preterm	75	37	33	7.4	1217	272	34.1
BILD_4389	1	Female	Term	33	35	38	8	1291	284	20.1
BILD_4390	1	Female	Preterm	95	39	41	8.5	1593	332	16.9
			Mean (STD)	**47 (21)**	**39 (7)**	**37 (3)**	**8 (1)**	**1437 (266)**	**312 (36)**	**21 (6)**

Table S3 Compounds contained in standard gas mixture used for quality control

MS polarity	m/z $[M+H]^+$	Name	Formula
Positive	59.0491414	Acetone	C_3H_6O
Positive	69.0698769	Isoprene	C_5H_8
Positive	73.0647915	2-Butanone	C_4H_8O
Positive	87.0804415	2-Pentanone	$C_5H_{10}O$
Positive	93.0698769	Toluene	C_7H_8
Positive	105.0698769	Styrene	C_8H_8
Positive	121.101177	Mesitylene	C_9H_{12}
Positive	137.1324771	α-Terpinene	$C_{10}H_{16}$

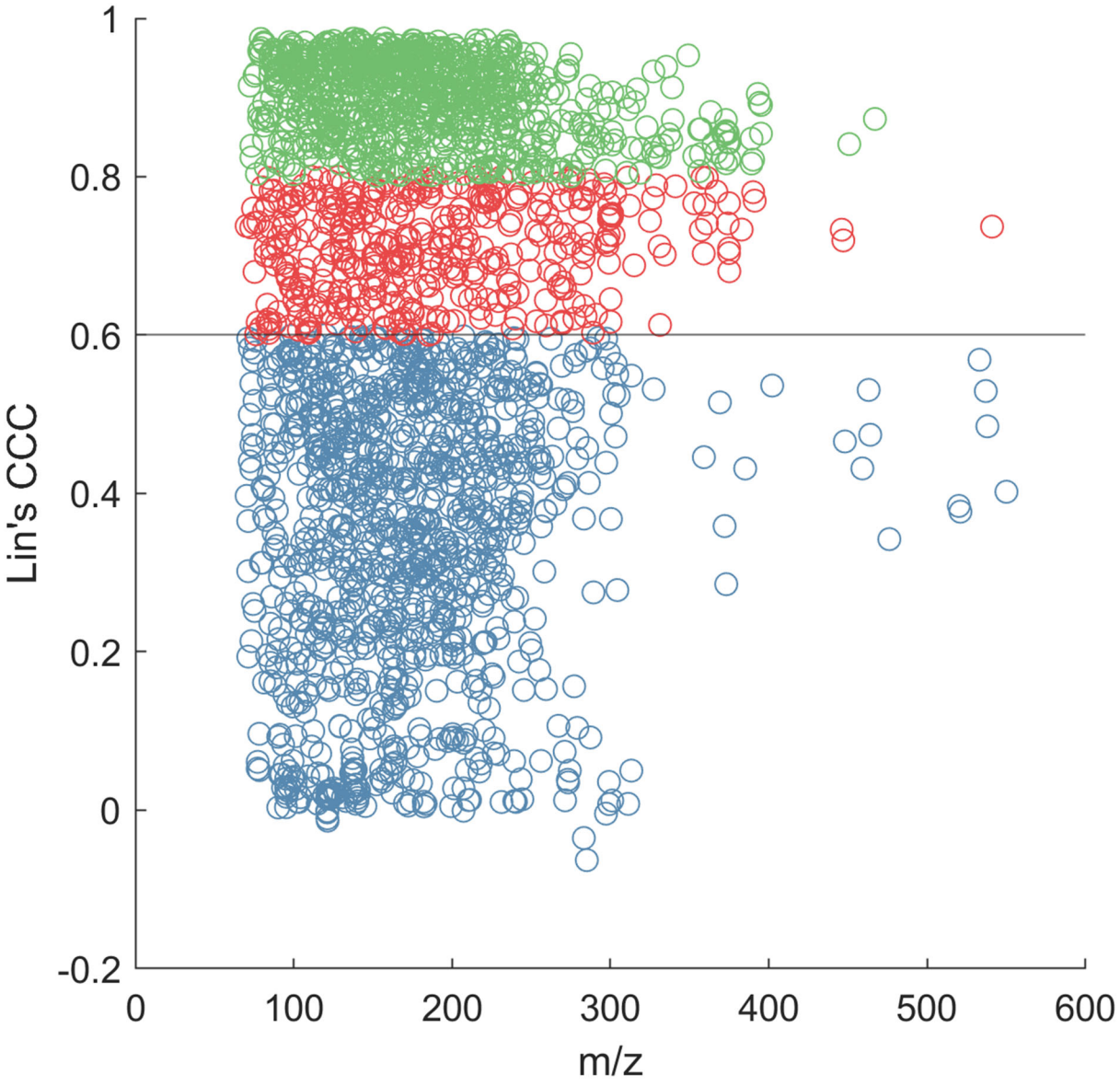

Figure S1 Lin's concordance correlation coefficient as a function of m/z for all measurements of adults. Highest quality data points are colored in green (Lin's CCC > 0.8), moderate in red (Lin's CCC 0.6-0.8) and poor quality in blue (Lin's CCC < 0.6)

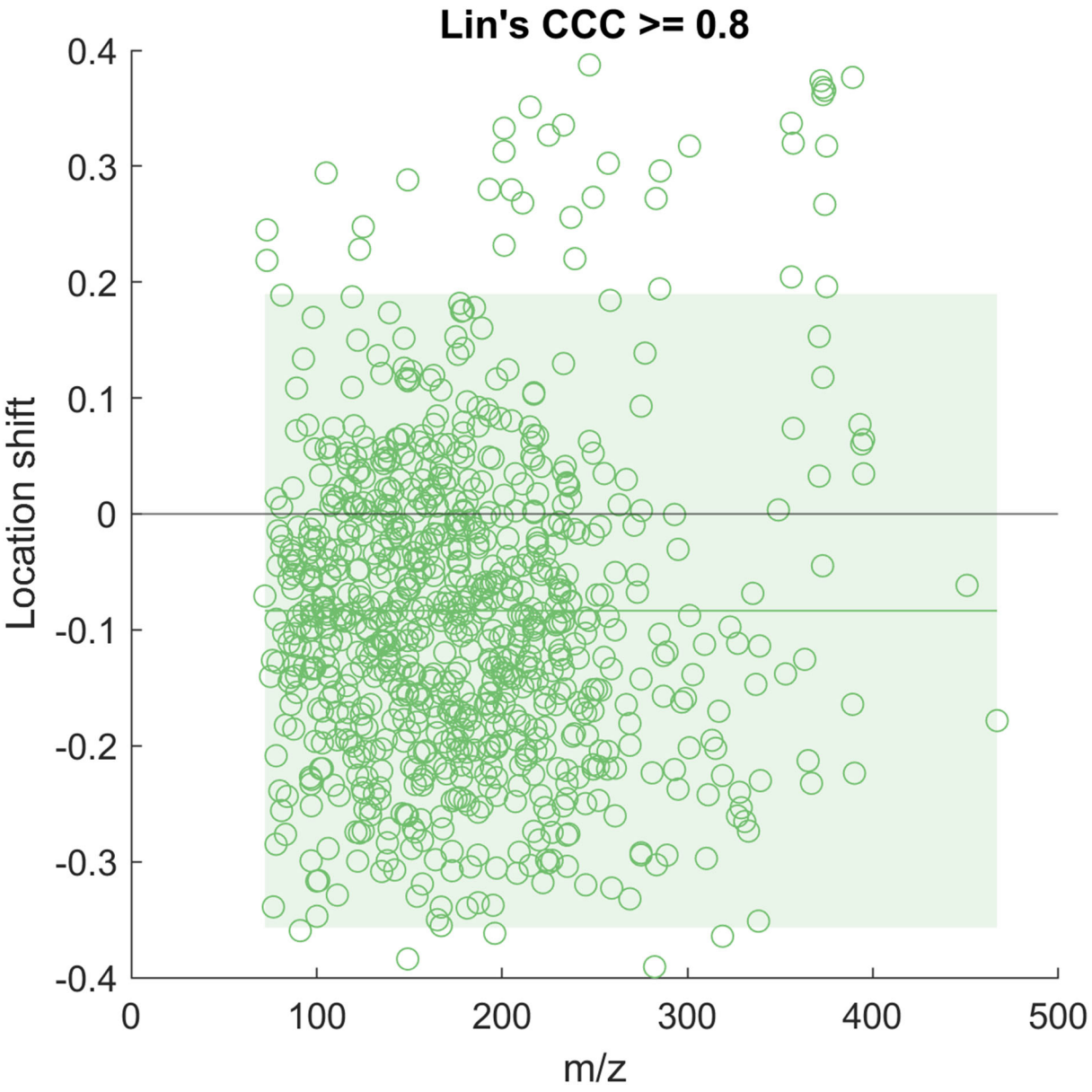

Figure S2 Location shift as a function of m/z for measurements of adults with Lin's CCC ≥ 0.8. Green line indicates the mean and band represents 95% confidence interval.

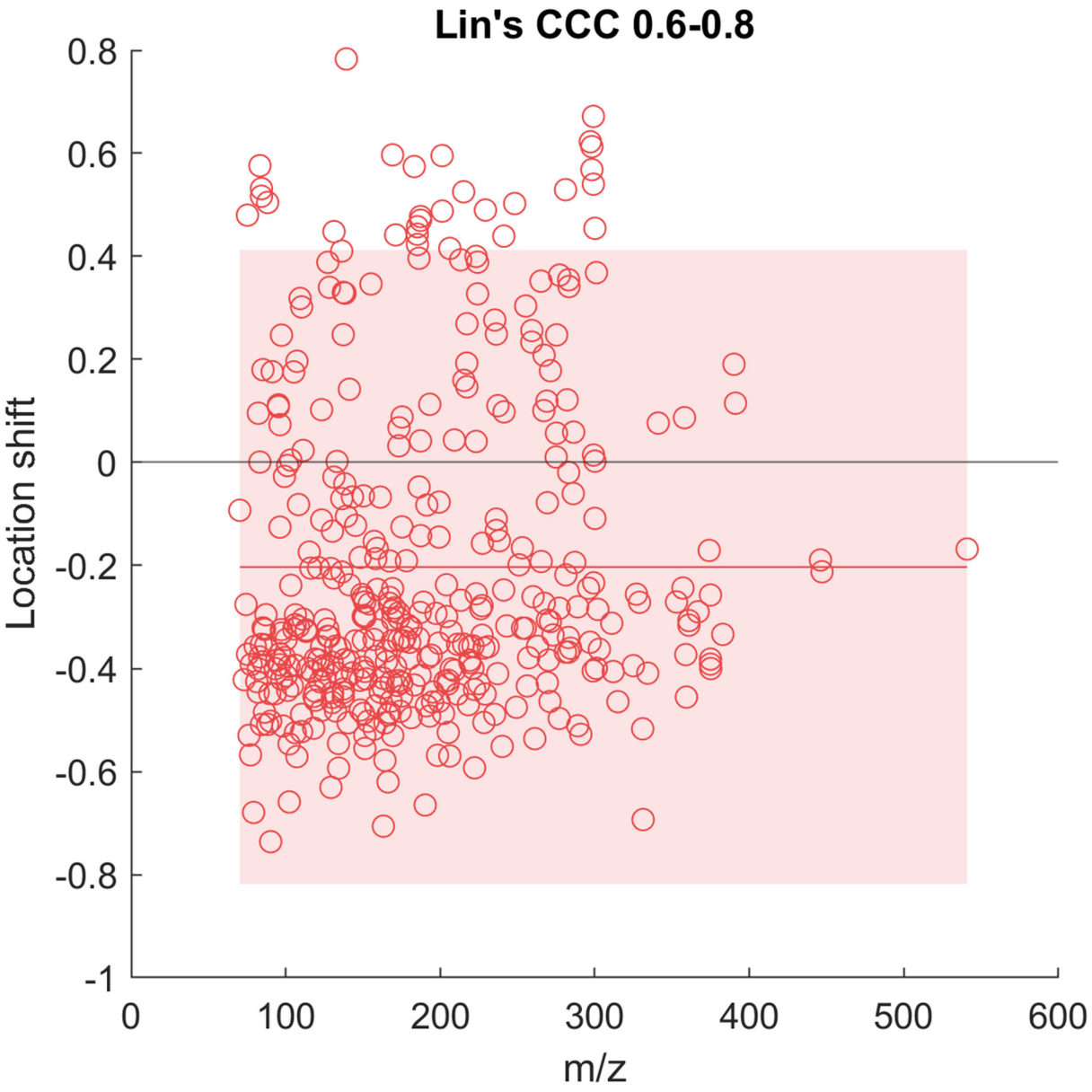

Figure S3 Location shift as a function of m/z for measurements of adults with Lin's CCC ≥ 0.6 and < 0.8. Red line indicates the mean and band represents 95% confidence interval.

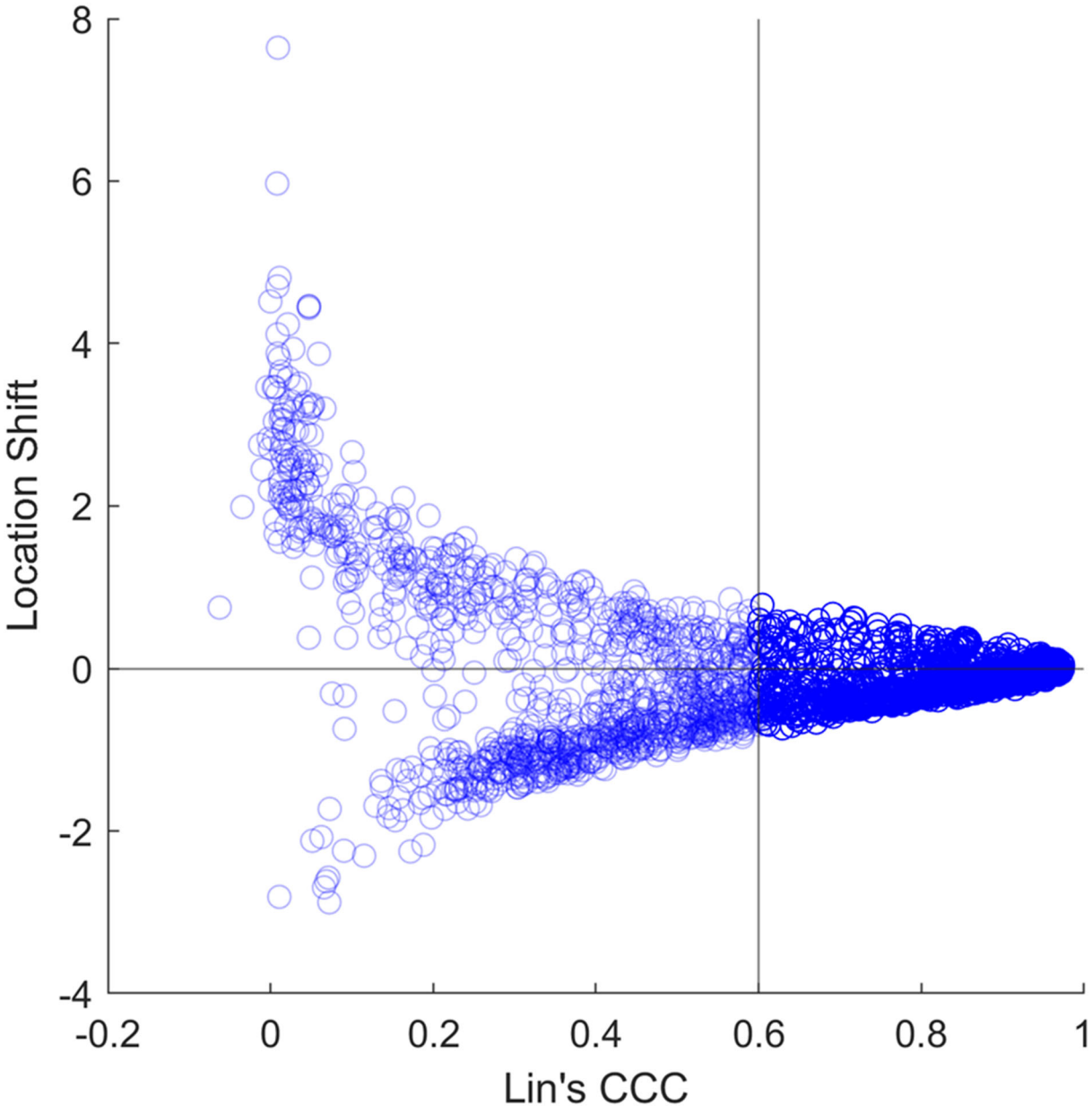

Figure S4 Location shift as a function of Lin's concordance correlation coefficient.

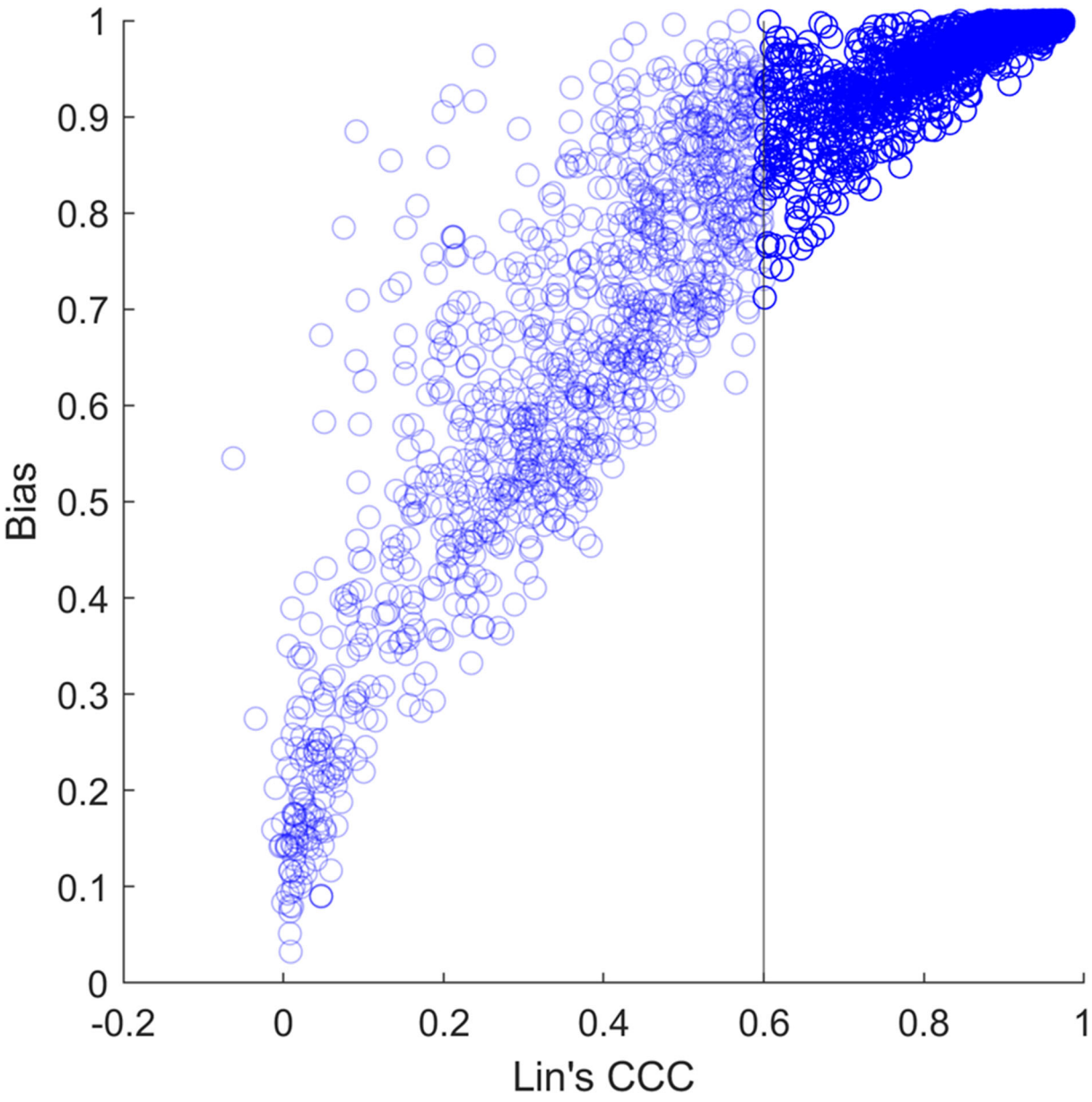

Figure S5 Bias as a function Lin's concordance correlation coefficient.

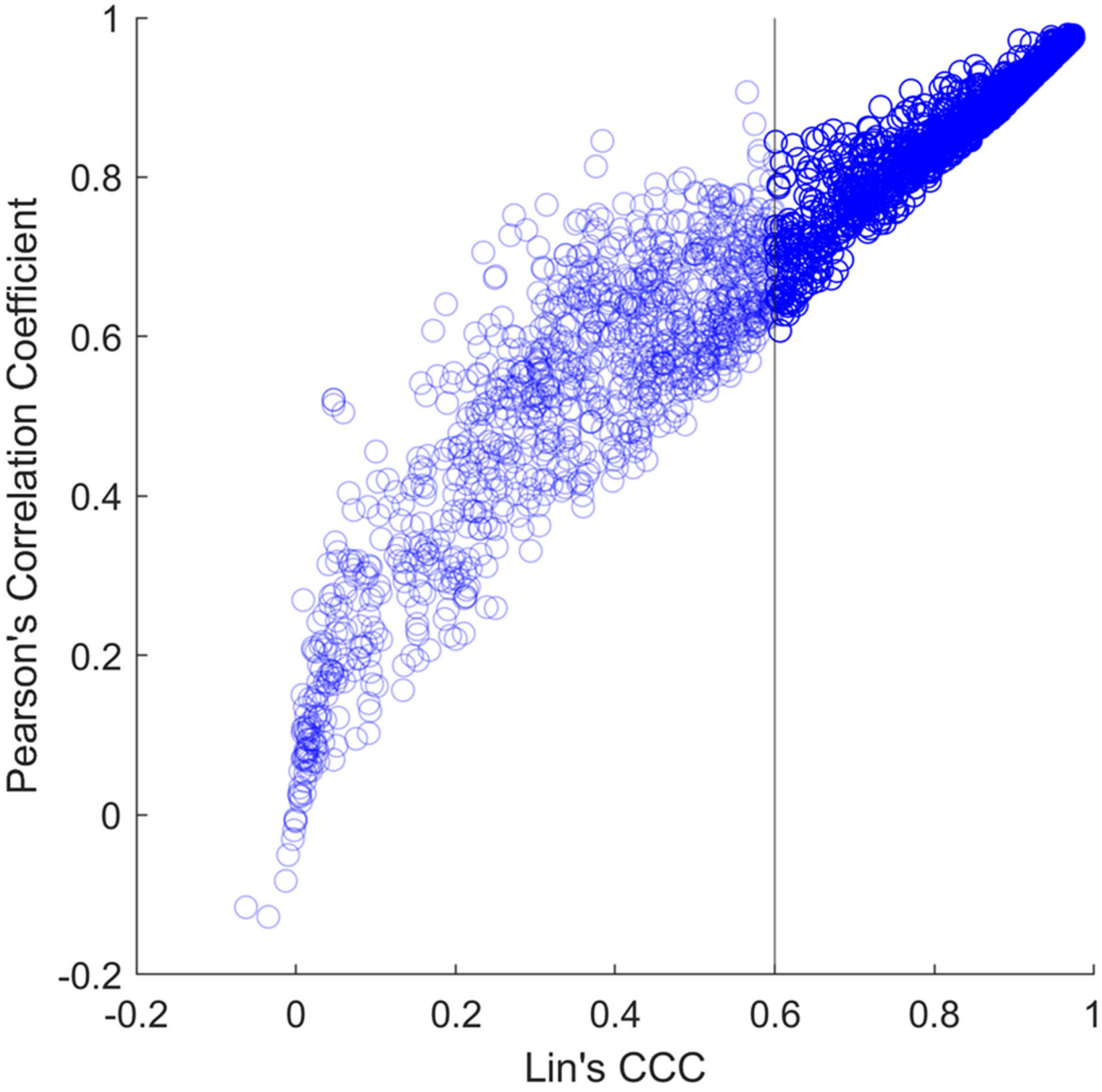

Figure S6 Pearson's correlation as a function of Lin's concordance correlation coefficient

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5 General discussion

This PhD thesis extends and corroborates evidence regarding the impact of air pollution on lung function in infancy and school-age and on inflammatory and metabolic profiles of preterm and term infants. We would like to answer the initially defined aims:

1. *To understand the complex network of interactions leading to impairment of lung function development in utero, in early life, and in later childhood*: We comprehensively reviewed the literature (Article I)
2. *To assess the effect of low-to-moderate pre- and postnatal exposure air pollution:*
    a. *with lung function at 44 weeks of postconceptional age (PCA)*
    b. *with lung function of healthy formerly term–born children at school-age*
    c. *to investigate specific windows of enhanced vulnerability pre- and postnatally*
    d. *to investigate the potential susceptibility of subgroups, such as preterm infants*

    (Article II and III)

Following our aims to further investigate the impact of environmental stressors on lung function throughout childhood, we first investigated the effects that air pollutants might have on lung function in preterm and term infants and school-aged children. Preterm infants represent a vulnerable subgroup that needs special attention, due to impaired capacity to deal with oxidative stress perinatally (10-13). We were the first to assess the association of air pollution and infant lung function in preterm compared to term infants, and were hence able to show detrimental effects of low-to-moderate air pollution levels on lung function at 44 weeks PCA in both populations. These effects were most pronounced when increases in air pollution levels occurred during a time window of fast lung development and growth, such as the second trimester of pregnancy. Further, as a proof of concept, moderate to late preterm infants (born 32 - 36 weeks GA) showed aggravated impairment of lung function in association with air

pollution. These infants additionally had increased levels of FeNO as an inflammatory response to air pollution, a known oxidative stressor to the airways.

Results from our study in school-aged, healthy term-born children underlined the findings of adverse effects of air pollution, in concentrations below the currently recommended thresholds, on lung function in childhood. Most pronounced effects were again seen, when higher air pollution levels occurred during windows of fast lung growth, such as pregnancy and the first and second year of life.

3. *To develop and standardize a collection and analysis platform for metabolic information retrieval of infants in exhaled breath by SESI-HRMS*
    a. *to prove feasibility of the off-line measuring technique by i) measuring key metabolites in comparison to the gold standard method; ii) investigating short-term repeatability; iii) investigating the in-/dependency of the collection method on breathing patterns; iv) exploring an exemplary relationship between oxidative stress markers measured off-line by SESI-HRMS and an independently measured, less specific marker of oxidative stress and inflammation (FeNO)* (Article IV)

Lung function and inflammatory markers, such as FeNO, are some of the methods used to measure the potential effects that air pollution might have on respiratory health in childhood. Oxidative stress markers specific to the respiratory system would can be another important aspect. However, there are currently no standardized procedures available to measure such in uncooperative patients. We therefore developed and standardized a technique to measure the metabolic profile of preterm and term infants by SESI-HRMS. The performance of the newly developed method in comparison to the gold standard technique and in terms of short-term repeatability was reassuring. The independence of target metabolites from minute ventilation further revealed that this technique was autonomous from breathing patterns. However, due to

the small sample size of the study, when exploring the correlation of specific markers of oxidative stress to inflammatory markers (FeNO) the results were inconclusive.

5.1 The complexity of host–environment interaction and respiratory disease in infancy and childhood

To understand the complex network of interactions leading to impairment of lung function, we comprehensively summarized literature on the risks and mechanisms of early-life factors influencing lung function trajectories (112). Understanding this complex interplay of environmental and genetic factors and their long-term effects on later respiratory morbidity and mortality is crucial for possible prevention and early treatment, including the prevention of prematurity and especially BPD or childhood asthma (6).

Pregnancy and the first years of life are time windows of increased vulnerability due to a phase of rapid lung growth during which the development and adaptation of the anti-oxidative, inflammatory, and immune systems are still ongoing (8), resulting in an increased plasticity of the respiratory system. Perinatal and environmental stimuli, such as prematurity and BPD (18) or exposure to air pollution (25, 28, 113) can consequently lead to impaired lung growth or secondarily, through inflammation and consequent remodeling processes, to structural changes, resulting in impaired lung function in early adulthood (114).

It is, however, still unclear if interventions can correct such abnormal lung function trajectories in childhood and at what ages they should be implemented. Nonetheless, preventative therapeutic measures to reduce exposure to air pollution likely reduce the impact on the prevalence of long-term lung functional sequelae (48), and therefore studies investigating the detrimental effects of air pollution levels (45, 113, 115) below current guidelines (116) should be incorporated in future health policies.

However, little is known about the dynamics of lung function impairment. Especially in premorbid populations, such as preterm infants, it is unclear whether an environmental insult over a limited time period during early life leads to temporary impairment of lung development (69), or sustained growth velocity impairment (20). In any case, even small effect size alterations in lung function trajectories by exposure to environmental toxicity in today's infants could lead to long-term respiratory morbidity in adults and the elderly (6, 8). In populations starting with impaired lung function in early childhood, such as asthmatic or preterm-born children, additional deterioration of lung function could have a substantial impact on faster lung function decline in adulthood (6, 8).

Given that the entire population is exposed, further research in the identification of the dose- and age-dependency of certain risk factors for subsequent preventative and early therapeutic options to avoid adverse effects of these factors is needed.

5.2 Novel impact in science: Air pollution and its effects during vulnerable time windows and on susceptible populations

To answer the questions of whether preterm infants are more susceptible to low-to-moderate air pollution and whether time windows of enhanced vulnerability exist, we compared associations of air pollution on lung function at 44 weeks PCA in preterm versus term infants. As a novelty and proof of principle, we demonstrated that groups of infants with pre-existing vulnerability, such as preterm infants, show increased impairment of lung function as a reaction to the detrimental effects of air pollution (115).

We specifically observed the negative associations between air pollution and lung function in the epidemiologically largest group of moderate to late preterm infants (32 – 37 weeks of GA) (115). These negative associations are potentially due to the fact that very immature infants are

born in the saccular phase of lung maturation process, depicting pulmonary insufficiency, and histopathological characteristics of arrested alveolarization and interstitial fibrosis in severe cases (59). These lung structural changes have been associated with severely altered infant lung function (19, 70). We assume, that the lack of association of air pollution on lung function in preterm infants born before 32 weeks of GA is therefore due to many other dominant developmental and perinatal therapeutic factors, such as longer supplementary oxygen or continuous positive airway pressure duration (19), which may outweigh the small effects that air pollution might have on overall lung mechanics and ventilatory needs (19, 70).

Our findings further support the hypothesis of increased susceptibility of preterm infants to oxidative stress, with an inflammatory response to air pollution reflected in altered FeNO levels (115). These findings are in line with observations in children with persistent respiratory symptoms showing increased FeNO levels after higher exposure to black carbon (29) and are supported by findings in asthmatic and non-asthmatic school-aged children with short-term exposure to $PM_{2.5}$ resulting in higher FeNO levels (35). At a cellular level, inflammation in preterm infants after birth could be related to persistent neutrophil activity and oxidative stress, which have both previously been identified as affecting FeNO levels (11, 38, 117). Both promote the oxygenation of nitrogen oxide (NO) to soluble NO metabolites, which have been found in increased concentrations in plasma and bronchoalveolar lavage fluids during the first month of life (118, 119). However, after peaking during this time window, the neutrophil activity seems to decrease thereafter (117). It is unclear how long this process persists after term.

These findings of adverse associations of air pollution and lung function in infancy are of special interest, given the low-to-moderate air pollution levels in Switzerland, which were below the annual thresholds for NO_2 and PM_{10} suggested by the World Health Organization (116). When detecting negative effects of exposure to air pollution at such low concentrations,

preterm infants, potentially exposed to even higher levels worldwide (116), might be at increased risk for lung function impairment.

The low-to-moderate air pollution levels in our studies might also be relevant when interpreting our findings in the population of healthy former term-born children at school-age (113). In this study we aimed to investigate the effects of low-to-moderate air pollution on lung function at six years of age and to assess, whether time- and dose-dependent responses to air pollution exist. Our results suggest that exposure to higher NO_2 levels, especially during the sensitive period of early lung development, such as pregnancy, and the first and second year of life, may be associated with reduced lung function at school-age. However, we observed only an association between air pollution levels and reduced FEV_1, but not with FRC_{pleth} at school-age (113). This finding could be explained by the breathing maneuvers used for lung function testing. For spirometry (to assess FEV_1) the child breathes at the mechanical limits of the respiratory system (flow limitation). This typically results in lower variability of forced flow lung function parameters. However, during body plethysmography, the child breathes closer to FRC. More adaptive variability of the airway resistance is given at FRC and thus small effects of air pollutants at low-to-moderate exposure levels might not be detectable. These physiological differences may therefore explain why the small impact of air pollution on lung function at school-age may only become measurable when assessed at the extreme, which is during forced breathing maneuvers, but not during regular FRC breathing (113). Nevertheless, our data from healthy former term-born children at six years of age (113) are consistent with the impact of low-level air pollution on airway obstruction in children at 8 years of age from a birth cohort in Sweden (45). Besides, air pollution is only one parameter resulting in reduced lung function, and other factors, such as toxins (e.g. ETS), as well as physiological decline over time, should be considered (6, 7).

Furthermore, to our knowledge, we were the first to investigate the association of both pre- and postnatal air pollution on lung function in infancy in preterm and term infants and at the age of

six years. We were consequently able to show, that the effect of air pollution may be time-dependent, with most impact during a phase of rapid lung development (120, 121); during the second trimester of pregnancy, and the first and second year of life.

5.3 Novel impact in methodology: A technique to study the metabolism of infants

We developed a novel off-line collection and subsequent high-resolution mass spectrometric analysis method for breath in a research setting. In the past, studies in adults in intensive care units have already demonstrated the possibility of measuring metabolic information in EBCs of intubated and therefore uncooperative patients (95). However, EBC is a time-consuming method, which is prone to dilution due to the exhaled vapor (80, 82) and despite broad investigation EBC has not been implemented into clinics (46).

As a novelty, we were the first to show the feasibility of off-line breath measurements by MS in infants and were thus able to measure relevant metabolites, suggestive of oxidative stress (e.g. 4-HNE) in infants' breath for the first time. The good performance of the newly developed off-line technique in comparison to the on-line approach was reassuring, with over 1000 detected mass spectral features of moderate to high quality and with target metabolites such as 4-HNE falling within this range (122), which was similar to comparison of off-line and on-line techniques by SIFT-MS (123).

Additionally, our study design with the correlation of specific metabolites with lung function parameters and FeNO levels in infancy has provided a deeper insight into the physiological background of metabolic measurements in breath. The independence of target metabolites (e.g. 4-HNE) from minute ventilation revealed that this technique was autonomous from breathing patterns, which is in contrast to EBC collection (46). The reasons for the negative correlation between FeNO and 4-HNE, however, are still unclear and given the technical standardization

approach of this project with a small study size, conclusions might be preliminary. However, one possible explanation could be the different cellular processes which underlie 4-HNE and FeNO, with 4-HNE reflecting lipid peroxidation caused by oxidative stress (47) and FeNO mainly being a surrogate marker of primarily eosinophilic inflammation of the airways (46).

The small sample size of infants measured (n = 16) was further not sufficient to enable comparison between subgroups of infants as described by others (13, 124). Filippone et al. (13) and Carraro et al. (124), however, reported differences between former preterm (with and without BPD) and healthy former term-born adolescents in regards to metabolic profiles measured by liquid chromatography MS (124) or different levels of target metabolites suggestive of oxidative stress (8-isoprostane) measured by enzyme-linked immunoassays (13) in EBCs. These results are suggestive of long-term metabolic abnormalities in the respiratory system (13, 124) after premature birth and further support our hypothesis of preterm infants showing a different metabolic response to oxidative stress, which may be associated with an ongoing respiratory disease later in life (13). However, sample sizes of more than 200 infants should be studied in order to obtain more conclusive results on the potential correlation between inflammatory markers, such as FeNO and target metabolic features detected in breath by SESI-HRMS in infancy (122).

5.4 Limitations to this thesis

Methodological limitations to this thesis need to be considered in the interpretation of our findings. As previously mentioned some limitations to our results exist, such as the *sample sizes, the temporal and spatial heterogeneity of exposure*, *the heterogeneity of the study population,* and *concomitant factors* affecting lung function.

There are several explanations why these study sizes occurred and what the consequences in terms of concluding on the findings are: On the one hand, within the comparison of preterm versus term infants, the stratification of our population by gestational age resulted in relatively small sample sizes of preterm subgroups. This approach (stratification in three groups; preterm born before or after 32 weeks of GA, and term infants), however, was necessary as preterm infants represent a very heterogenous group in terms of respiratory morbidity and mortality (62, 67, 70). Very immature infants (born before 32 weeks of GA) show lung structural changes associated with severely altered infant lung function (19, 70) due to dominant developmental and perinatal therapeutic factors, such as longer supplementary oxygen or continuous positive airway pressure duration (19) needing a multivariable statistical approach. The small effects of air pollution on overall lung mechanics and ventilatory needs might therefore not be seen in these populations (19, 70). The small sample size in the study on air pollution and lung function at school-age, on the other hand, was an issue as we were not able to perform further subgroup analyses, such as asthmatic versus non-asthmatic children, as reported by others (45). Thus, we cannot conclude on the effects of air pollution on lung function at school-age in potentially more susceptible populations.

In terms of exposure, the used estimation models of air pollutants did not include satellite data, due to the early recruitment of participants (1999) when modeling techniques for satellite data were not yet available. Therefore, our studies were based on well-established and previously validated PM_{10} exposure values. Although $PM_{2.5}$ effects may biologically be more detrimental,

there is a high collinearity between $PM_{2.5}$ and PM_{10} exposure. The available timeframe from the air pollution models (data available from 1999 until 2016) additionally represent a limitation to track individual lung function growth from infancy until school-age. In order to track lung functional growth deficits, we would potentially need a large sample size to assess effects of low-to-moderate air pollution levels on longitudinal lung function measurements. Tracking lung function throughout childhood therefore remains a future project to be conducted within the BILD cohort.

Due to the observational design of our studies we are further not able to prove causality. This is of utmost importance in order to avoid misunderstandings when interpreting our results. We additionally aimed to adjust for potential confounders based on statistical methods and previous literature, however, residual confounding may persist. Confounding is one of the major challenges in observational research and confounding might lead to false associations or false conclusions.

Drawing conclusions on the correlation of inflammatory and oxidative stress markers from our newly developed measuring technique, would be too preliminary due to the relatively small sample size of the study. Further, with increasing storage time, dilution, interaction of metabolites with each other and with the Nalophan bag can occur (88). We therefore aimed to reduce time between sampling and measurement by SESI-HRMS to a maximum of 10 min (distance from sampling site to the SESI-HRMS). Within these short time periods of 10 min we did not see any effect (data not shown).

5.5 Relevance

In conclusion, the results of this thesis have a highly relevant impact on population health. Our findings provide new insights on lung function development and metabolic profiles early in life and may contribute to better understanding of the effects of air pollution on lung function in vulnerable populations, such as preterm infants. Since recent evidence has shown that early-life lung function impairment may track through the lifespan, and poor lung function trajectories from childhood to late senescence are important early-life risk factors for chronic respiratory disease in adults (5, 8, 112, 125), prevention in utero and early life is of utmost importance (4-6, 8, 54, 112, 125). Even though the effect size at an individual level might be small, the effect on a population basis (attributable risk) is of major concern (126). In preterm infants, which are a heterogeneous group with the risk of respiratory morbidity later in life, the determination of such risk factors and vulnerable time windows and subsequent prevention of such in utero and postnatally can especially play a crucial role in the prevention of chronic respiratory disease in adults.

The successfully developed and standardized measuring technique to capture information on metabolic profiles of non-cooperative patients is a milestone. Metabolomics and especially analysis of such by MS open a future possibility of gaining insight into the mechanisms underlying lung disease and provide a more personalized medicine. This technique may enable the identification of potential therapeutic targets and capture information on metabolic response to therapeutic interventions (e.g. drugs with a narrow therapeutic window) in children and adults not capable of performing the necessary breath maneuvers for on-line measurements or for centers without the necessary analytical tools.

5.6 Conclusion and outlook

For the projects conducted during this thesis, different recommendations for future actions can be described. Within a population of preterm and term-born children living in Switzerland, we were able to show that even low-to-moderate air pollution levels, especially during periods of fast lung development, are associated with impaired lung function in infancy and school-age. From a public health perspective, our findings should therefore encourage more stringent health policies on air pollution thresholds. This is especially of note, when considering our findings in the preterm population, in which we showed even more pronounced associations of air pollution levels during pregnancy on infant lung function than in term infants. These findings are suggestive of susceptible populations, starting with impaired lung function, who might be at increased risk for further lung function decline due to environmental stimuli. However, in order to confirm our findings in this potentially more vulnerable subgroup and other susceptible populations, large multicenter cohorts and high-risk populations (e.g. high prevalence of maternal atopy, bigger sample size of preterm groups) with individual tracking of individual lung function growth are needed.

Impaired lung function is the result of the perinatal and environmental insults, the mechanisms leading to this result, however, are still unclear. Basically, air pollution can impair lung growth or it can act through oxidative stress, inflammation, and remodeling, which then impairs lung development. In order to better understand these mechanisms, one must have better methods to measure inflammation, and oxidative stress response and associated cell death. One approach would be to measure oxidative stress response in cord blood, however this reflects processes within the whole body and is not lung-specific. Therefore, we have set a milestone for a more personalized medicine approach in uncooperative patients with the development and standardization of the off-line collection and analysis platform for metabolic information retrieval. Initially, this technique could be used for research purposes (e.g. external validation

in multicenter studies) with the aim of being subsequently implemented into clinics for the benefit of clinical decision making. We opt for the off-line technique in various clinical settings, in which cooperation is not given, and have already implemented the technique in research settings to monitor diabetic patients administered in an intensive care unit due to ketoacidosis or to monitor the dose-response of antiepileptic drugs. However, in regards to this thesis, this technique should be used to provide further biological background information on metabolic responses to environmental stimuli, such as exposure to air pollution, to support our findings.

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7 Appendix

Symptom score	Daytime symptoms (cough, wheeze, or breathing difficulties)	Night-time symptoms (cough, wheeze, or breathing difficulties)
0	None	None
1	Slight; no treatment given	Slight; sleep not disturbed
2	Required treatment but no outside help	Sleep disturbed once; no help required
3	Severe; required help from GP	Sleep disturbed more than once or child needed help
4	Very severe; admitted to hospital	Sleep very disturbed or GP called

Table E1 Standardized symptom scores used in weekly phone calls (102)

7.1 Effect of breastfeeding duration on lung function, respiratory symptoms and allergic diseases in school-age children

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Pediatric Pulmonology

https://doi.org/10.1002/ppul.24733

Accepted: 06 March 2020

Received: 18 September 2019 | Accepted: 6 March 2020

DOI: 10.1002/ppul.24733

ORIGINAL ARTICLE: OUTCOMES

PEDIATRIC PULMONOLOGY WILEY

Effect of breastfeeding duration on lung function, respiratory symptoms and allergic diseases in school-age children

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Funding information
Schweizerischer Nationalfonds zur Förderung der Wissenschaftlichen Forschung, Grant/Award Number: 320030B_182871

Abstract

Background: A positive effect of breastfeeding on lung function has been demonstrated in cohorts of children with asthma or risk for asthma. We assessed the impact of breastfeeding on lung function and symptoms at the age of 6 years in an unselected, healthy birth cohort.

Methods: We prospectively studied healthy term infants from the Bern-Basel Infant Lung Development (BILD) cohort from birth up to 6 years. Any breastfeeding was assessed by weekly phone calls during the first year of life. Risk factors (eg, smoking exposure, parental history of allergic conditions, and education) were obtained using standardized questionnaires. The primary outcomes were lung function parameters measured at 6 years of age by spirometry forced expiratory volume in 1 second, body plethysmography (functional residual capacity [FRC_{pleth}], the total lung capacity [TLC_{pleth}], and the effective respiratory airway resistance [R_{eff}]) and fractional exhaled nitric oxide (FeNO). Secondary outcomes included ever wheeze (between birth and 6 years), wheeze in the past 12 months, asthma, presence of allergic conditions, atopic dermatitis, rhinitis, and positive skin prick test at the age of 6 years.

Results: In 377 children the mean breastfeeding duration was 36 weeks (SD 14.4). We found no association of breastfeeding duration with obstructive or restrictive lung function and FeNO. After adjustment for confounders, we found no associations of breastfeeding duration with respiratory symptoms or the presence of allergic conditions.

Conclusion: This study found no evidence of an association between breastfeeding and comprehensive lung function in unselected healthy children with long-term breastfeeding. Our findings do not support the hypothesis that the duration of breastfeeding has a direct impact on lung function in a healthy population with low asthmatic risk.

KEYWORDS

allergic disease, breastfeeding, lung function, school-age children

Olga Gorlanova and Rhea Appenzeller contributed equally to this work.

1 | INTRODUCTION

Breastfeeding has a variety of beneficial effects for children.[1] Many studies have provided clear evidence that breastfeeding reduced the risk of respiratory morbidity in early life.[2-7] The effect of breastfeeding on lung function and atopic diseases including asthma in childhood is less consistent. A meta-analysis by Dogaru et al showed a protective association between breastfeeding and asthma with the strongest effect in the first 2 years of life,[8,9] whereas another meta-analysis reported a decreased risk of asthma in children aged 5 to 18 years, but in children from studies with insufficient adjustment for confounders. Furthermore, the effect of breastfeeding on other allergic disease is still conflicting.[9-11]

There is some evidence that breastfeeding could improve school-aged lung function, but the positive association was seen predominantly in studies with a relatively high number of children with atopic/asthmatic mothers[12-16] or from subgroups of children of asthmatic mothers,[17] suggesting that the relationship between breastfeeding and lung function might be mediated by atopic disease. Indeed, the ALSPAC cohort of healthy unselected children found no effect of breastfeeding on bronchial responsiveness.[18] Few studies are available linking breastfeeding with fractional exhaled nitric oxide (FeNO)[19] or more comprehensive lung function. Taken together, results seem to be heterogeneous and influenced by risk factors, categorization, and duration of breastfeeding.

From a mechanistic point of view, it is unclear whether the protective effect of breastfeeding is related to inflammatory mechanisms in asthma and subsequent remodeling and impaired lung growth or whether breastfeeding directly affects lung functional development. To address the latter hypothesis, we aimed to assess the effect of breastfeeding duration on lung function at 6 years of age in a prospective unselected birth cohort study of primarily healthy children with an appropriate adjustment for confounders and comprehensive lung function outcomes such as spirometry, plethysmography, and FeNO. Secondary aims were clinical markers of respiratory and allergic diseases at the age of 6 years, such as ever wheeze, wheeze in the past 12 months, presence of allergic conditions, atopic dermatitis, rhinitis, and positive skin prick test.

2 | METHODS

2.1 | Study design and subjects

Data were obtained from the ongoing prospective Basel-Bern Infant Lung Development (BILD) birth cohort, collected since 1999 in Switzerland. Pregnant women were recruited antenatally in four maternity hospitals and practices of gynecologists in the region of Bern. Unselected healthy children were followed up at 6 years after enrollment.[20] Exclusion criteria for the study were preterm delivery (<37 weeks) and significant perinatal disease, including respiratory distress and known major birth defects. Assessments were undertaken at ages 1 and 6 years. The assessment in the first year of life comprised clinical examination at the age 1 month in the study clinic, weekly phone interviews and information from perinatal records. At the age of 6 years, parents were mailed a questionnaire with questions on allergy and respiratory symptoms as well as environmental exposure and were offered a visit to the study clinic. During the follow-up visit the history of wheeze episodes between birth and age 6, including their frequency, severity and trigger factors were recorded by trained study physicians using standardized questionnaires. Children also underwent a lung function measurement and a skin prick test. This study focuses on the follow-up assessments conducted between August 2005 and April 2018. The Ethics Committee of the Region of Bern approved the study and written consent was obtained at enrollment and again at follow-up.

2.2 | Exposure: Breastfeeding

During the first year of life, mothers were asked weekly by telephone interview with a study nurse about their breastfeeding status until they completely stopped breastfeeding. *Any breastfeeding* was treated as a continuous variable in weeks.

2.3 | Primary outcome: Lung function at 6 years

Spirometry and body plethysmography was performed at the age of 6 years using MasterLab setup (Jaeger, Wurzburg, Germany) according to current ERS/ATS guidelines.[21] The primary spirometry outcome was forced expiratory volume in 1 second according to European Respiratory Society (ERS)/American Thoracic Society (ATS) criteria.[22]

Body plethysmography measurements were done to assess the functional residual capacity (FRC_{pleth}), the total lung capacity (TLC_{pleth}), and the effective respiratory airway resistance (R_{eff}). FRC_{pleth} and TLC_{pleth} were assessed according to European standards[23] and R_{eff} was determined as the mean of at least five separate specific resistance loops.[24] FeNO was used as a measure of eosinophilic airway inflammation and measured online (CLD88sp FeNO analyser, ECO MEDICS, Duernten, Switzerland). Compliant to the ATS/ERS recommendations, the mean of two or three reproducible FeNO values has been reported.[25]

2.4 | Secondary outcome: Clinical data

Standardized questions on key clinical outcomes (eg, wheeze, atopic dermatitis, and rhinitis) were adapted from the International Study on Asthma and Allergy in Childhood (ISAAC) questionnaire.[26]

Ever wheeze was obtained from a physician-administered questionnaire and defined as present if the question "Has your child ever had wheezing or whistling at any time in the past?" was answered positively. *Current wheeze* was defined as present if the question "Has your child had wheezing or whistling in the past 12 months?" was answered positively.

According to the GINA guidelines 2018,[27] *asthma* was defined as present if there was cough/wheezing/difficult or heavy breathing in the absence of an apparent respiratory infection in the last 12 months in combination with an asthma-medication (inhaled corticosteroids or ß-agonists) used in the last 12 months and/or positive past history of allergic conditions (atopic dermatitis and/or rhinitis/rhinoconjunctivitis) and/or a positive family history of allergic conditions.

Rhinitis/rhinoconjunctivitis was defined as parent-reported prolonged sneezing, runny, or blocked nose accompanied by ocular itching and tearing without a common cold in the last 12 months, according to international standards.[28,29]

Based on modified Hanifin and Rajka criteria,[30] *atopic dermatitis* at 6 years was defined as present if three of four major criteria were met: (a) pruritus in the last 12 months, (b) typical morphology and distribution, (c) chronic dermatitis, and (d) personal or family history of atopy allergic conditions (asthma, rhinitis and/or atopic dermatitis). Infants were defined as having atopic dermatitis in the first year of life if they had at least one of the following occurrences in the first year of life: (a) pruritus or/and rashes (eg, redness, dryness, and papules) with the distribution in at least two typical regions; (b) recurrent dermatitis/rashes within the first year of life; (c) doctor diagnosed atopic dermatitis or treatment with topical steroids. We excluded skin lesions caused by cradle cap and seborrheic dermatitis.

The presence of allergic conditions was defined as the presence of asthma and/or rhinitis/rhinoconjunctivitis and/or atopic dermatitis.

A skin prick (at 6 years) was defined as positive for at least one of the following measured allergens: dog dander, cat dander, dermatophagoides pteronyssinus, mixed tree pollens, mixed grass pollens, alternaria tenuis. The test was defined as positive if a weal diameter was bigger than the histamin in any of the tested allergens compared to a valid negative and a valid positive (histamine ≥3 mm) control.[31]

2.5 | Risk factors

Other risk factors included parental history of allergic conditions (defined as asthma, rhinitis, or atopic dermatitis), mode of delivery (vaginal or cesarean), maternal educational level as a marker of socioeconomic status, older siblings, maternal smoking during pregnancy, and parental smoking during the first year of life.

2.6 | Statistical analysis

All variables were examined in relation to their ranges, distributions, means, standard deviations, outliers, and logical errors. For later analysis FeNO was log-transformed. The relationship between breastfeeding and outcomes were tested for possible nonlinearity. We found no evidence for the curvilinear relation of breastfeeding to lung function and clinical outcomes. To investigate the association of breastfeeding with pulmonary function measures, we first performed linear regression analysis with standard adjustments for age, sex, and height (baseline model). Second, we used linear regression analysis with additional adjustments for gestational length, parental history of allergic conditions, and maternal smoking during pregnancy (adjusted model). Estimates are presented as change or a percent change (for back-transformed outcomes) in the lung function parameters per week of any breastfeeding with their 95% confidence intervals (CIs). To exclude the possible effect mediation by a respiratory infection in early life, we performed a sensitivity analysis with adjustment for a number of weeks with respiratory symptoms accessed during the 1 year of life. We did not investigate the possible effect of modification by maternal asthma because of the low number of children with asthmatic mothers.

The association of breastfeeding with secondary outcomes was assessed using, first, univariable logistic regression, and then after adjustment for sex, maternal smoking during pregnancy, parental history of allergic conditions, and maternal education. Ever wheeze was additionally adjusted for the presence of older siblings. Results are presented as odds ratios (ORs) with 95% CIs.

All data processing and analyses were performed in STATA 15.0 (Stata Cooperation, College Station, TX) and R (Version 3.31).[32] Significance was defined by a *P* value less than .05 for two-sided tests.

3 | RESULTS

3.1 | Participants

Between 1999 and 2012, 458 children were enrolled in the BILD study. Among these, 377 (82%) had a documented follow-up visit between 2005 and 2018. A total of 8 (2%) children did not meet the inclusion criteria as described above, 73 (16%) we lost to follow-up. The characteristics of included vs non-included children are shown in Table S1. There was a significant difference between included and non-included children for maternal smoking during pregnancy and parental smoking during the first year of life, and the mean duration of breastfeeding was significantly shorter in the non-included than in the included children.

A total of 32 (8%) participants did not show for the follow-up lung function test at 6 years, but sent the questionnaire back. A total of 279 (74%) of 377 had available data on FeNO measurements. The quality control of lung function resulted in 204 children (54%) with spirometry and 263 children (70%) with body plethysmography data (Figure S1). Table 1 shows the anthropometric data, potential risk factors, spirometry, FeNO, and body plethysmography data for the whole study population.

TABLE 1 Characteristic of children included in the study

Characteristic	Complete data	Values
Anthropometric data		
Gestational age, mean (SD), weeks	377	40 (1.17)
Gestational length, mean (SD), cm	377	55 (2.17)
Gestational weight, mean (SD), kg	377	22 (3.26)
Male sex, n (%)	377	204 (54)
Age at follow-up, mean (SD), years	345	6.03 (0.28)
Length at follow-up, mean (SD), cm	345	117.29 (5.45)
Weight at follow-up, mean (SD), kg	345	22.17 (3.26)
Risk factors		
Cesarean section, n (%)	377	61 (16)
Maternal smoking during pregnancy, n (%)	377	30 (8)
Parental smoking during first year of life, n (%)	377	75 (20)
Maternal history of allergic conditions[a], n (%)	377	123 (33)
Maternal asthma[b], n (%)	377	37 (10)
Paternal history of allergic conditions[a], n (%)	377	132 (35)
Paternal asthma[b], n (%)	377	67 (18)
Maternal education[c], n (%)	376	
Low		93 (25)
Middle		132 (35)
High		151 (40)
Presence of older siblings, n (%)	377	200 (47)
Exposure		
Breastfeeding		
≥ 1 wk, n (%)	377	372 (98.7%)
No. of week with breastfeeding, mean (SD)	375	36.5 (13.9)
Primary outcomes		
Spirometry		
FEV_1, mean (SD), L	204	1.28 (0.20)
FEV1, % predicted, mean (SD)	204	100.3 (11.3)
FVC, mean (SD), L	82	1.4 (0.26)
FVC, % predicted, mean (SD)	82	100.8 (12.3)
FeNO, mean (SD), ppb	279	7.81 (6.98)
Body plethysmography		
FRC_{pleth}, mean (SD), L	260	1.07 (0.19)
TLC_{pleth}, mean (SD), L	198	2.05 (0.33)
R_{eff}, mean (SD), kPa*s/L	263	0.68 (0.20)
Secondary outcomes		
Asthma, n (%)	345	18 (5)
Ever wheezing, n (%)	345	72 (21)
Current wheezing, n (%)	345	18 (5)
Presence of allergic conditions, n (%)	346	100 (29)
Rhinoconjunctivitis, n (%)	372	71 (19)
Atopic dermatitis at 6 y, n (%)	366	38 (11)
Positive skin prick test, n (%)	302	40 (13)

[a]Defined as self-reported doctor-diagnosed asthma, atopic dermatitis, or allergic rhinoconjunctivitis.
[b]Defined as self-reported doctor-diagnosed asthma.
[c]Categorized into low (less than 4 years of apprenticeship), middle (4 years of apprenticeship and above), and high (tertiary education).

3.2 | Breastfeeding prevalence

Overall, five children (1%) were not breastfed at all, 78 (21%) were breastfed less than 6 months. The mean (SD) duration of breastfeeding for those who received breastfeeding was 36.5 (13.9) weeks. The duration of breastfeeding according to exposure characteristics at birth is shown in Table S2. Maternal smoking during pregnancy was only significantly associated with shorter duration of breastfeeding.

3.3 | Association of breastfeeding with lung function and clinical symptoms

The duration of breastfeeding was not associated with lung function (Table 2). There were no substantial differences in baseline and adjusted models. Additional adjustments for maternal education or respiratory symptoms in the first year of life did not change the effect estimates (data not shown).

In a univariable model, we found significant evidence for a 2% reduction in the presence of allergic conditions for each week of breastfeeding (Table 2). However, after control for confounders, we found no evidence for association of breastfeeding with any of secondary clinical outcomes (Table 2).

4 | DISCUSSION

In this prospective cohort study of primarily healthy unselected children followed from birth until school age, we found no significant effect of breastfeeding duration on lung functional outcomes, if adjusted for known confounders. The physiological relevance of the findings was strengthened by the consistency across several functional outcomes at school age. We found no evidence of airway obstruction, altered residual or end-expiratory volume nor restricted total lung capacity. With respect to secondary outcomes, we observed that a longer duration of breastfeeding was associated with a reduced risk of atopic dermatitis in girls. For every week mothers continued breastfeeding, the risk of having atopic dermatitis was reduced by 4%. There was no significant effect of breastfeeding duration on other clinical data such as asthma, ever wheeze, current wheeze, rhinitis or positive skin prick test. Sensitivity analysis of lung function outcomes excluding children who developed asthma during preschool age showed consistent results (Table S3).

Primary outcomes: In contrast to asthma cohort studies,[12,13,15,17] we found no effect of breastfeeding on lung function outcomes in unselected, primarily healthy children. Similarly, we could not demonstrate an effect of breastfeeding on FeNO, a marker of eosinophilic inflammation in asthma, as suggested by others.[19] Studies investigating the effect of breastfeeding on lung function are heterogeneous with regard to age (mixed preschool and school-age populations), risk factors, duration, and categorization of breastfeeding. Overall, there is evidence that breastfeeding has the most consistent beneficial effect on FVC.[33] However, of the three cohort studies reporting on the effect of breastfeeding on FVC, two were based on the Isle of White cohort and one on The Tucson Children's Respiratory Study with enrollment periods from 1980 to 1984 and from 1989 to 1990, respectively.[16,34] FVC is highly cooperation-dependent in preschoolers, strict quality control criteria in our cohort did not allow us to collect a high enough sample size (Table 1) to confirm data from the literature. Furthermore, in comparison to our

TABLE 2 Association of breastfeeding with lung function and clinical data

	Baseline model[a]			Adjusted model[b]		
Breastfeeding, week	**N**	**Coeff (95%CI)**	***P* value**	**N**	**Coeff (95%CI)**	***P* value**
Spirometry						
FEV_1, mL	204	−0.46 (−1.88; 0.94)	.513	204	−0.23 (−1.65; 1.19)	.747
Body plethysmography						
FRC_{pleth}, mL	260	−0.3 (−1.91; 1.31)	.714	260	−0.14 (−1.75; 1.47)	.866
TLC_{pleth},mL	198	0.59 (−2.09; 3.27)	.667	198	0.77 (−1.87; 3.41)	.564
R_{eff}, mL	263	1.36 (−0.41; 3.12)	.131	263	1.36 (−0.42; 3.15)	.134
FeNO, ppb	276	1.00 (0.99; 1.01)	.725	276	1.00 (0.99;1.01)	.604
	Univariable model			**Adjusted model[c]**		
Breastfeeding, week	**N**	**OR (95%CI)**	***P* value**	**N**	**OR (95%CI)**	***P* value**
Clinical data						
Asthma	345	0.99 (0.96; 1.02)	.397	345	0.99 (0.97; 1.03)	.816
Ever wheezing	345	0.99 (0.97; 1.01)	.298	345	1.00 (0.98; 1.01)	.634
Current wheezing	345	0.99 (0.96; 1.02)	.542	345	1.00 (0.97; 1.03)	.976
Presence of allergic conditions	346	0.98 (0.96; 1.00)	**.018**	346	0.99 (0.97; 1.00)	.068
Rhinoconjunctivitis	372	0.98 (0.97; 1.00)	.071	371	0.99 (0.97; 1.01)	.194
Atopic dermatitis	366	0.98 (0.96; 1.00)	.080	365	0.99 (0.96; 1.01)	.187
Positive skin prick test	302	0.99 (0.97; 1.01)	.379	302	0.99 (0.97; 1.02)	.579

Note: Effect is reported per week of breastfeeding.

Abbreviations: 95% CI, 95% confidence interval; Coeff, regression coefficient; N, complete data; OR, odds ratio.

[a]Adjusted only for anthropometric data (age, height, and sex).

[b]Adjusted for sex, maternal smoking during pregnancy, and parental atopy.

[c]Adjusted for sex, maternal smoking during pregnancy, parental atopy, and maternal education. Ever wheezing was adjusted additionally for older siblings.

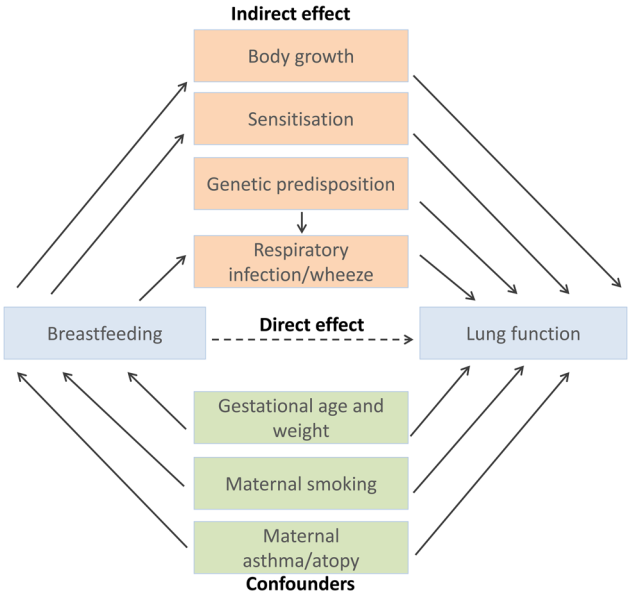

FIGURE 1 The potential causal pathway (in concordance to Waidyatillake et al[33]). We hypothesized that the effect of breastfeeding on lung function is weak and might be mediated by reducing the airway inflammation and allergic sensitization in early childhood, and also a complex interaction between breastfeeding and genetic variants on the respiratory infection.[36,37] Moreover, breastfeeding may positively influence body growth that can lead to better lung function. In addition, it is possible that reported associations between breastfeeding and lung function are due to confounding factors [Color figure can be viewed at wileyonlinelibrary.com]

primarily healthy cohort, these cohorts (ALSPAC, the Isle of White, and Tucson Children's Respiratory Study) are characterized by the lower duration of breastfeeding and higher prevalence of asthmatic/atopic mothers and maternal smoking. In addition, depending on the enrollment period, changes in environmental factors, the prevalence of respiratory infection and medical care may contribute to both changes in breastfeeding duration and lung functional growth. Our findings are, however, in line with the subgroup of healthy offspring of non-asthmatic mothers, and the ALSPAC and PROBIT studies, which did not find an effect of breastfeeding on lung function.[18,35] Our findings are consistent with the hypothesis, that breastfeeding has no direct strong impact on lung functional development. Combining knowledge from other studies and our findings, we may speculate that the impact of breastfeeding on lung functional development may be a secondary effect mediated by susceptibility to early viral infections or chronic inflammatory processes at preschool age, such as described in asthma (Figure 1).

Secondary outcomes: In contrast to studies reporting an association between breastfeeding and allergic disease, in our unselected, primarily healthy cohort we found no effect of breastfeeding on asthma and respiratory symptoms, nor on the presence of allergic conditions. This may be related to the limited sample size, which would not allow us to assess small and probably clinically less relevant effects. Another reason for contrasting results is the low prevalence of children with asthma and wheeze in our cohort compared to many other cohorts.[38,39] We have, however, found a weak protective effect on the development of atopic dermatitis. In several studies, it has been shown that breastfeeding protected children from atopic dermatitis.[40,41] Other authors could not find a protective effect at any age, from infancy through adolescence,[42,43] instead, breastfeeding was associated with increased atopic dermatitis,[44-46] especially in the subgroup of children with no heredity for atopy. Consistent with that, in a systematic review and meta-analysis of 18 prospective studies, the protective effect of breastfeeding was higher in the subgroup with a positive family history of atopy.[38] Contrary to that, Kull et al showed, based on the BAMSE birth cohort, that exclusive breastfeeding for 4 months or more reduced the risk for atopic dermatitis at 4 years by about 20%, irrespective of sensitization to common food or inhalant allergens or parental allergic diseases.[41]

4.1 | Strength and limitation

Our cohort is a very homogeneous, primarily healthy, cohort. The findings remain robust even if children with asthma were removed from the analysis. However, our healthy population also sets our study apart and is one of our strengths. There are limitations in terms of the generalizability of our findings. Considering Tables 1 and S1, the sample analyzed might not fully represent the entire population of Bern. The participants tended to come from a higher socioeconomic class and the results from the included/non-included comparison analysis with loss of less-breastfed children suggest a population bias regarding the average duration of breastfeeding. The high prevalence of breastfeeding in our study makes it impossible to provide risk estimates for breastfeeding per se (breastfed children vs non-breastfed children) and makes comparisons between studies difficult. A further difficulty was quantifying the addition of formula supplementation (partial breastfeeding). This made it difficult to assess exposure dose and might have hidden a greater benefit of exclusive breastfeeding compared to partial breastfeeding.

Furthermore, to avoid recall bias we prospectively asked mothers on a weekly basis, whether or not they were breastfeeding and identified the exact time point when mothers completely weaned their children.

5 | CONCLUSION

Although we found significant breastfeeding effects on respiratory symptoms in the first 6 months of life within the same cohort,[36] this study suggests that in unselected primarily healthy children with low risk for asthma, breastfeeding duration has no relevant effect on comprehensive lung function and FeNO in healthy school-aged children.

ACKNOWLEDGMENTS

We would like to thank our study participants and their families for their participation. We also thank also Karine Bell Hugentobler for proofreading the manuscript. Furthermore, we thank the BILD study nurses for their invaluable assistance and support.

CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

BASEL BERN INFANT LUNG DEVELOPMENT (BILD) COHORT, CURRENT STUDY GROUP

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Gorlanova O, Appenzeller R, Mahmoud YS, et al. Effect of breastfeeding duration on lung function, respiratory symptoms and allergic diseases in school-age children. *Pediatric Pulmonology*. 2020;55:1448–1455. https://doi.org/10.1002/ppul.24733

Online Supplement

Table S1: Anthropometric data of the study participants compared to non-included children

	Included	Non-included	p-value
Number of children	377	81	
Anthropometric data			
Male sex, n (%)	204 (54)	44 (54)	0.973
Gestational age, mean (sd), weeks	40 (1.2)	39 (1.9)	0.974
Length at birth, mean (sd), cm	49.6 (2.0)	48.9 (2.2)	0.998
Weight at birth, mean (sd), g	3392 (441)	3283 (511)	0.974
Risk factors			
Caesarean section, n (%)	61 (84)	13 (17)	0.887
Maternal smoking during pregnancy, n (%)	30 (8)	16 (20)	**0.001**
Parental smoking during first year of life, n (%)	75 (20)	26 (34)	**0.008**
Maternal history of allergic conditions[a], n (%)	123 (33)	27 (34)	0.790
Maternal asthma, n (%)	37 (10)	10 (13)	0.450
Paternal history of allergic conditions[a], n (%)	132 (35)	31 (39)	0.486
Paternal asthma, n (%)	35 (9)	12 (15)	0.120
Maternal education [b]			
low, n (%)	151 (40)	28 (40)	**0.020**
middle, n (%)	132 (35)	16 (23)	
high, n (%)	93 (25)	26 (37)	
Presence of older siblings, n (%)	200 (53)	45 (57)	0.526
Exposure			
Breastfeeding, mean (sd), weeks	36 (14)	26 (18)	**<0.001**

Values are mean (standard deviation) or number (percentage). [a] defined as self-reported, doctor-diagnosed asthma, rhinitis or atopic dermatitis; [b] categorized into low (less than four years of apprenticeship), middle (four years of apprenticeship and above) and high (tertiary education).

Table S2: Duration of breastfeeding stratified by exposure, N=377

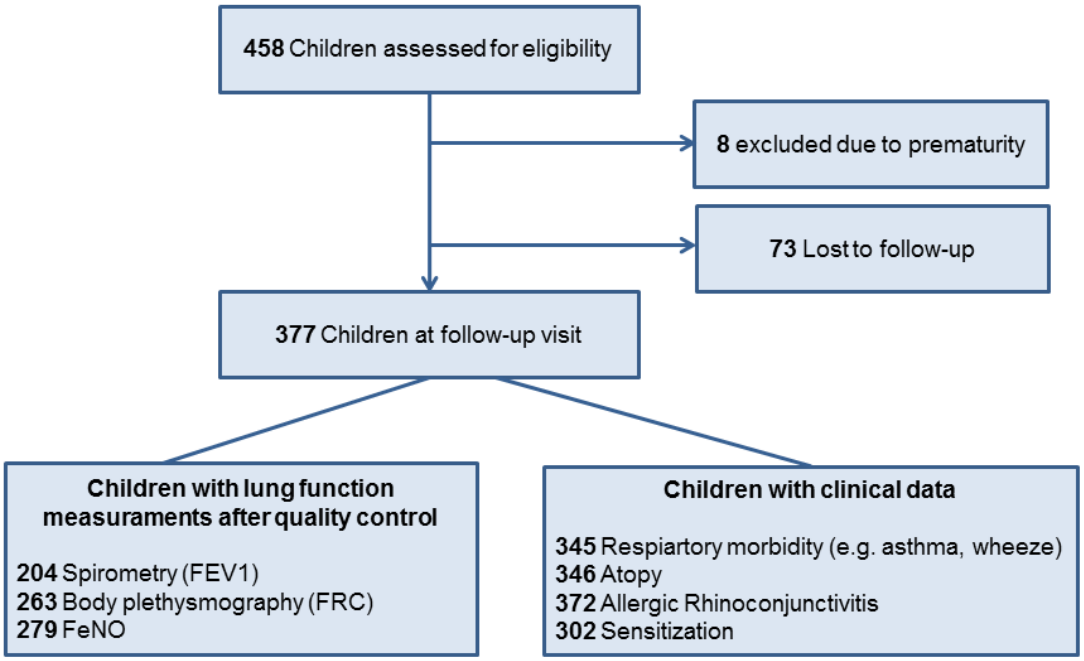
	N (%)	Median in weeks	IQR in weeks
Study population	377		
Sex			
Male	204 (54)	39	27-47
Female	173 (46)	36	29-52
Gestational age			
≤ 40 weeks	218 (58)	36	26-47
>40 weeks	159 (42)	40	29-52
Parental history of atopy			
No	164 (44)	38.5	30-47.5
Yes	213 (56)	36	27-51
Maternal age at enrollment			
≥ *25y*	367 (97)	39	27-49
< *25y*	10 (3)	37	13-50
Maternal socioeconomic status[1]			
Low	93 (25)	36	26-48
Middle	132 (35)	35	24.5-47.5
High	151 (40)	40	30-51
Maternal smoking during pregnancy	347 (92)	**38**	28-50
No	30 (8)	**32**	30-41
yes			
Parental smoking during first year of life			
No	302 (80)	37	27-48
Yes	75 (20)	36	23-52
Older siblings			
No	177 (47)	37	27-51
yes	200 (53)	37	28-48

[1] Data were available for n=376. [2] Data were available for n=236

in bold shown the significant difference (p-value<0.05)

Table S3: Association of breastfeeding with lung function in non-asthmatic children

	Adjusted model[a]		
Breastfeeding (wks)	N	Coeff (95%CI)	p-value
Spirometry			
FEV_1, ml	195	-0.28 (-1.76; 1.20)	0.713
Bodyplethysmography			
FRC_{pleth}, ml	245	-0.04 (-1.68; 1.60)	0.958
$TLC_{pleth,}$ml	185	1.20 (-1.47; 3.88)	0.377
R_{eff}, ml	248	1.17 (-0.70; 3.04)	0.219
FeNO, ppb	266	1.00 (0.99;1.01)	0.556


458 Children assessed for eligibility
8 excluded due to prematurity
73 Lost to follow-up
377 Children at follow-up visit
Children with lung function measurements after quality control
204 Spirometry (FEV1)
263 Body plethysmography (FRC)
279 FeNO
Children with clinical data
345 Respiartory morbidity (e.g. asthma, wheeze)
346 Atopy
372 Allergic Rhinoconjunctivitis
302 Sensitization

7.2 Standardization procedures for real-time breath analysis by secondary electrospray ionization high-resolution mass spectrometry

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Analytical and Bioanalytical Chemistry

https://doi.org/10.1007/s00216-019-01764-8

Accepted: 6 March 2019

Analytical and Bioanalytical Chemistry (2019) 411:4883–4898
https://doi.org/10.1007/s00216-019-01764-8

RESEARCH PAPER

Standardization procedures for real-time breath analysis by secondary electrospray ionization high-resolution mass spectrometry

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Received: 3 December 2018 / Revised: 25 February 2019 / Accepted: 6 March 2019 / Published online: 15 April 2019

Abstract

Despite the attractiveness of breath analysis as a non-invasive means to retrieve relevant metabolic information, its introduction into routine clinical practice remains a challenge. Among all the different analytical techniques available to interrogate exhaled breath, secondary electrospray ionization high-resolution mass spectrometry (SESI-HRMS) offers a number of advantages (e.g., real-time, yet wide, metabolome coverage) that makes it ideal for untargeted and targeted studies. However, so far, SESI-HRMS has relied mostly on lab-built prototypes, making it difficult to standardize breath sampling and subsequent analysis, hence preventing further developments such as multi-center clinical studies. To address this issue, we present here a number of new developments. In particular, we have characterized a new SESI interface featuring real-time readout of critical exhalation parameters such as CO_2, exhalation flow rate, and exhaled volume. Four healthy subjects provided breath specimens over a period of 1 month to characterize the stability of the SESI-HRMS system. A first assessment of the repeatability of the system using a gas standard revealed a coefficient of variation (CV) of 2.9%. Three classes of aldehydes, namely 4-hydroxy-2-alkenals, 2-alkenals and 4-hydroxy-2,6-alkedienals—hypothesized to be markers of oxidative stress—were chosen as representative metabolites of interest to evaluate the repeatability and reproducibility of this breath analysis analytical platform. Median and interquartile ranges (IQRs) of CVs for CO_2, exhalation flow rate, and exhaled volume were 3.2% (1.5%), 3.1% (1.9%), and 5.0% (4.6%), respectively. Despite the high repeatability observed for these parameters, we observed a systematic decay in the signal during repeated measurements for the shorter fatty aldehydes, which eventually reached a steady state after three/four repeated exhalations. In contrast, longer fatty aldehydes showed a steady behavior, independent of the number of repeated exhalation maneuvers. We hypothesize that this highly molecule-specific and individual-independent behavior may be explained by the fact that shorter aldehydes (with higher estimated blood-to-air partition coefficients; approaching 100) mainly get exchanged in the airways of the respiratory system, whereas the longer aldehydes (with smaller estimated blood-to-air partition coefficients; approaching 10) are thought to exchange mostly in the alveoli. Exclusion of the first three exhalations from the analysis led to a median CV (IQR) of 6.7 % (5.5 %) for the said classes of aldehydes. We found that such intra-subject variability is in general much lower than inter-subject variability (median relative differences between subjects 48.2%), suggesting that the system is suitable to capture such differences. No batch effect due to sampling date was observed, overall suggesting that the intra-subject

Published in the topical collection *Young Investigators in (Bio-)Analytical Chemistry* with guest editors Erin Baker, Kerstin Leopold, Francesco Ricci, and Wei Wang.

Kapil Dev Singh and Georgi Tancev contributed equally to this work.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00216-019-01764-8) contains supplementary material, which is available to authorized users.

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variability measured for these series of aldehydes was biological rather than technical. High correlations found among the series of aldehydes support this notion. Finally, recommendations for breath sampling and analysis for SESI-HRMS users are provided with the aim of harmonizing procedures and improving future inter-laboratory comparisons.

Keywords Breath metabolomics · Fatty aldehydes · Secondary electrospray ionization high-resolution mass spectrometry · Oxidative stress · Standardization procedures · Variability

Introduction

Mass spectrometry is a pivotal technique in clinical chemistry laboratories and will continue its expansion to support clinical decision-making [1]. One of such potential future applications is the analysis of exhaled breath metabolites for clinical diagnosis and therapeutic monitoring [2]. However, such an endeavor requires standardized protocols, performed in multicenter studies leading to conclusive evidence, before regulatory authorities can approve a clinical test. In this regard, transitioning from promising research results to concrete clinical applications proves to be a challenge, leading to few routinely used clinical breath tests [3].

A number of analytical techniques have emerged over the last five decades, aiming to address this challenge, being the earliest one gas chromatography-mass spectrometry (GC-MS) [4, 5]. GC-MS and its improved modern variants such as GC×GC-Time of flight remain to be the workhorse platform capable of mapping the yet largely unknown breath metabolome [6]. However, one important limitation of GC-MS is the requirement of sample preparation, which leads to lengthy analyses and poses at the same time additional difficulties to standardize procedures and to preserve chemically uncompromised breath specimens [7]. Since breath constitutes a virtually unlimited source of information, real-time techniques such as proton-transfer-reaction mass spectrometry (PTR-MS) [8] and selected-ion flow-tube mass spectrometry (SIFT-MS) [9] emerged to conveniently capture this information. Such convenient online monitoring of exhaled metabolites is obviously of great advantage. However, it comes at the price of limited sensitivity—as no sample pre-concentration is possible—and limited selectivity—as no chromatographic separation prior to mass analysis is possible. A third real-time mass spectrometric alternative is secondary electrospray ionization-mass spectrometry (SESI-MS) [10]. In contrast to PTR-MS and SIFT-MS, ionization of exhaled metabolites takes place at atmospheric pressure in SESI-MS. The benefit of doing so is twofold: (i) the ionization probability increases with pressure [11] and (ii) it allows to conveniently interface the ionization stage with virtually any pre-existing atmospheric pressure ionization mass analyzer, including ultra-high-resolution (> 100,000) MS such as Orbitrap. This results in sensitive and selective, yet real-time, analysis of trace vapor species. As a result, despite being the most recently proposed mass spectrometric alternative for real-time gas analysis, it is steadily gaining interest across different research groups [10, 12–26]. However, most of the published SESI-MS studies rely on lab-built instrumentation, making it difficult to standardize procedures for this technique. Following ongoing efforts to standardize exhaled breath collection and subsequent analysis for other analytical platforms [27–34], we present here a series of instrumental developments aiming to standardize breath analysis procedures and to provide recommendations for SESI-HRMS users interested in breath analysis. To do so, we characterized a series of new instrumentation with a focus on a panel of three classes of exhaled aldehydes.

Material and methods

We investigated the exhaled breath composition of healthy subjects by SESI-HRMS. The breath analysis platform consisted of three main components. The first one was a newly developed interface (Exhalion, FIT, Spain), which measures CO_2 (%), pressure drop (mbar), exhalation flow rate (L/min), and exhaled volume (L) in real time to guide the exhalation maneuver. Downstream, the exhaled breath is ionized in an ion source (Super SESI, FIT, Spain). Ionized breath metabolites were then analyzed in real time by a high-resolution mass spectrometer (Q Exactive Plus, Thermo Fisher Scientific, Germany). Figure 1 a shows a picture of the breath analysis platform.

Exhalation maneuver monitoring and guiding (Exhalion)

The breath interface Exhalion was constructed with the aim of assisting in the control and reproducibility of exhalation maneuver. Exhalion consists of the following elements: a disposable standard antibacterial/antiviral medical grade filter. In this study, commercially available spirometry filters (MicroGard™, Vyaire Medical, USA; 3 cm ID; filters 99.98% of bacteria and 99.92% of viruses) were used as a mouthpiece. Downstream, the filter is connected to an autoclavable interface, housing a calibrated flow restriction. By measuring the pressure drop through the calibrated restriction (range 0 to 20 mbar, accuracy 2.5%, precision 0.1 mbar), Exhalion determines the flow rate (range 0 to 15 L/min, accuracy 2.5%), and total exhaled volume (the latter is automatically estimated by detecting the onset of the exhalation and integrating flow rate over time). Capnography data is measured side-stream (range 0 to 20%, accuracy 5% of the reading), with

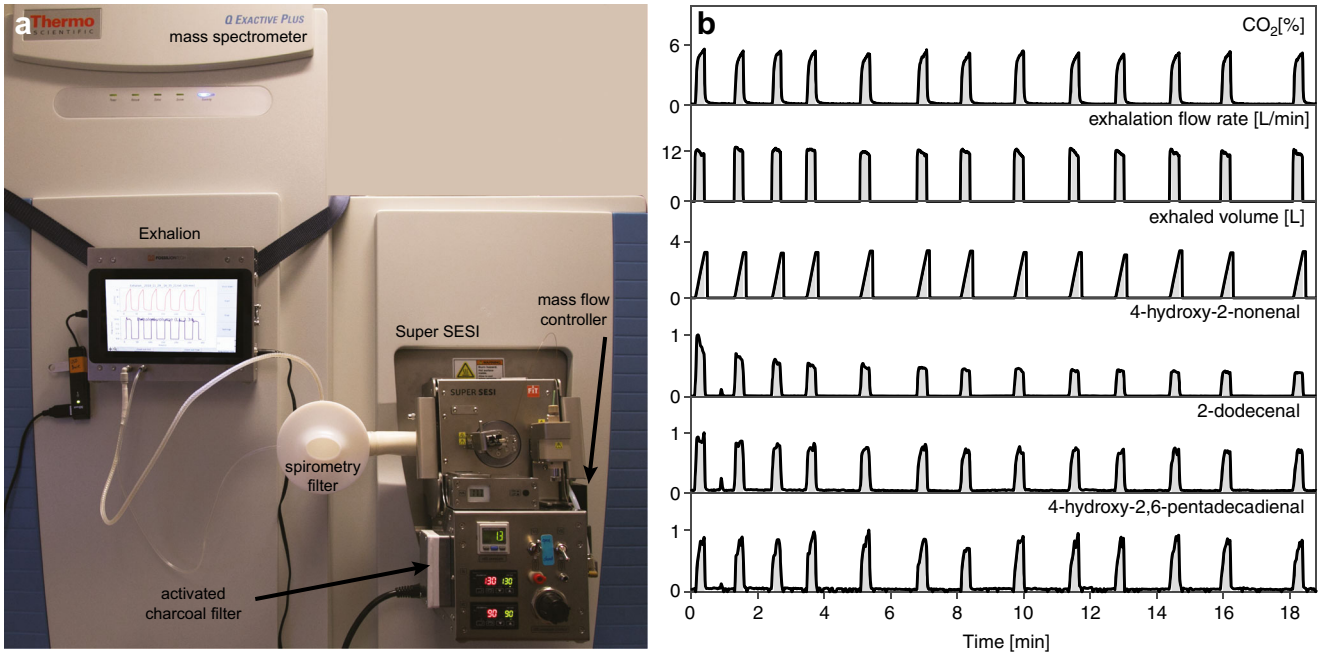

Fig. 1 Real-time breath analysis using SESI-HRMS. **a** SESI-HRMS analytical platform located in a clinical setting (University Children's Hospital Basel) dedicated for real-time breath analysis. The system features three main elements: (i) exhalation interface, which provides feedback to the participants on the exhalation maneuver; (ii) ion source, which efficiently ionizes exhaled metabolites, and (iii) high-resolution mass spectrometer. **b** Real-time analysis by simultaneous monitoring of CO_2, physical exhalation parameters (exhalation flow rate and exhaled volume), and relative intensities of three representative aldehydes from one experiment. 13 consecutive exhalations within 20 min for one subject are shown (see ESM Fig. S4 for zoomed-in view of the first exhalation).

an approximate flow rate of 0.5 L/min. Absolute pressure measurement is also integrated and is used to compensate for the effect of barometric variations on CO_2 and flow readings. Time and other parameters are measured at a rate of 1.5 Hz, and stored in a text file. Finally, a main module, incorporating a touch screen, a micro-computer, all sensors, and a dedicated firmware to run autonomously, is used to process all the data from flow restriction and capnograph in real time. All routines to seamlessly calibrate the sensors are integrated into the firmware. The main module and the flow restriction interface are connected with two tubes (1/8" OD, for CO_2 and pressure measurement). Nafion tubing was used to prevent condensation. The dead volume of the side-stream tubing and the sensors was below 5 cm^3, which provides an upper limit for the CO_2 reading delay of 0.5 s. The total dead volume was dominated by the mouthpiece filter, as Exhalion was designed to minimize this contribution. The Exhalion device was connected downstream with the ionization device (Super SESI).

Secondary electrospray ionization (Super SESI)

The Super SESI source was optimized for breath analysis and integrates all components required to control the ionization of the sample flow. A fraction of the total exhaled flow is passed to the ionizer, which features a sampling line connected to an ionization chamber whereby a nano-electrospray (0.1% ammonium formate in water) ionizes the metabolites present in breath. We used a 20-μm ID TaperTip (New Objective, USA) silica capillary emitter. The Super SESI pressure was set to 1.3 bar to drive the liquid through the capillary. The steady-state reading of the nano-amperemeter indicated that a stable spray was formed (typically 130 nA). The sampling line temperature was set to 130 °C and the ion chamber temperature was set to 90 °C. In addition, the sampling line and the ionization chamber core were silica-coated to minimize analyte adsorption onto the system walls. Super SESI uses a flow of clean nitrogen (filtered through a built-in activated charcoal filter) to sweep the ionizer when there is no sample input. It was set to provide an excess of 0.4 L/min over the flow ingested by the mass spectrometer (precise reading and control of this is integrated into the Super SESI). The exhaust mass flow controller was then set to 0.7 L/min so that the fraction of breath entering the ionizer was fixed at 0.3 L/min regardless of potential exhalation pressure fluctuations. The dead volume of the sample line and the ionizer was approximately 10 cm^3. At this flow, the time required for breath to reach and sweep the ionizer is 2 s.

High-resolution mass spectrometry (Q Exactive Plus)

The Super SESI source was directly coupled to the Q Exactive Plus MS and was recognized as an ESI source (sheath gas flow rate 60, auxiliary gas flow rate 2, spray voltage 3.5 kV, capillary temperature 275 °C, and S-lens RF level 55.0). The MS was operated directly via Q Exactive Tune software (version 2.9) in

full MS mode (polarity positive, scan range 100 to 400 *m/z*, microscans 4, ACG target 10^6, and maximum injection time 500 ms) with a resolution of 140,000 (at *m/z* 200). The MS was externally calibrated on a weekly basis using a commercially available calibration solution (Pierce™ Triple Quadrupole, extended mass range) and internally calibrated by enabling lock masses (*m/z* 149.02332, 279.15909, 355.06993, 371.10123, and 391.28429), which correspond to common background mass spectrometric contaminants [35, 36].

Subjects

Three male and one female healthy subjects (33 ± 8 years, mean ± SD) were enrolled in the study, each subject provided at least 49 exhalations. All measurements were performed during weekdays at any given time between 8 a.m. and 6 p.m. Fig. S1 (see Electronic Supplementary Material, ESM) shows the measurement scheduling distribution for all participants, indicating no significant bias towards a specific time window for any given subject. The sample size and number of replicates resulted from estimating the within-subject standard deviation, following the approach described by Bland and Altman [37]. Shortly, the precision with which one can estimate within-subject standard deviation depends on both the number of subjects and the number of observations per subject. Details are described in the ESM (Table S1). The subjects provided prolonged exhalations, whereby the subjects inspired to total lung capacity and expired at a constant flow rate. This expiration maneuver was repeated at least six consecutive times with breaks of at least 10 s in-between replicate exhalations. Typically, the total exhaled volume per exhalation was 3 L. To guide the maneuver, the subjects could monitor in real time their CO_2 level, exhalation flow rate, and exhaled volume on the Exhalion touch screen. Fig. S2 (see ESM) shows a picture of how a subject would perform the breath test.

Data analysis

Raw data from the MS and Exhalion device were exported and processed using MATLAB (version 2018a, MathWorks Inc., USA). Briefly, raw MS data were converted into mzXML file format using ProteoWizard's msConvertGUI [38]. Afterwards, each spectrum from all files was aligned and calibrated using the RAFFT algorithm implemented in MATLAB [39]. Then *mspeaks* and *ksdensity* functions of MATLAB were used to appropriately pick and extract the final feature list of 2,255 features. Molecular formulae were generated based on the accurate mass by considering C, H, N, and O [40]. A number of studies suggest using CO_2- and volume-controlled sampling maneuvers as a standardization procedure [34, 41–44]. Following the recommendations to use this physiological parameter to normalize breath analysis data, we normalized signal intensities by considering exhalation windows where the CO_2 concentrations rose above 3%. In particular, we computed the signal intensity for each of these 2,255 features during each exhalation by using the time corresponding to 3% or more of the CO_2 signal (from Exhalion) to define a single exhalation event. Finally, the integrated area under the curve (AUC) for all features was normalized by the exhaled volume in the exhalation (calculated by integrating the flow over time in a particular exhalation from Exhalion data). We will henceforth refer to this normalized AUC as breath-signal.

For Fig. 2 and Figs. S6-S8 (see ESM), we first normalized the breath-signal of metabolites from each experiment (containing 6 exhalations) to the maximum. Then, normalized breath-signals of metabolites were averaged across different experiments to obtain the final "mean normalized breath-signal" of metabolites for each subject along with their 95% confidence interval (CI).

Intra-subject variability for each feature was estimated by calculating the coefficient of variation (CV, expressed as percentage) of the replicate exhalations (this analysis led to Table 1). Inter-subject variability was evaluated by performing one-way analysis of variance (ANOVA; grouped by subjects), followed by a multiple comparison (post hoc) test, using the Bonferroni method, to determine whether pairs of group means were significantly different (this analysis led to Table 2).

Gas standard generation (ReGaS2)

To monitor the stability of the ionization, a reactive gas standard generator (ReGaS2) developed by the Swiss Federal Institute of Metrology (METAS) [45], was used. This device releases a flow with stable concentrations of trace gases and can be used to standardize gas sensors. In our case, β-pinene at a concentration of 92.7 ppb in air was used as target vapor (carrier flow of 1 L/min and dilution flow of 0.5 L/min at an oven temperature of 41 °C).

Results and discussion

Technical variability measured using β-pinene vapors

Before discussing the biological variability measured in human breath, we gauged the typical technical variability to be expected for our SESI-HRMS system. In order to do so, we infused a continuous stream of air seeded with 92.7 ppb of β-pinene, simulating an exhalation maneuver. Upon injection of the standard, the mass spectrum was dominated by the expected protonated β-pinene at *m/z* 137.1326 ($C_{10}H_{17}$), along with some oxidized species ($C_{10}H_{15}O$ and $C_{10}H_{17}O_2$; ESM Fig. S3). SESI-MS is known to detect trace species down to the

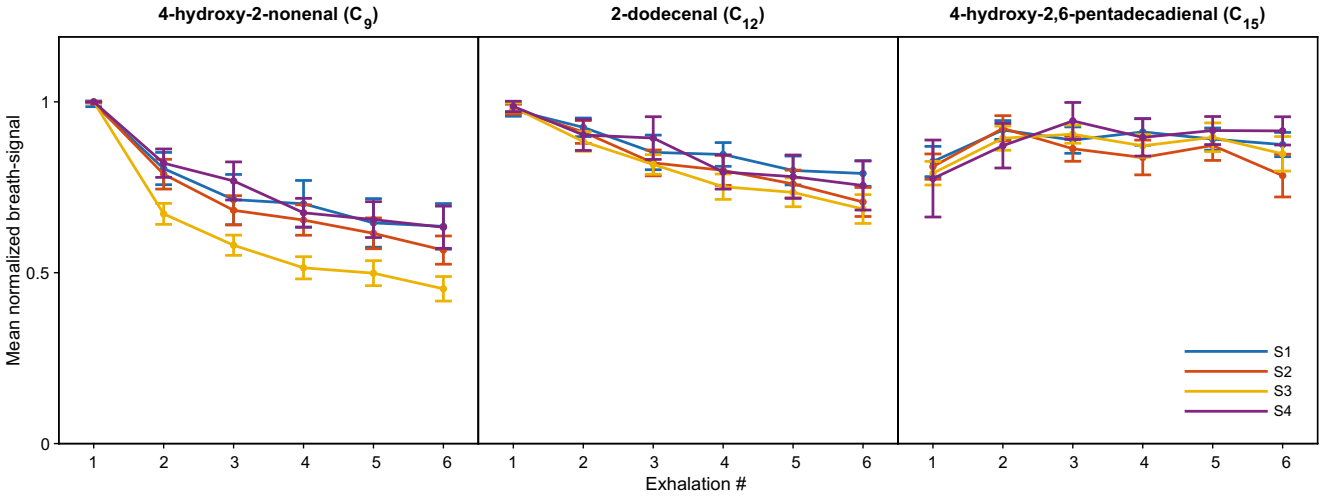

Fig. 2 Aldehydes show a subject-independent and molecule-dependent exhalation pattern. Data shown is the mean normalized breath-signal with errors bars representing 95% CIs for three selected aldehydes from four subjects (denoted as S1–S4) in 104 experiments as a function of exhalation number. Lighter species show a systematic decaying trend across consecutive exhalations, which is subject independent.

sub-ppt range [22]. For this reason, and not surprisingly, 92.7 ppb of β-pinene nearly saturated the detector of the Orbitrap mass analyzer. Because the dynamic range of our mass analyzer is five orders of magnitude (signal intensity

Table 1 Intra-subject variability in the breath-signal for the series of aldehydes studied in this work. The median and IQR values of the CVs (expressed as percentage) measured for the four subjects for the 27 aldehydes studied are listed; *DBE* double bond equivalent

m/z ($[M+H]^+$)	Metabolite				Coefficient of variation (%)							
					S1 ($N=25$)		S2 ($N=29$)		S3 ($N=37$)		S4 ($N=13$)	
	Formula (M)	Name	DBE	Mass error (ppm)	Median	IQR	Median	IQR	Median	IQR	Median	IQR
143.1066	C8H14O2	4-Hydroxy-2-octenal	2	−0.32	6.2	6.7	7.5	4.2	6.3	6.0	4.3	2.7
157.1223	C9H16O2	4-Hydroxy-2-nonenal	2	0.16	6.8	6.0	8.7	4.6	7.8	7.4	4.9	2.4
171.1379	C10H18O2	4-Hydroxy-2-decenal	2	−0.15	7.3	5.3	7.5	4.8	7.3	5.8	4.3	2.2
185.1537	C11H20O2	4-Hydroxy-2-undecenal	2	0.30	6.8	4.8	7.5	4.2	6.9	6.5	4.4	3.8
199.1694	C12H22O2	4-Hydroxy-2-dodecenal	2	0.58	7.6	8.5	8.0	4.7	7.7	6.6	4.6	3.6
213.1851	C13H24O2	4-Hydroxy-2-tridecenal	2	0.68	7.6	6.7	7.9	4.7	6.5	5.5	4.7	2.5
227.2005	C14H26O2	4-Hydroxy-2-tetradecenal	2	−0.11	5.6	4.5	7.2	3.9	6.6	5.6	5.5	4.0
241.2162	C15H28O2	4-Hydroxy-2-pentadecenal	2	−0.11	4.8	5.1	6.1	5.0	6.1	3.8	5.7	3.2
255.2319	C16H30O2	4-Hydroxy-2-hexadecenal	2	−0.02	6.2	5.0	5.1	5.0	6.0	5.8	7.1	3.4
127.1118	C8H14O	2-Octenal	2	0.07	6.5	3.8	6.3	4.2	7.3	8.5	7.4	6.5
141.1275	C9H16O	2-Nonenal	2	0.42	7.4	5.2	7.5	3.4	7.1	6.3	5.8	3.9
155.1429	C10H18O	2-Decenal	2	−0.66	6.0	7.0	6.6	5.4	6.0	5.9	4.0	4.7
169.1587	C11H20O	2-Undecenal	2	−0.01	6.6	7.0	8.1	4.2	7.4	5.6	5.7	5.3
183.1744	C12H22O	2-Dodecenal	2	0.49	6.7	4.9	7.7	5.2	6.9	6.3	5.4	2.2
197.1901	C13H24O	2-Tridecenal	2	0.56	6.5	7.3	7.0	3.5	7.5	7.0	5.5	4.6
211.2055	C14H26O	2-tetradecenal	2	−0.53	7.5	5.7	7.8	4.9	6.8	5.9	7.1	4.9
225.2213	C15H28O	2-Pentadecenal	2	−0.09	7.6	7.7	7.8	4.1	7.4	5.2	6.8	5.0
239.2369	C16H30O	2-Hexadecenal	2	−0.05	8.3	7.0	12.8	12.2	8.4	7.2	8.0	8.4
141.0910	C8H12O2	4-Hydroxy-2,6-octadienal	3	0.25	5.4	5.8	7.3	3.6	5.7	4.8	4.5	3.0
155.1066	C9H14O2	4-Hydroxy-2,6-nonadienal	3	−0.62	5.4	7.6	7.7	3.8	6.7	6.5	5.3	2.8
169.1223	C10H16O2	4-Hydroxy-2,6-dodecadienal	3	0.09	7.4	6.9	7.9	4.5	5.8	5.1	5.4	3.8
183.1380	C11H18O2	4-Hydroxy-2,6-undecadienal	3	0.46	7.1	6.3	7.8	3.5	7.7	5.7	4.5	3.0
197.1537	C12H20O2	4-Hydroxy-2,6-dodecadienal	3	0.58	7.6	5.5	7.7	4.5	5.3	4.3	4.8	3.0
211.1692	C13H22O2	4-Hydroxy-2,6-tridecadienal	3	−0.41	7.0	7.3	7.7	5.0	6.0	5.6	5.1	4.3
225.1849	C14H24O2	4-Hydroxy-2,6-tetradecadienal	3	−0.07	6.6	6.5	6.6	5.3	6.0	6.2	5.1	3.4
239.2005	C15H26O2	4-Hydroxy-2,6-pentadecadienal	3	−0.11	7.4	6.6	11.7	13.5	5.8	4.7	5.3	6.2
253.2162	C16H28O2	4-Hydroxy-2,6-hexadecadienal	3	−0.02	8.1	9.2	4.6	4.4	6.3	5.5	5.6	3.8

Table 2 Pairwise inter-subject variability in the breath-signal for the series of aldehydes studied in this work. Table shows the relative differences in aldehyde breath-signals between all subject pairings together with the lower and upper bounds (LB and UB) of the 95% CI and *p*-values

m/z ([M+H]$^+$)	Metabolite				S1 vs S2				S1 vs S3				S1 vs S4			
	Formula (M)	Name	DBE	Mass error (ppm)	% change	LB	UB	*p* value	% change	LB	UB	*p* value	% change	LB	UB	*p* value
143.1066	$C_8H_{14}O_2$	4-Hydroxy-2-Octenal	2	−0.32	−30.1	−54.6	−5.6	7.98×10^{-3}	−10.4	−33.6	12.9	1	−3.0	−33.7	27.7	1
157.1223	$C_9H_{16}O_2$	4-Hydroxy-2-Nonenal	2	0.16	−42.4	−67.9	−17.0	1.17×10^{-4}	−6.6	−30.7	17.6	1	−14.2	−46.1	17.7	1
171.1379	$C_{10}H_{18}O_2$	4-Hydroxy-2-Decenal	2	−0.15	−22.5	−56.0	11.0	4.41×10^{-1}	−23.0	−54.7	8.8	3.28×10^{-1}	18.6	−23.4	60.6	1
185.1537	$C_{11}H_{20}O_2$	4-Hydroxy-2-Undecenal	2	0.30	−56.4	−96.0	−16.9	1.28×10^{-3}	3.0	−34.5	40.5	1	−69.7	−119.2	−20.2	1.54×10^{-3}
199.1694	$C_{12}H_{22}O_2$	4-Hydroxy-2-Dodecenal	2	0.58	−30.4	−56.6	−4.3	1.37×10^{-2}	−4.2	−29.0	20.6	1	−11.5	−44.3	21.3	1
213.1851	$C_{13}H_{24}O_2$	4-Hydroxy-2-Tridecenal	2	0.68	−33.8	−56.7	−10.8	8.40×10^{-4}	−1.2	−23.0	20.6	1	−18.5	−47.2	10.3	5.22×10^{-1}
227.2005	$C_{14}H_{26}O_2$	4-Hydroxy-2-Tetradecenal	2	−0.11	−60.0	−88.5	−31.6	7.74×10^{-7}	1.6	−25.4	28.6	1	−84.0	−119.6	−48.4	3.89×10^{-8}
241.2162	$C_{15}H_{28}O_2$	4-Hydroxy-2-Pentadecenal	2	−0.11	−44.0	−67.6	−20.4	1.35×10^{-5}	2.9	−19.5	25.3	1	−38.2	−67.8	−8.7	4.44×10^{-3}
255.2319	$C_{16}H_{30}O_2$	4-Hydroxy-2-Hexadecenal	2	−0.02	−36.2	−62.9	−9.5	2.53×10^{-3}	10.8	−14.5	36.1	1	−5.3	−38.7	28.2	1
127.1118	$C_8H_{14}O$	2-Octenal	2	0.07	−73.7	−125.0	−22.4	1.18×10^{-3}	4.9	−43.8	53.5	1	−56.5	−120.7	7.8	1.19×10^{-1}
141.1275	$C_9H_{16}O$	2-Nonenal	2	0.42	−82.7	−114.8	−50.7	2.34×10^{-9}	14.8	−15.6	45.3	1	−100.8	−141.0	−60.7	5.73×10^{-9}
155.1429	$C_{10}H_{18}O$	2-Decenal	2	−0.66	20.1	−11.8	52.1	5.56×10^{-1}	89.3	59.0	119.6	1.85×10^{-11}	78.7	38.6	118.7	4.30×10^{-6}
169.1587	$C_{11}H_{20}O$	2-Undecenal	2	−0.01	−88.0	−144.0	−31.9	3.16×10^{-4}	5.1	−48.0	58.3	1	−139.7	−209.9	−69.5	3.28×10^{-6}
183.1744	$C_{12}H_{22}O$	2-Dodecenal	2	0.49	−57.9	−95.8	−20.0	4.83×10^{-4}	9.8	−26.1	45.8	1	−99.4	−146.8	−51.9	9.72×10^{-7}
197.1901	$C_{13}H_{24}O$	2-Tridecenal	2	0.56	−39.3	−68.0	−10.7	2.16×10^{-3}	11.9	−15.3	39.1	1	−27.4	−63.4	8.5	2.54×10^{-1}
211.2055	$C_{14}H_{26}O$	2-Tetradecenal	2	−0.53	−50.3	−78.3	−22.3	2.88×10^{-5}	−5.2	−31.8	21.4	1	−78.0	−113.1	−42.9	2.06×10^{-7}
225.2213	$C_{15}H_{28}O$	2-Pentadecenal	2	−0.09	−47.7	−81.0	−14.5	1.19×10^{-3}	−13.1	−44.7	18.4	1	−60.3	−102.0	−18.7	1.06×10^{-3}
239.2369	$C_{16}H_{30}O$	2-Hexadecenal	2	−0.05	−35.5	−67.6	−3.4	2.19×10^{-2}	−3.6	−34.1	26.8	1	−6.1	−46.3	34.1	1
141.0910	$C_8H_{12}O_2$	4-Hydroxy-2,6-Octadienal	3	0.25	−39.9	−65.2	−14.6	2.96×10^{-4}	9.9	−14.1	33.9	1	−23.6	−55.3	8.1	2.88×10^{-1}
155.1066	$C_9H_{14}O_2$	4-Hydroxy-2,6-Nonadienal	3	−0.62	−54.8	−83.4	−26.3	7.14×10^{-6}	7.0	−20.1	34.0	1	−43.6	−79.3	−7.8	8.54×10^{-3}
169.1223	$C_{10}H_{16}O_2$	4-Hydroxy-2,6-Dodecadienal	3	0.09	−72.4	−99.8	−45.0	1.06×10^{-9}	−10.0	−36.0	16.0	1	−24.3	−58.6	10.0	3.58×10^{-1}
183.1380	$C_{11}H_{18}O_2$	4-Hydroxy-2,6-Undecadienal	3	0.46	−95.4	−179.2	−11.7	1.66×10^{-2}	1.3	−78.1	80.7	1	−166.9	−271.8	−62.0	2.55×10^{-4}
197.1537	$C_{12}H_{20}O_2$	4-Hydroxy-2,6-Dodecadienal	3	0.58	−50.3	−91.0	−9.6	7.41×10^{-3}	−20.8	−59.4	17.8	9.01×10^{-1}	−74.6	−125.6	−23.7	9.04×10^{-4}
211.1692	$C_{13}H_{22}O_2$	4-Hydroxy-2,6-Tridecadienal	3	−0.41	−51.6	−76.1	−27.2	7.93×10^{-7}	−5.5	−28.7	17.7	1	−37.4	−68.0	−6.7	$8.53\ 10^{-3}$
225.1849	$C_{14}H_{24}O_2$	4-Hydroxy-2,6-Tetradecadienal	3	−0.07	−74.0	−108.8	−39.1	6.82×10^{-7}	3.3	−29.7	36.4	1	−90.0	−133.7	−46.3	1.42×10^{-6}
239.2005	$C_{15}H_{26}O_2$	4-Hydroxy-2,6-Pentadecadienal	3	−0.11	−31.3	−54.1	−8.4	2.22×10^{-3}	6.4	−15.2	28.1	1	−55.5	−84.1	−26.8	6.02×10^{-6}
253.2162	$C_{16}H_{28}O_2$	4-Hydroxy-2,6-Hexadecadienal	3	−0.02	−24.0	−47.1	−1.0	3.61×10^{-2}	8.7	−13.1	30.6	1	−21.0	−49.9	7.8	3.16×10^{-1}

Table 2 (continued)

					S1 vs S2				S1 vs S3				S1 vs S4			
					% change	LB	UB	*p* value	% change	LB	UB	*p* value	% change	LB	UB	*p* value
143.1066	$C_8H_{14}O_2$	4-Hydroxy-2-Octenal	2	−0.32	15.1	−2.0	32.3	1.15×10^{-1}	20.8	−2.3	43.8	1.02×10^{-1}	6.7	−19.6	32.9	1
157.1223	$C_9H_{16}O_2$	4-Hydroxy-2-Nonenal	2	0.16	25.2	8.9	41.4	3.88×10^{-4}	19.8	−2.1	41.7	9.96×10^{-2}	−7.2	−35.4	21.0	1
171.1379	$C_{10}H_{18}O_2$	4-Hydroxy-2-Decenal	2	−0.15	−0.4	−25.2	24.5	1	33.5	0.1	67.0	4.89×10^{-2}	33.8	1.6	66.0	3.43×10^{-2}
185.1537	$C_{11}H_{20}O_2$	4-Hydroxy-2-Undecenal	2	0.30	38.0	15.0	60.9	1.33×10^{-4}	−8.5	−39.4	22.4	1	−74.9	−123.1	−26.8	3.59×10^{-4}
199.1694	$C_{12}H_{22}O_2$	4-Hydroxy-2-dodecenal	2	0.58	20.1	1.9	38.3	2.25×10^{-2}	14.5	−10.0	39.0	6.84×10^{-1}	−7.0	−36.6	22.7	1
213.1851	$C_{13}H_{24}O_2$	4-Hydroxy-2-Tridecenal	2	0.68	24.4	8.8	39.9	3.41×10^{-4}	11.4	−9.5	32.4	8.72×10^{-1}	−17.1	−43.9	9.7	5.36×10^{-1}
227.2005	$C_{14}H_{26}O_2$	4-Hydroxy-2-Tetradecenal	2	−0.11	38.5	22.4	54.7	2.70×10^{-8}	−14.9	−36.7	6.8	4.02×10^{-1}	−87.0	−121.1	−52.9	3.49×10^{-9}
241.2162	$C_{15}H_{28}O_2$	4-Hydroxy-2-Pentadecenal	2	−0.11	32.6	17.7	47.4	3.17×10^{-7}	4.0	−16.0	24.0	1	−42.3	−71.0	−13.6	8.10×10^{-4}
255.2319	$C_{16}H_{30}O_2$	4-Hydroxy-2-Hexadecenal	2	−0.02	34.5	16.7	52.3	6.02×10^{-6}	22.7	−1.3	46.7	7.39×10^{-2}	−18.0	−53.4	17.3	1
127.1118	$C_8H_{14}O$	2-Octenal	2	0.07	45.2	18.4	72.1	9.54×10^{-5}	9.9	−26.2	46.0	1	−64.5	−128.2	−0.8	4.54×10^{-2}
141.1275	$C_9H_{16}O$	2-Nonenal	2	0.42	53.4	37.4	69.3	8.66×10^{-14}	−9.9	−31.4	11.5	1	−135.8	−180.3	−91.4	4.58×10^{-12}
155.1429	$C_{10}H_{18}O$	2-Decenal	2	−0.66	86.7	50.3	123.0	2.79×10^{-8}	73.3	24.4	122.2	6.47×10^{-4}	−100.2	−454.2	253.7	1
169.1587	$C_{11}H_{20}O$	2-Undecenal	2	−0.01	49.5	22.4	76.6	2.03×10^{-5}	−27.5	−64.0	8.9	2.69×10^{-1}	−152.7	−222.5	−82.9	3.14×10^{-7}
183.1744	$C_{12}H_{22}O$	2-Dodecenal	2	0.49	42.9	21.1	64.7	4.29×10^{-6}	−26.3	−55.6	3.1	1.07×10^{-1}	−121.1	−170.7	−71.4	1.39×10^{-8}
197.1901	$C_{13}H_{24}O$	2-Tridecenal	2	0.56	36.8	18.1	55.5	4.21×10^{-6}	8.5	−16.6	33.7	1	−44.7	−83.1	−6.2	1.38×10^{-2}
211.2055	$C_{14}H_{26}O$	2-Tetradecenal	2	−0.53	30.0	13.1	46.9	3.76×10^{-5}	−18.4	−41.2	4.4	1.92×10^{-1}	−69.2	−100.6	−37.7	2.71×10^{-7}
225.2213	$C_{15}H_{28}O$	2-Pentadecenal	2	−0.09	23.4	3.0	43.9	1.59×10^{-2}	−8.5	−36.1	19.0	1	−41.7	−76.5	−7.0	9.93×10^{-3}
239.2369	$C_{16}H_{30}O$	2-Hexadecenal	2	−0.05	23.5	2.0	45.0	2.45×10^{-2}	21.7	−7.3	50.7	2.80×10^{-1}	−2.4	−39.0	34.2	1
141.0910	$C_8H_{12}O_2$	4-Hydroxy-2,6-Octadienal	3	0.25	35.6	19.2	52.1	4.11×10^{-7}	11.7	−10.5	33.8	9.56×10^{-1}	−37.2	−70.4	−4.0	1.95×10^{-2}
155.1066	$C_9H_{14}O_2$	4-Hydroxy-2,6-Nonadienal	3	−0.62	39.9	23.2	56.6	2.84×10^{-8}	7.3	−15.3	29.8	1	−54.3	−90.5	−18.1	6.40×10^{-4}
169.1223	$C_{10}H_{16}O_2$	4-Hydroxy-2,6-Dodecadienal	3	0.09	36.2	21.7	50.6	6.08×10^{-9}	27.9	8.5	47.3	1.20×10^{-3}	−13.0	−42.4	16.4	1
183.1380	$C_{11}H_{18}O_2$	4-Hydroxy-2,6-Undecadienal	3	0.46	49.5	10.6	88.4	5.38×10^{-3}	−36.6	−88.9	15.8	3.80×10^{-1}	−170.5	−270.7	−70.2	8.13×10^{-5}
197.1537	$C_{12}H_{20}O_2$	4-Hydroxy-2,6-Dodecadienal	3	0.58	19.6	−5.0	44.2	2.06×10^{-1}	−16.2	−49.3	16.9	1	−44.6	−84.4	−4.8	1.96×10^{-2}
211.1692	$C_{13}H_{22}O_2$	4-Hydroxy-2,6-Tridecadienal	3	−0.41	30.4	15.8	45.1	1.18×10^{-6}	9.4	−10.3	29.1	1	−30.3	−57.7	−2.9	2.22×10^{-2}
225.1849	$C_{14}H_{24}O_2$	4-Hydroxy-2,6-Tetradecadienal	3	−0.07	44.4	26.2	62.6	1.36×10^{-8}	−9.2	−33.7	15.3	1	−96.6	−139.1	−54.0	1.20×10^{-7}
239.2005	$C_{15}H_{26}O_2$	4-Hydroxy-2,6-Pentadecadienal	3	−0.11	28.7	12.9	44.6	$2.31 \times 10_{-5}$	−18.4	−39.7	2.9	1.32×10^{-1}	−66.2	−95.0	−37.3	8.76×10^{-8}
253.2162	$C_{16}H_{28}O_2$	4-Hydroxy-2,6-Hexadecadienal	3	−0.02	26.4	9.5	43.3	3.35×10^{-4}	2.4	−20.3	25.2	1	−32.6	−62.5	−2.8	2.43×10^{-2}

10^4–10^9 a.u.), the limit of detection is expected to be at around 1 ppt, which is consistent with previous SESI-MS quantification studies [46]. When we started the delivery of β-pinene, the signal of the protonated analyte raised sharply to reach a plateau. We measured the stability of the signal intensity detection during 1 h. When the delivery of β-pinene was stopped, the signal intensity dropped abruptly to baseline level, indicating no carryover effects, at least for this particular compound (inset Fig. S3, see ESM). The CV of β-pinene signal intensity during an hour of continuous delivery of the vapor was found to be 2.3%. We therefore conclude that technical CVs within 3% are to be expected for our SESI-HRMS platform.

Replicate exhalations: intra- and inter-subject variability

In total, the four participants provided 648 exhalations (n = 171 for subject 1, n = 174 for subject 2, n = 225 for subject 3 and n = 78 for subject 4). These measurements were subdivided into 104 single experiments (N = 25 for subject 1, N = 29 for subject 2, N = 37 for subject 3, and N = 13 for subject 4) each containing 6 to 13 exhalations (replicates) performed within 10 to 20 min. The aim was to examine the variability across these replicates, considering that the technical variability, as mentioned above, was found to be in the range of 3%. Figure 1 b shows one such representative experiment whereby a subject provided 13 consecutive exhalations during 19 min (ESM Fig. S4 shows a zoomed-in view of the first exhalation, where the time traces can be inspected in greater detail).

The vast majority of the features typically detected by SESI-HRMS in human breath remain to be positively identified. However, over the last years, we have made a substantial effort to systematically identify the molecular structure for some of these metabolites by combining real-time breath MS/MS analysis and UPLC-MS/MS analysis of exhaled breath condensate [47–53]. Given the clinical importance of aldehydes, as potential surrogates of oxidative stress [54–59], we will concentrate in discussing our findings for a series of three classes of fatty aldehydes [48]: 4-hydroxy-2-alkenals ($C_xH_{2x-2}O_2$), 2-alkenals ($C_xH_{2x-2}O$), and 4-hydroxy-2,6-alkadienals ($C_xH_{2x-4}O_2$) with chain lengths ranging from C_8 to C_{16}. These 27 representative aldehydes were used as benchmarking metabolites. For reference, Fig. 1 b shows the time traces of three such representative exhaled aldehydes and Fig. S5 (see ESM) shows the time traces for the 27 aldehydes of interest from the same experiment. The gray areas in Fig. 1 b and Fig. S4 (see ESM) represent the time windows whereby CO_2 levels were above 3%.

Visual inspection of CO_2 and exhalation parameters from Fig. 1b suggests a high repeatability across replicate measurements. Indeed, computed mean ± SD for this particular experiment yielded a CO_2 level of 4.7 ± 0.1%, an exhalation flow rate of 11.7 ± 0.3 L/min and an exhaled volume of 2.6 ± 0.1 L (i.e., excluding 0.5–0.6 L of breath not containing at least 3% of CO_2) for the considered windows. Median CVs (IQRs) for CO_2, exhalation flow rate, and exhaled volume based on all 104 experiments were 3.2% (1.5%), 3.1% (1.9%), and 5.0% (4.6%), respectively. The overall picture for the aldehydes was somehow more complex. While 4-hydroxy-2,6-pentadecadienal in Fig. 1 b shows a relatively constant behavior across all exhalations (akin to CO_2), 2-dodecenal drops over time during consecutive exhalations and the decay is even more pronounced for 4-hydroxy-2-nonenal, whose signal intensity decays by ~35% during the first three exhalations, to then reach a steady state. Interestingly, we observed this behavior systematically for these particular molecules among all participants. Figure 2 shows the mean normalized breath-signal (see "Material and methods" for details) and the corresponding 95% CI from all experiments for the four participants as a function of exhalation number for the three selected representative compounds shown in Fig. 1 b. It clearly shows that the dynamics for each compound are subject independent and, interestingly, seem to depend on the aldehyde chain length. For example, signal intensity drops between the first and the sixth exhalation for 4-hydroxy-2-nonenal is around 50%, for 2-dodecenal the drop is around 30%, whereas for 4-hydroxy-2,6-pentadecadienal signal intensity remains stable (or even increases after the first exhalation). This trend was systematically observed for all the aldehydes from the three classes (ESM Figs. S6-S8).

Location within the respiratory system where the gas exchange occurs may explain the molecule-dependent exhalation traces

The signal intensity decaying behavior as a function of chain length can be rationalized by the dependency with Ostwald blood-air partition coefficient ($\lambda_{b:a}$), which is the most important factor in determining the location within the respiratory system where the gas exchange occurs [60]. Soluble gases with $\lambda_{b:a} > 100$ exchange almost exclusively within the airways (with the bronchial blood), whereas those with $10 < \lambda_{b:a} < 100$ exchange partially in the airways and in the alveoli, and those with a $\lambda_{b:a} < 10$ nearly exclusively exchange in the alveoli (with the pulmonary blood) [61]. Therefore, CO_2 ($\lambda_{b:a} = 3$) exchanges in the alveoli [62]. Figure 1 b shows that the CO_2 level does not decrease as the participant provides consecutive exhalations and this was the trend observed across all measurements. The same trend is observed for the longest aldehydes, which in turn have the lowest $\lambda_{b:a}$ from the series. The predicted $\lambda_{b:a}$ by Kramer et al. [63] suggests that, indeed, shorter aldehydes have a greater $\lambda_{b:a}$. For example, the predicted $\lambda_{b:a}$ for 2-hexenal was 111, therefore exchanges almost exclusively in the airways. In contrast, 2-undecenal

has a predicted $\lambda_{b:a} = 39$; hence, it exchanges partially in the airways and in the alveoli. It is expected that even longer aldehydes (> C_{14}), such as those studied in this work, will have a $\lambda_{b:a}$ approaching the critical value of 10 (i.e., almost exclusively exchanged in the alveoli). This trend can be observed in Fig. S9 (see ESM), which shows predicted $\lambda_{b:a}$ as a function of the number of carbon atoms from the aldehydes, based on data by Kramer et al. [63]. Thus, we hypothesize that the longest aldehydes studied here (C_{14}–C_{16}) exchange exclusively in the alveoli, and for this reason show a similar behavior as CO_2. In contrast, the smaller aldehydes exchange mainly in the airways, leading to a decrease during prolonged consecutive exhalations. For example, it has been estimated that ethanol, which has a high blood solubility ($\lambda_{b:a} = 1{,}803$), can show a 20% lower concentration than alveolar air after a complete prolonged exhalation [62]. Reinforcing this idea, we found that the signal intensity as a function of exhaled volume during a single exhalation, varies significantly depending on the aldehyde chain length and therefore on their $\lambda_{b:a}$. Figure 3 a displays signal intensity profiles of the aldehydes as a function of exhaled volume for a representative first and last exhalation in an experiment (same experiment as Fig. 1 b). It clearly shows how the C_9 metabolites reach a maximum intensity at ~ 0.7 L to then decrease. In contrast, the exhalation profile for the longest aldehydes (C_{12} and C_{15}) tends to increase systematically with exhaled volume (similarly to CO_2 profiles). We hypothesize that, as the exhalation maneuver is repeated, the net influx towards bronchial circulation exceeds that outwards. Thus, the partial pressure cannot re-equilibrate in the short lapse in-between exhalations, leading to a constant non-linear decay across the repeated measurements. For 4-hydroxy-2-dodecanal, we observed a deviation from the decaying pattern (Fig. 3 a and ESM Fig. S5). The underlying reason might be that this particular *m/z* channel is dominated by an isomer of 4-hydroxy-2-dodecanal. It is important to note at this point that this is a limitation of SESI-HRMS, as discrimination of isomers is sacrificed by the possibility of performing real-time analysis.

In order to further connect the theoretical explanation as to why $\lambda_{b:a}$ ultimately modulates the decay in signal intensity due to gas exchange in the airways, Fig. 3 b (and ESM Fig. S10) shows the experimental average breath-signal difference between the last and first exhalation, as a function of predicted $\lambda_{b:a}$. These $\lambda_{b:a}$ values were estimated by fitting the $\lambda_{b:a}$ for all aldehydes reported by Kramer et al. [63] (ESM Fig. S9). It reveals a clear trend, whereby for the longest chain (C_{16}) the difference tends to increase during the repeated measurements. This is especially evident for 4-hydroxy-2-hexadecenal (ESM Fig. S6). In contrast, as the chain length decreases (and thus $\lambda_{b:a}$ increases), the breath-signal difference decreases to finally reach a plateau of Δ −20% to −40% at C_{11}. The fact that this clear trend occurs in the transition boundaries between $10 < \lambda_{b:a} < 100$ suggests that indeed this may be due to the different regions of the respiratory

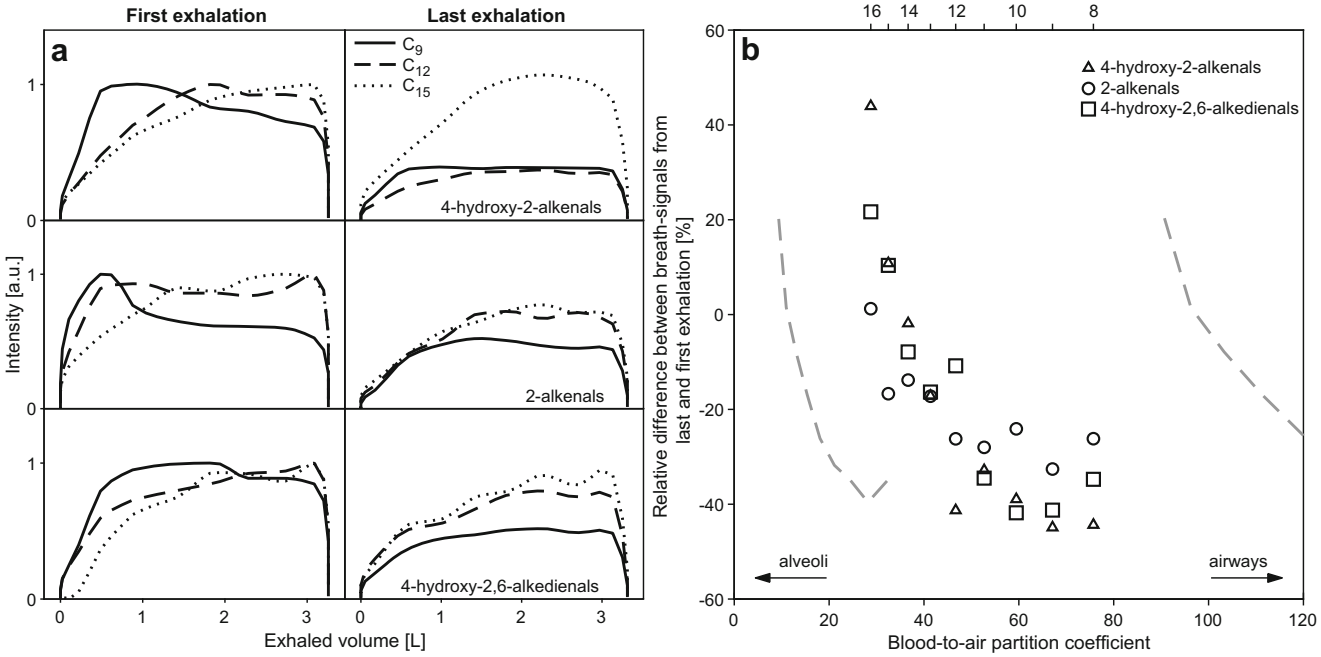

Fig. 3 Dependency of exhalation profile of breath metabolites with blood-to-air partition coefficient. **a** Exhalation profiles of short and long aldehydes as a function of exhaled volume is consistent with the hypothesis that the shorter aldehydes exchange mostly in the airways, while longer aldehydes exchange in the alveoli. 4-hydroxy-2-dodecenal shows a deviating pattern that may be caused by an interfering peak. **b** Relative difference between breath-signals from last and first exhalation as a function of predicted blood-to-air partition coefficient. A number of carbon atoms for molecules are shown at the top and gray dashed curves shows the 95% CI from $\lambda_{b:a}$ estimation. A large partition coefficient is associated with a strongly decaying pattern (also see ESM Fig. S10, with *x*-axis on $\log_{10}$ scale, showing the complete range for 95% CIs)

system where these series of compounds exchange: from alveoli for C_{16} to airways for C_8, with a mixed exchange situation for intermediate species. Further work is required to confirm this hypothesis and whether this could be further exploited to infer physiological information of the respiratory system, for example, complementing other tests such as the multiple-breath washout test to measure abnormal ventilation distribution between well- and poorly ventilated lung regions.

Despite that the first exhalation may reflect more accurately systemic concentrations for metabolites with high blood-air partition coefficients, we recommend to sample at least ten replicate exhalations and compute breath-signals considering only the last three exhalations, thus capturing the steady state. When doing so in the example shown in Fig. 1 b, the median CV (IQR) for the 27 aldehydes was 4.1% (1.5%), which approaches the technical variability of ~ 3% measured with standard β-pinene vapors. However, for pediatric patients and patients suffering from respiratory diseases, this may prove difficult. For this reason, in order to determine an upper bound of expected variability, we have evaluated here the variability of breath metabolites across all subjects considering only six exhalations and excluded the first three maneuvers to the breath-signal for metabolites. When doing so, we found that the median CV (IQR) for the aldehydes studied here was 6.7% (5.5%). Table 1 lists the intra-subject CVs for the 27 aldehydes studied here.

Flow dependency

Some studies indicate that the exhalation maneuver itself can in some cases alter the metabolic profile, hence providing misleading results [32]. For this reason, we further investigated whether the exhalation flow rate of our protocol had an impact on the breath-signal of the exhaled metabolites. Flow resistance of the device was as low as 3 mbar × min/L, mean ± SD exhalation flow rates of all the experiments performed in this study ($N = 104$) was 10.6 ± 0.9 L/min (ESM Fig. S11) and typical exhaled volumes were in the order of 3 L (i.e., 15–20 s of exhalation). It is important to note that this maneuver is far less invasive and easy to perform than a classical spirometry, whereby the forced expiratory volume in one second (FEV_1) can typically be 4 L in adults. This implies exhalation flow rates around 25 times higher than the maneuver used in our experiments. It has been shown that such forced expiration maneuvers can lead to substantial changes in exhaled CO_2 and other metabolites [32]. The fact that no significant changes in the CO_2 levels were observed suggests that the maneuver does not induce hyperventilation [42]. In order to determine whether there was any dependency with the exhalation flow rate, we explored the impact of exhaling at two flow rates, one at the lower end and another one at the upper end of the distribution of exhalation flow rates measured for all participants (ESM Fig. S11). Figure 4 a shows the comparison of two measurements from the same subject at a lower flow rate (9.8 ± 0.1 L/min) and consecutively at a higher flow rate (12.0 ± 0.3 L/min). Bland-Altman plot for log-transformed variables shows that the breath-signal of metabolites is independent of the exhalation flow rate. The mean of $\log_{10}$(ratio) was found to be − 0.09. As expected, only ~ 4% of low-intensity ions lie outside the mean ± 1.96 × SD bands. We therefore conclude that the range of flow rates between 9 and 12 L/min are suitable for breath metabolomics using our particular configuration.

Antibacterial/antiviral spirometry filter

Patient and operator safety and hygiene are crucial factors to take into account in the clinics. For this reason, the interface between the patient and the breath analysis platform is through a disposable barrier filter, as the ones routinely used for pulmonary function testing. This is a new element incorporated in this device to allow for measuring patients with suspected respiratory infectious diseases. Until now, our system featured a mouthpiece filter used for alcohol breath tests, which would not be suitable to investigate contagious respiratory diseases. In a separate set of experiments, we examined whether these aerosol filters may have an impact on the detected metabolites. To do so, we compared the breath-signal of the same subject exhaling through the filter and subsequently exhaling without the filter. Figure 4 b shows the resulting comparison, represented as a Bland-Altman plot for log-transformed variables. There appears to be a small bias towards lower intensities by the use of the filter, as the mean of $\log_{10}$(ratio) was found to be − 0.17. Moreover, only 4.6% of the signals fell outside the mean ± 1.96 × SD boundaries. Globally, these results are consistent with previous studies suggesting that SESI-MS breath spectra using and removing aerosol filters look alike [64]. We therefore conclude that, while the antibacterial/antiviral filters incorporated in our system may partially suppress some signal intensities, they represent a good compromise to protect the system and the operator from pathogens and to preserve the quality of the mass spectral readout of exhaled metabolites.

Instrumental time drift

Instrumental time drifts and batch effects are a common problem in untargeted metabolomics [65, 66]. This can be especially critical in clinical studies as patient recruitment typically runs over several months/years. In order to assess whether our system showed any significant batch effect due to the date of measurement, we visualized our data using principal components analysis (PCA). Figure 5 shows the resulting plot for the first two components, whereby the labels on the left-hand side correspond to a total of 17 measuring days spanning across 1 month. No clustering according to measuring day is evident,

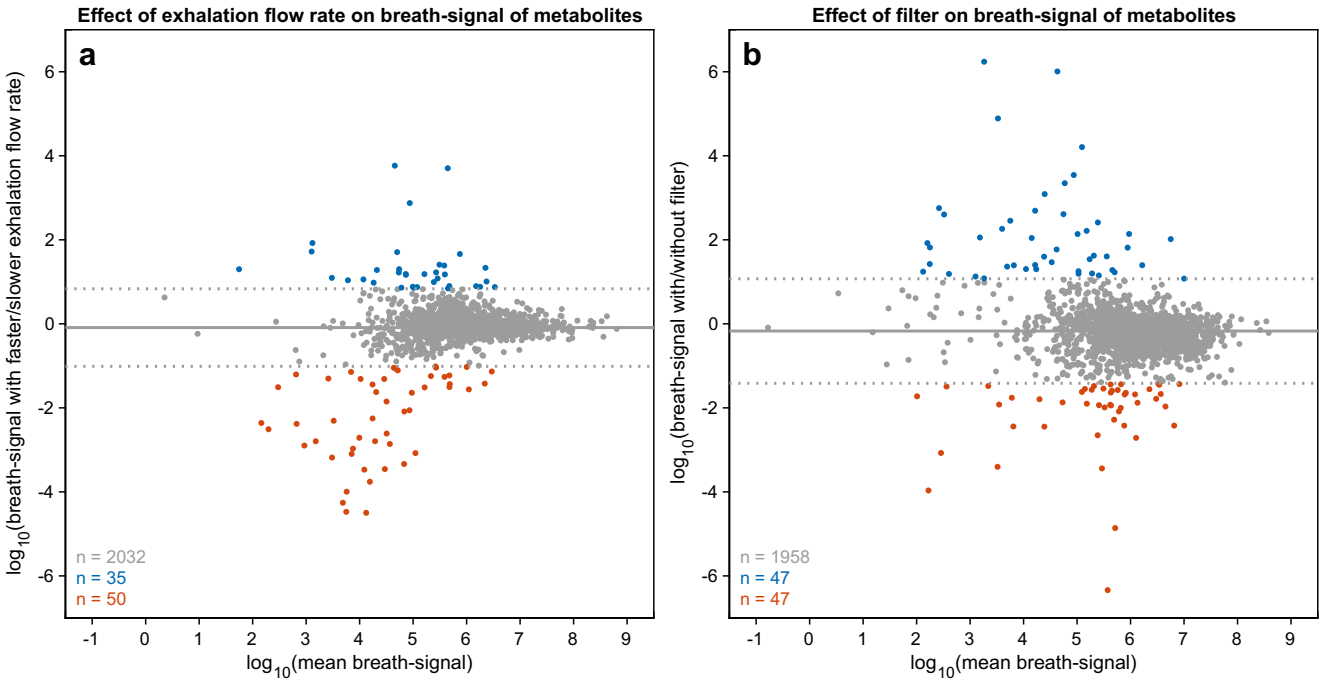

Fig. 4. Evaluation of breath mass spectra at varying exhalation flow rates and using spirometry filters. **a** Breath-signals of exhaled metabolites are independent of exhalation flow rate. As seen by the comparison of signals from two experiments with slower (9.8 ± 0.1 L/min) and faster (12.0 ± 0.3 L/min) exhalation flow rates. **b** Use of filters does not significantly affect the breath-signals of exhaled metabolites. As seen by the comparison of signals from two experiments with and without the presence of an antibacterial/antiviral filter. In both panels, solid gray horizontal line represents the mean and dotted gray horizontal lines represent mean ± 1.96 × SD.

suggesting that the variance explained by these two components (48.6% in total) cannot be attributed to a batch effect. Note that no special cleaning procedures, apart from flushing the ion source with hot nitrogen overnight, were performed during this month of operation. In contrast, on the right-hand side of Fig. 5, the same score plot is shown whereby the labels now indicate the subject number. Grouping based on the subject number is much more evident. For example, subjects 1

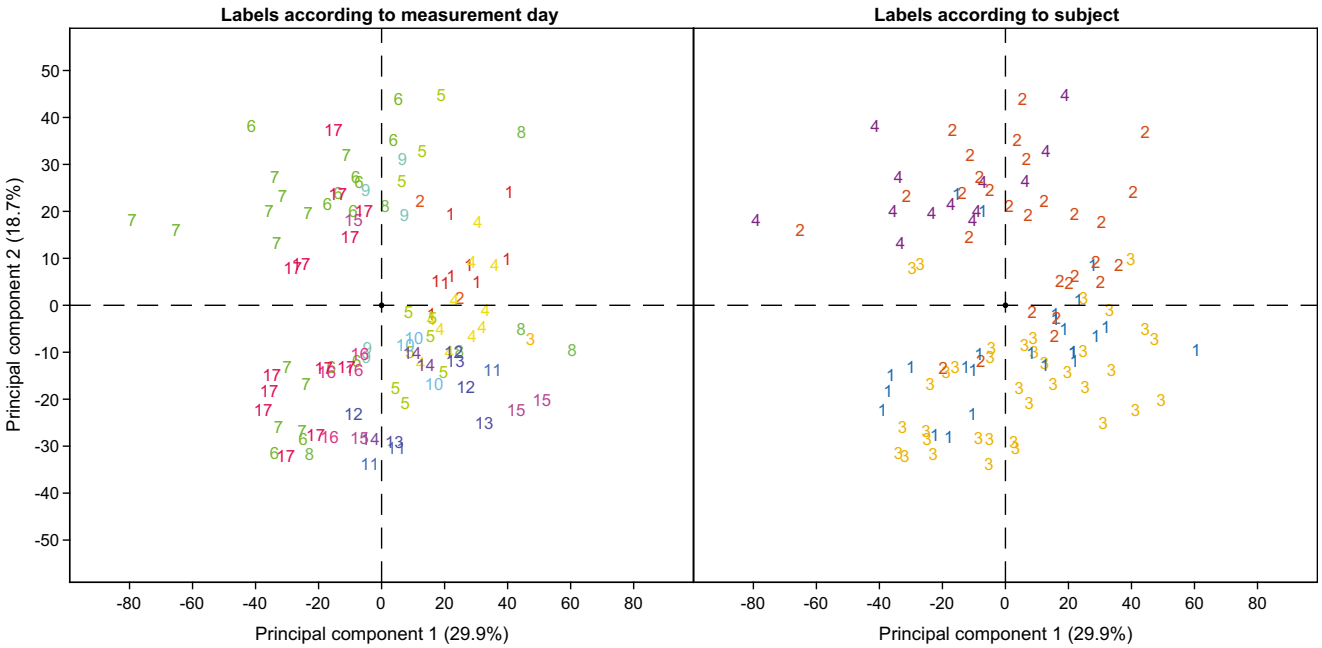

Fig. 5 Variability of SESI-HRMS breath mass spectra are dominated by inter-individual differences, rather than by batch effect**.** PCA score plot of all measurements with labels according to measuring day (left) and subject number (right). Grouping according to subject number is more evident than by measurement date.

and 3 cluster together suggesting a significantly different exhaled metabolic phenotype than subjects 2 and 4. This is also consistent with previous studies suggesting the existence of stable individual-specific metabolic traits [67–69]. The same picture emerged when we considered the 27 representative aldehydes (ESM Fig. S12). In order to provide a more objective assessment of whether significant differences exist across subjects for these metabolites, we conducted an ANOVA test followed by post hoc multiple comparison using a Bonferroni method (Table 2).

This univariate approach revealed significant differences in the breath-signal of exhaled aldehydes. Overall, the median (IQR) relative difference between individuals (considering only those $p \leq 0.05$) was 48.2% (39.3%). This is consistent with inter-subject variability in blood concentrations for these particular compounds. For example, Mak et al. [70] reported CVs for 4-hydroxy-nonenal from eight healthy individuals of 95.8%. In our case, mean differences between subject 1 and 2 were of 42.4% for this particular compound. It is therefore evident that the inter-subject biological variability is greater than intra-subject variability, and is consistent with the variability expected in blood levels.

Fatty aldehydes as surrogate markers of oxidative stress

Fatty aldehydes were chosen as metabolite models for this study as they are related to lipid peroxidation and oxidative stress. Oxidative stress is the trigger for the production of fatty aldehydes, such as 4-hydroxy-2-nonenal, in human metabolism [71]. Abnormally elevated values (factor two to three as compared to controls) of some of the aldehydes studied here have been associated with pathologies such as congestive heart failure [70]. Strong associations between series of metabolites, i.e., in terms of correlations, might be an indication for a common metabolic pathway, as already shown previously for series of omega-oxidation end-products of aliphatic fatty acids [52, 72] and aminoacids [73]. In an attempt to visualize whether an interplay between the different series of fatty aldehydes may be captured by breath analysis, we computed correlation coefficients across all measurements. A first indication suggesting that these metabolites are indeed metabolically connected is given by the fact that all of them showed positive correlations (ESM Figs. S13-15). Thus, all measured subjects had consistent (high or low) breath-signals for all 27 metabolites. One could argue that this might be an artifact as a

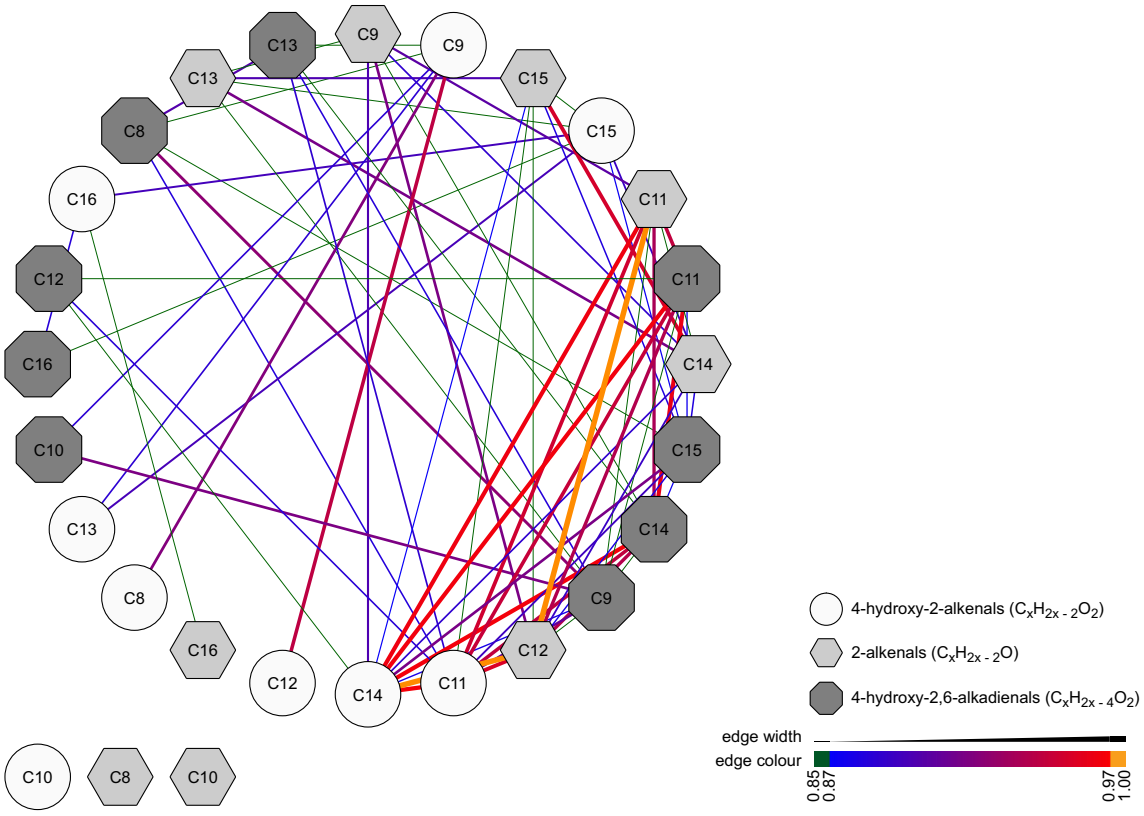

Fig. 6. Positive correlation among aldehydes suggests a common origin of mechanism of generation. Correlation network (considering Spearman's $r \geq 0.85$) with an average node degree of 4 ± 2. Note that 4-hydroxy-2-decenal, 2-octenal and 2-decenal do not pass the correlation cutoff and hence are shown at the bottom-left side. Node shape and color are based on the classes of aldehydes, whereas edge width and color depends on the correlation coefficient, as shown in the legend at the bottom-right side.

result of different performance of the system during the different days (i.e., consistently high- or low-intensity mass spectra). However, this can be ruled out as we found that these aldehydes consistently correlated with each other, but not with the rest of the over 2,000 features considered in the breath mass spectra (ESM Fig. S16). Only around 2% of the pairwise correlations for all features correlated with $r \geq 0.85$ with the aldehydes. We therefore conclude that the observed associations for these families of compounds should encode a biological meaning. Figure 6 shows the resulting correlation network for the aldehydes. Most of the aldehydes are indeed linked with a mean ± SD degree of 4 ± 2 ($r \geq 0.85$). This is to be expected from the metabolic point of view, as aliphatic aldehydes in humans are largely produced by a cascade of catabolic metabolism of several lipids [71]. In particular, peroxidative cleavage of polyunsaturated fatty acids by reactive oxygen species is the mechanism behind a complete series of aldehydes as those studied, including short- and medium-chain aldehydes, or hydroxy-alkenals.

Conclusions

Summing up, we presented here a series of instrumental developments aiming to standardize sampling and analysis of expired metabolites by real-time SESI-HRMS. This analytical platform was tested using a constant infusion of β-pinene vapors in the ppb range resulting in a technical variability within 3%. We then tested the system during a series of repeated breath measurements from four healthy individuals. Real-time display of CO_2, exhalation flow rate, and exhaled volume to the subjects during the exhalation maneuver enabled a variability for these variables within 5%. We found no evidence that the exhalation maneuvers would induce hyperventilation, nor that the exhalation flow rates and mouthpiece filter used would have any significant impact on the quality of the metabolic breath print. We also did not find any evidence of obvious batch effect. However, despite these indications of exhalation maneuver control and reproducibility, we observed a systematic decay in the signal intensity of the shorter aldehydes across all measurements for all subjects. This compound-specific and individual-independent pattern has been rationalized as a result of the different locations of the respiratory system where the aldehydes may exchange. We hypothesize that shorter aldehydes exchange within the airways (with the bronchial blood), and longer ones primarily in the alveoli (with the pulmonary blood). Although the first exhalation may correlate better with systemic aldehyde concentrations, we recommend the collection of at least six replicate exhalations per subject and exclude the first three from the analysis. Caution should be taken when interpreting results from such measurements, especially for shorter species. Taking into account these measures, we found intra-subject variabilities is in general much lower than inter-subject variability for the aldehydes studied (6.7% vs 48.2%). Such inter-subject differences are consistent with reported variability of such aldehydes in blood. Moreover, we found that all 27 aldehydes strongly positively correlated with each other, which is to be expected due to their common metabolic origin in humans. Overall, we conclude that this breath analysis platform and procedures described herein meet the required standards to conduct breath metabolomics studies in multi-center clinical studies. Further work to interrogate exhaled breath using this analytical platform in two different clinical settings is ongoing.

Acknowledgements This work is part of the Zurich Exhalomics project under the umbrella of University Medicine Zurich/*Hochschulmedizin Zürich*. Amelia Imolesi and Maya Weber are gratefully acknowledged for their administrative and experimental support.

Funding This study was funded by the Fondation Botnar, Switzerland (Professorship of Pablo Sinues), and the Swiss National Science Foundation (grants number 320030_173168 and PCEGP3_181300). J. Usemann was supported by research fellowship of the University Children's Hospital Basel.

Data availability The raw mass spectra and Exhalion files of the real-time breath measurements are available from the MetaboLights (https://www.ebi.ac.uk/metabolights) repository (accession number MTBLS842).

Compliance with ethical standards

This study was approved by the Ethics Committee for Northwest/Central Switzerland (Determination of Optimal Procedures for Analysis of Expired Breath by Secondary Electrospray Ionization-Mass Spectrometry; 2018-01324). All participants signed an informed consent form.

Conflict of interest G. Jaumà, P. Barreiro, M. Macia Santiago and G. Vidal de Miguel have a financial interest in Exhalion and Super SESI, as these devices are commercialized by Fossil Ion Technology S.L., Malaga, Spain. K.D. Singh, G. Tancev, F. Decrue, J. Usemann, R. Appenzeller, U. Frey, and P. Sinues declare no conflict of interest.

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Online Supplement

Standardization procedures for real-time breath analysis by secondary electrospray ionization high-resolution mass spectrometry

Kapil Dev Singh, Georgi Tancev, Fabienne Decrue, Jakob Usemann, Rhea Appenzeller, Pedro Barreiro, Gabriel Jaumà, Miriam Macia Santiago, Guillermo Vidal de Miguel, Urs Frey, Pablo Sinues

Sample Size Calculation

The width of the 95% confidence interval (CI) for the population within-subject standard deviation (SD) is defined as:

$$Width\ of\ 95\%\ CI = \frac{1.96 * SD}{\sqrt{2m(n-1)}} \quad (1)$$

Where, m is the number of subjects and n is the number of repeated measurements per subject. Thus, one can estimate it to within some fraction of the population value.

$$Width\ of\ 95\%\ CI = \frac{1.96 * SD}{\sqrt{2m(n-1)}} = p * SD \quad (2)$$

This is an equation with two unknown quantities: *m* and *n*. There are multiple combinations of *m* and *n* which will give the required precision (*p*). **Table S1** shows the solution of equation 2 for six different scenarios considering three, four, and five subjects and target precision of 5% and 10%. In this study we aimed to enroll 3 to 5 subjects to achieve a precision of at least 10% (*i.e.* 65 to 39 measurements per subject).

Table S1 Sample size calculation table based on solution for equation 2. At the end we enrolled 4 subjects with at least 49 exhalations to achieve at least 10% precision

Number of	Number of replicates (n) per subject	
Subjects (m)	5% precision	10% precision
3	257	65
4	193	**49**
5	155	39

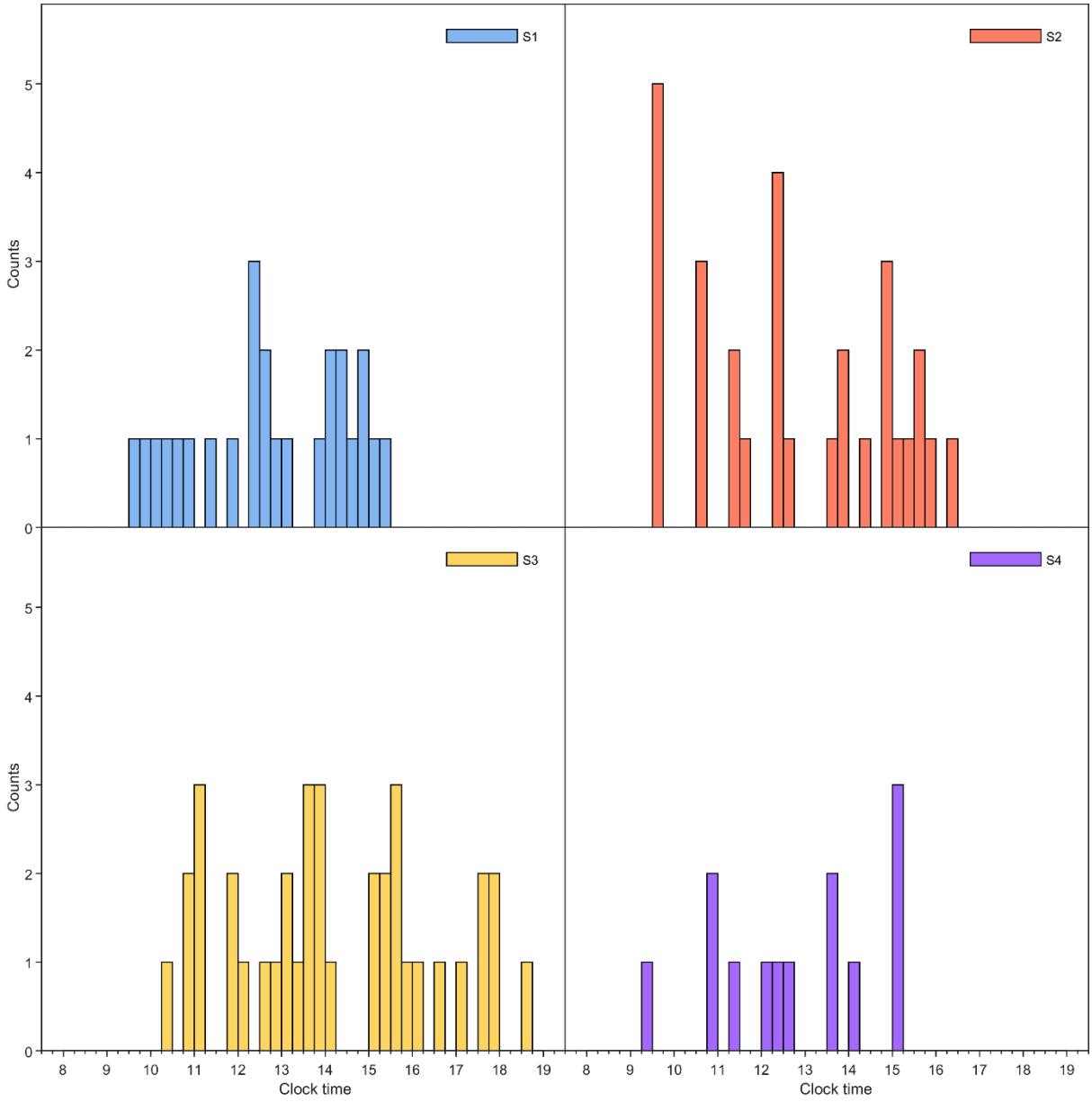

Fig. S1 Inter-individual difference observed are unlikely to be explained by circadian rhythms, as the sampling time was similar for all participants. The histograms depict the sum of all measurements as a function of measurement time for each subject

Fig. S2 A pictorial representation of a subject exhaling into the real-time breath analysis platform used in this study

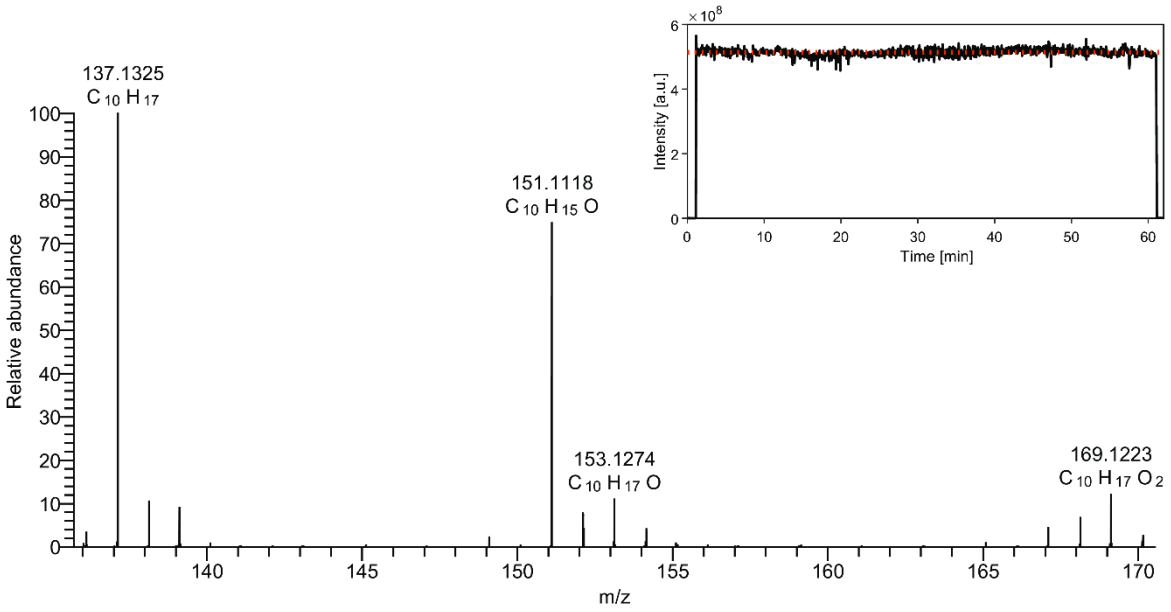

Fig. S3 Technical CV as measured by using β-pinene standards is within 3%. The mass spectrum shows the expected protonated β-pinene at *m/z* 137.1326 ($C_{10}H_{17}$), along with some oxidized species ($C_{10}H_{15}O$, $C_{10}H_{17}O$, and $C_{10}H_{17}O_2$). Note that high resolution and accuracy of Q Executive Plus enables unambiguous molecular formulae assignment. Inset shows the summed ion current for β-pinene and the oxidized species

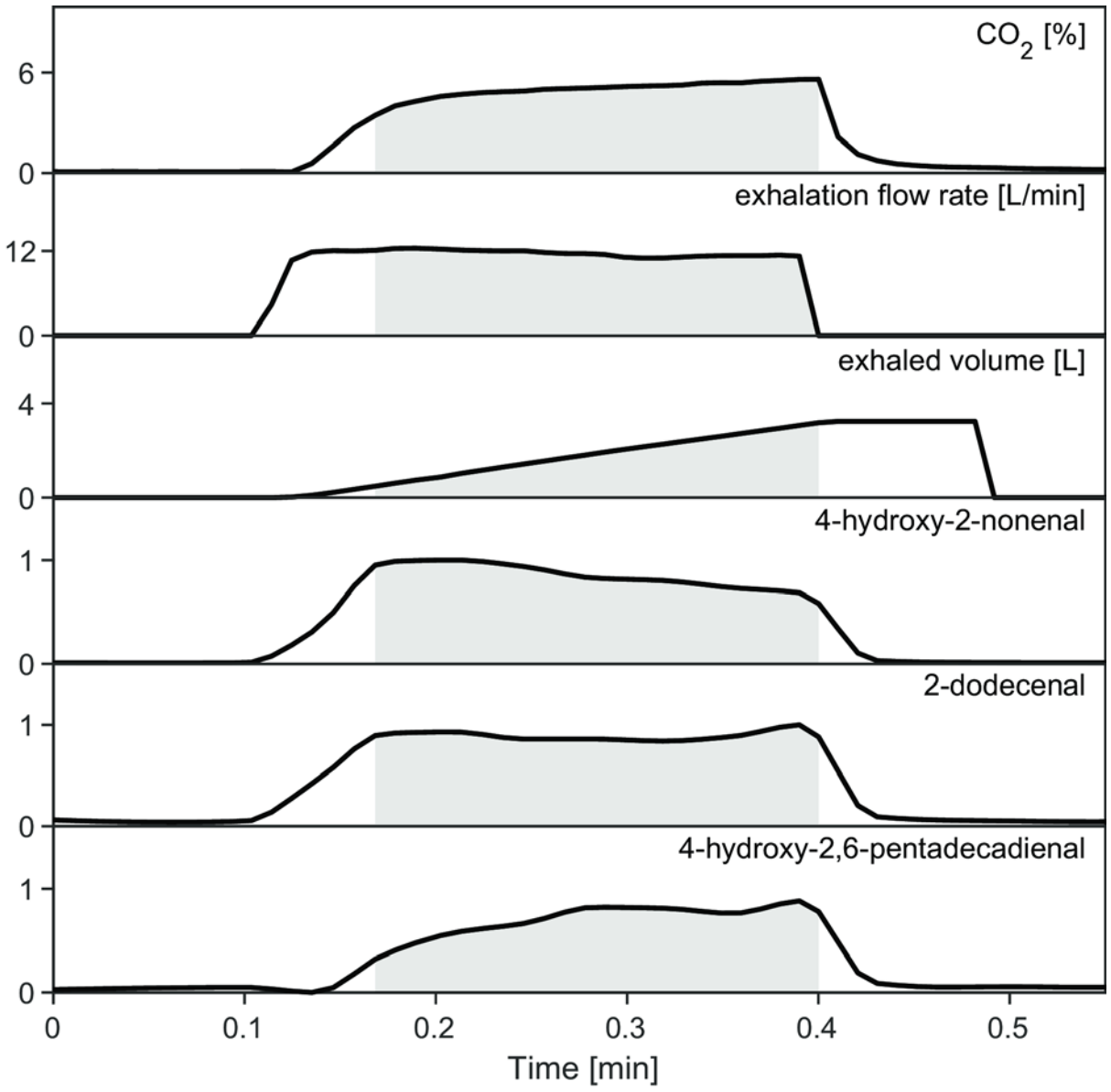

Fig. S4 Zoomed-in view of first exhalation from Fig. 1B. The grey areas here and also in **Fig. 1B**, show the time windows whereby CO_2 levels were above 3%. Note that the exhaled volume (computed by real-time integration of the exhalation flow rate) holds its value for few seconds after the end of the exhalation. This does not have any effect on analysis because we calculated the exhaled volume based on the bounds within 3% CO_2

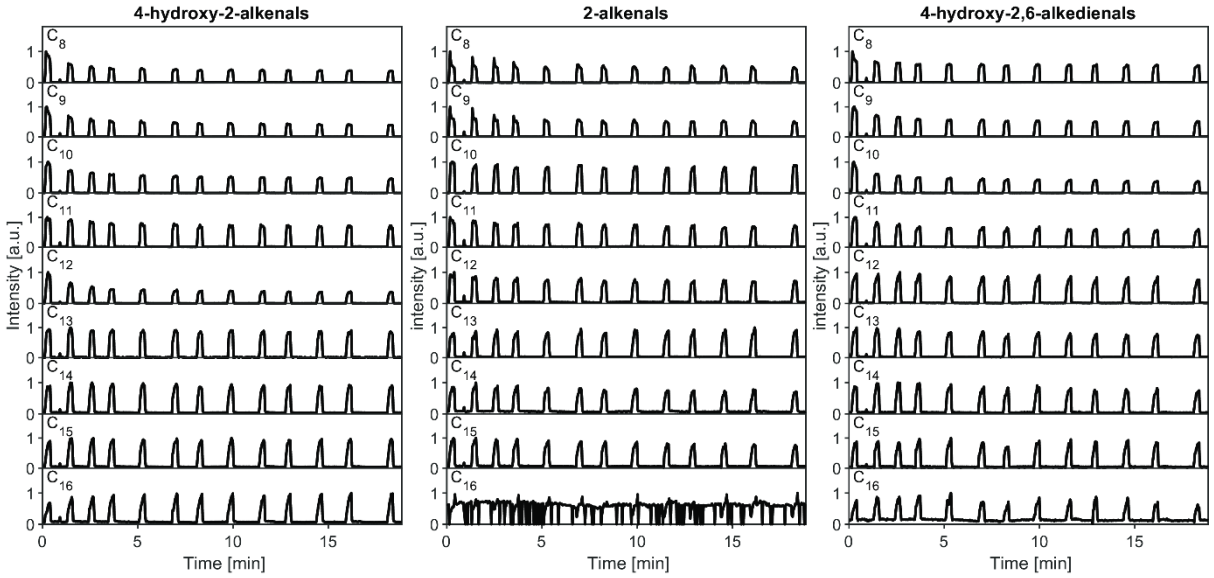

Fig. S5 Selected aldehydes show a chain length-dependent and class-independent decay of signal intensity over time within an experiment. Note the deviation of 4-hydroxy-2-dodecenal from the regular pattern, which might be due to an interfering peak. 2-hexadecenal (C_{16}) was not detected in this particular experiment

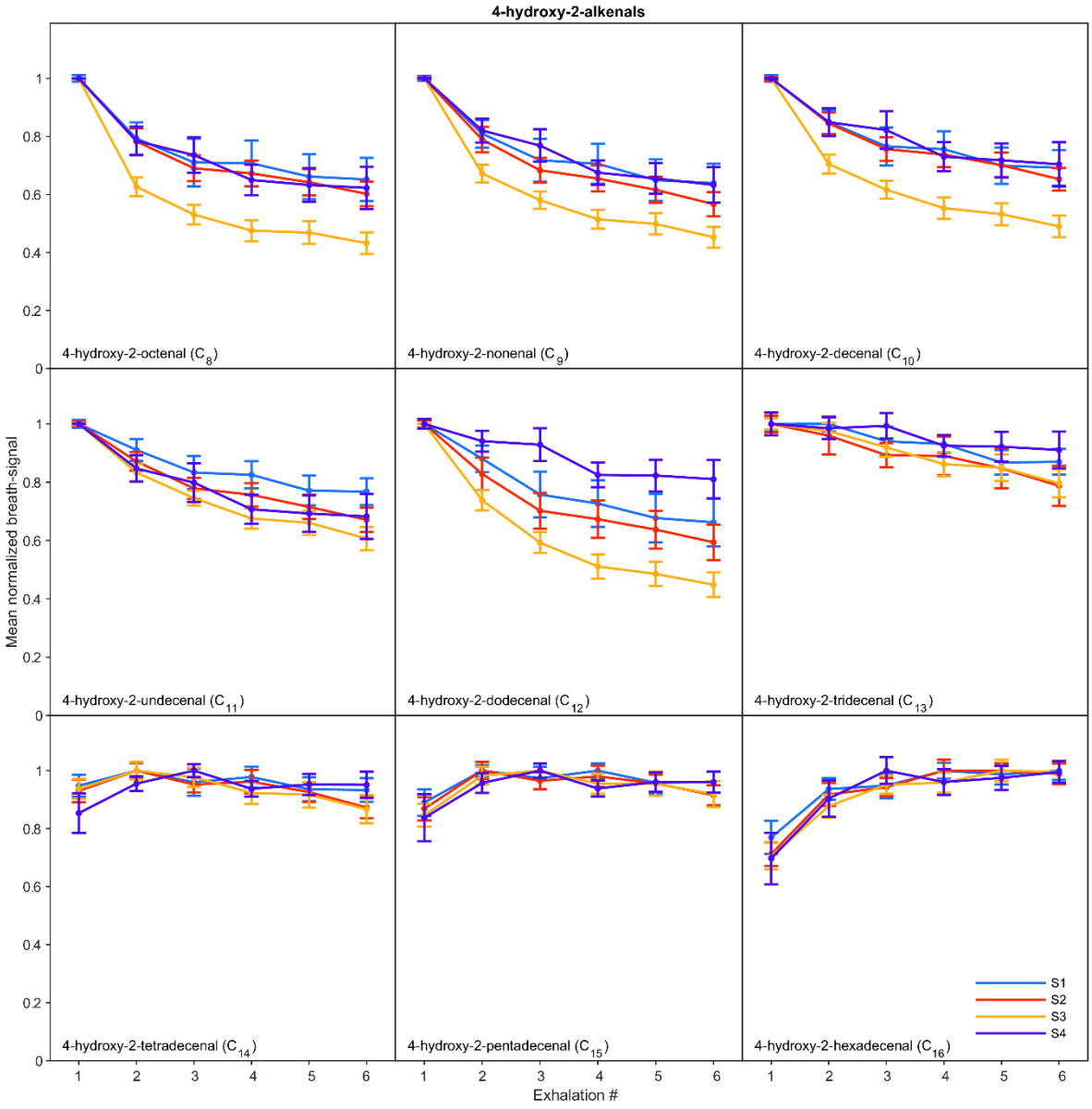

Fig. S6 Mean normalized breath-signal with error bars representing 95% CIs for 4-hydroxy-2-alkenals from four subjects in 104 experiments. Note the deviation of 4-hydroxy-2-dodecenal from the regular pattern, which might be due to an interfering peak

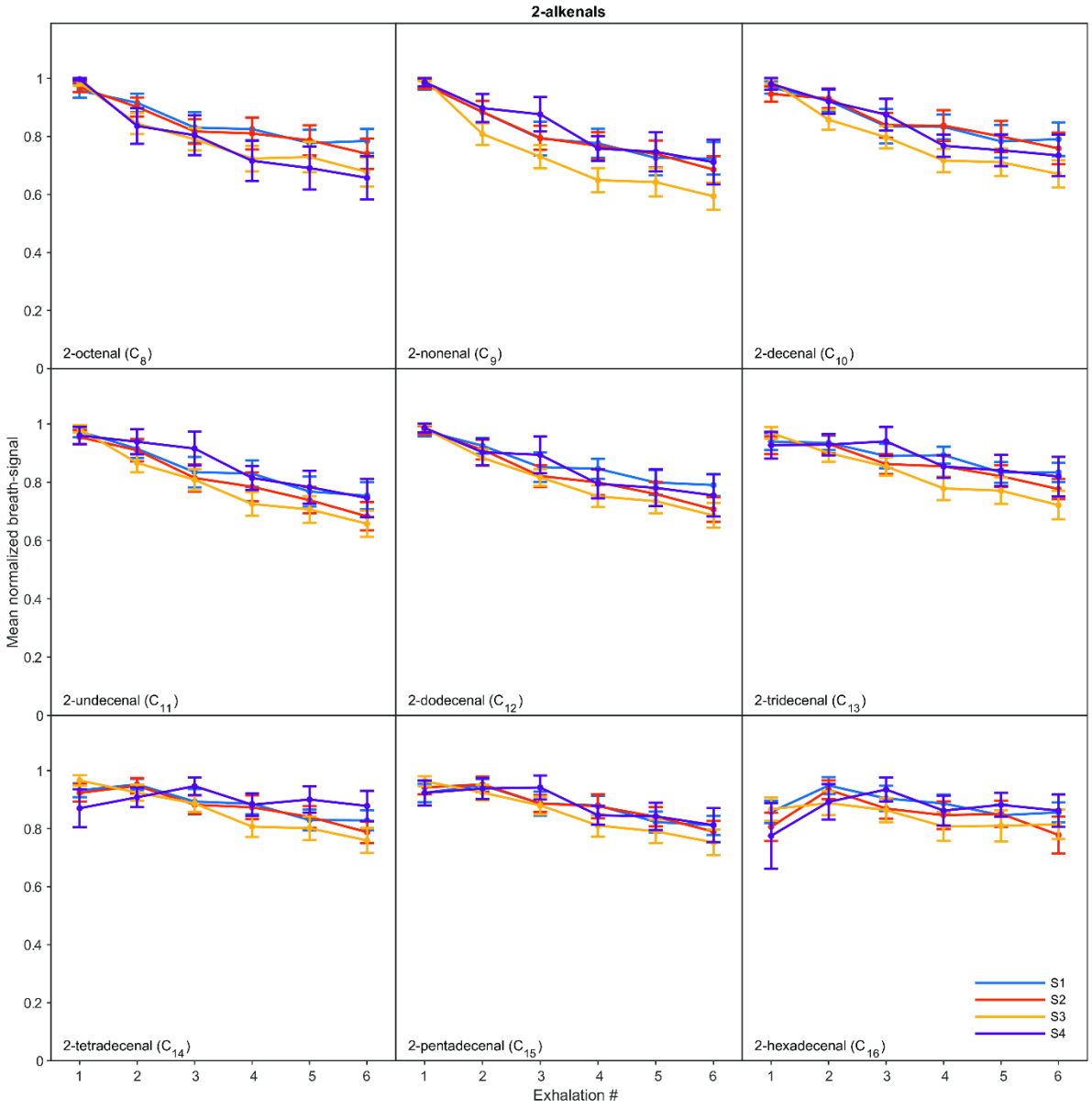

Fig. S7 Mean normalized breath-signal with error bars representing 95% CIs for 2-alkenals from four subjects in 104 experiments

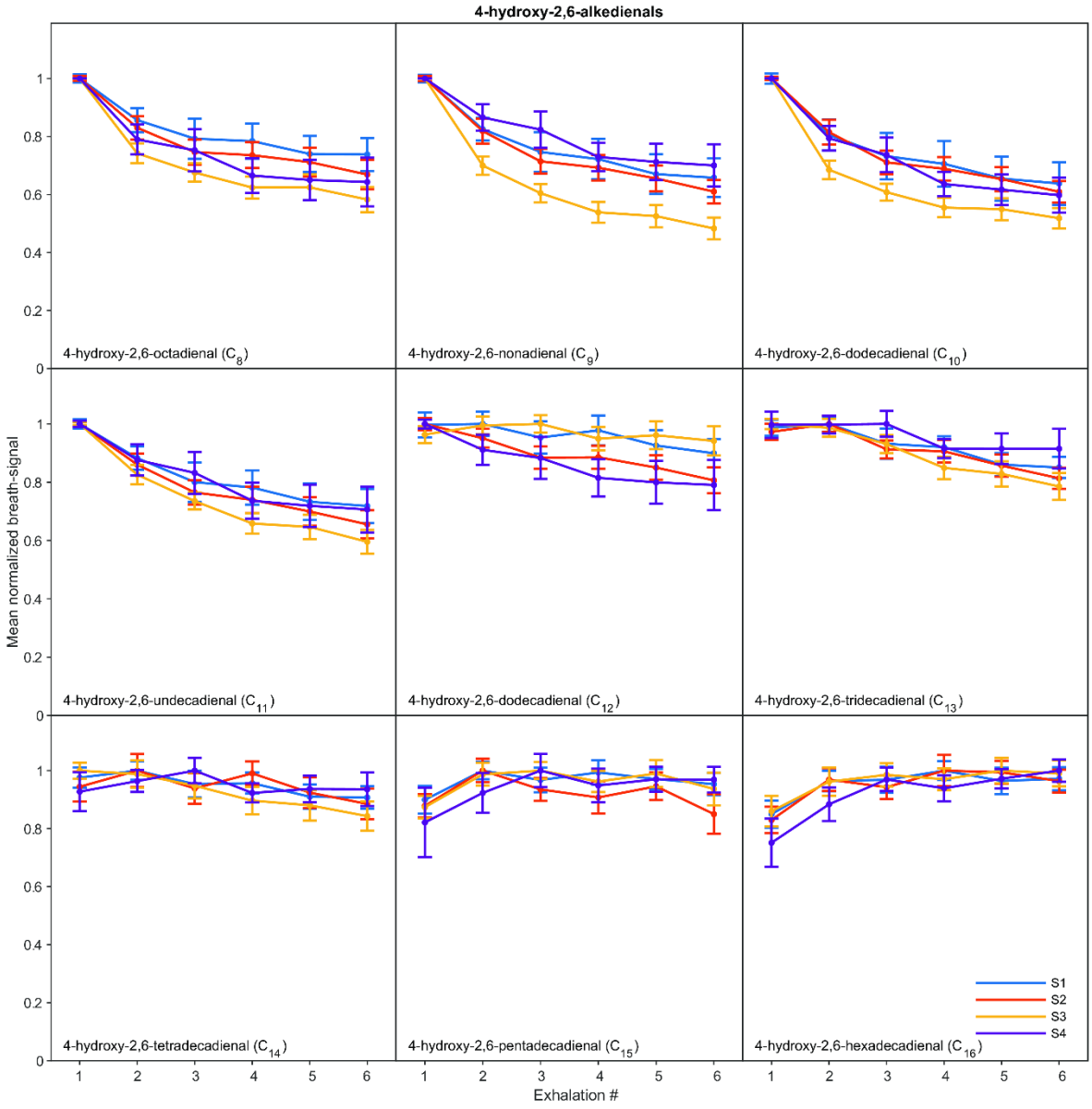

Fig. S8 Mean normalized breath-signal with error bars representing 95% CIs for 4-hydroxy-2,6-alkedienals from four subjects in 104 experiments

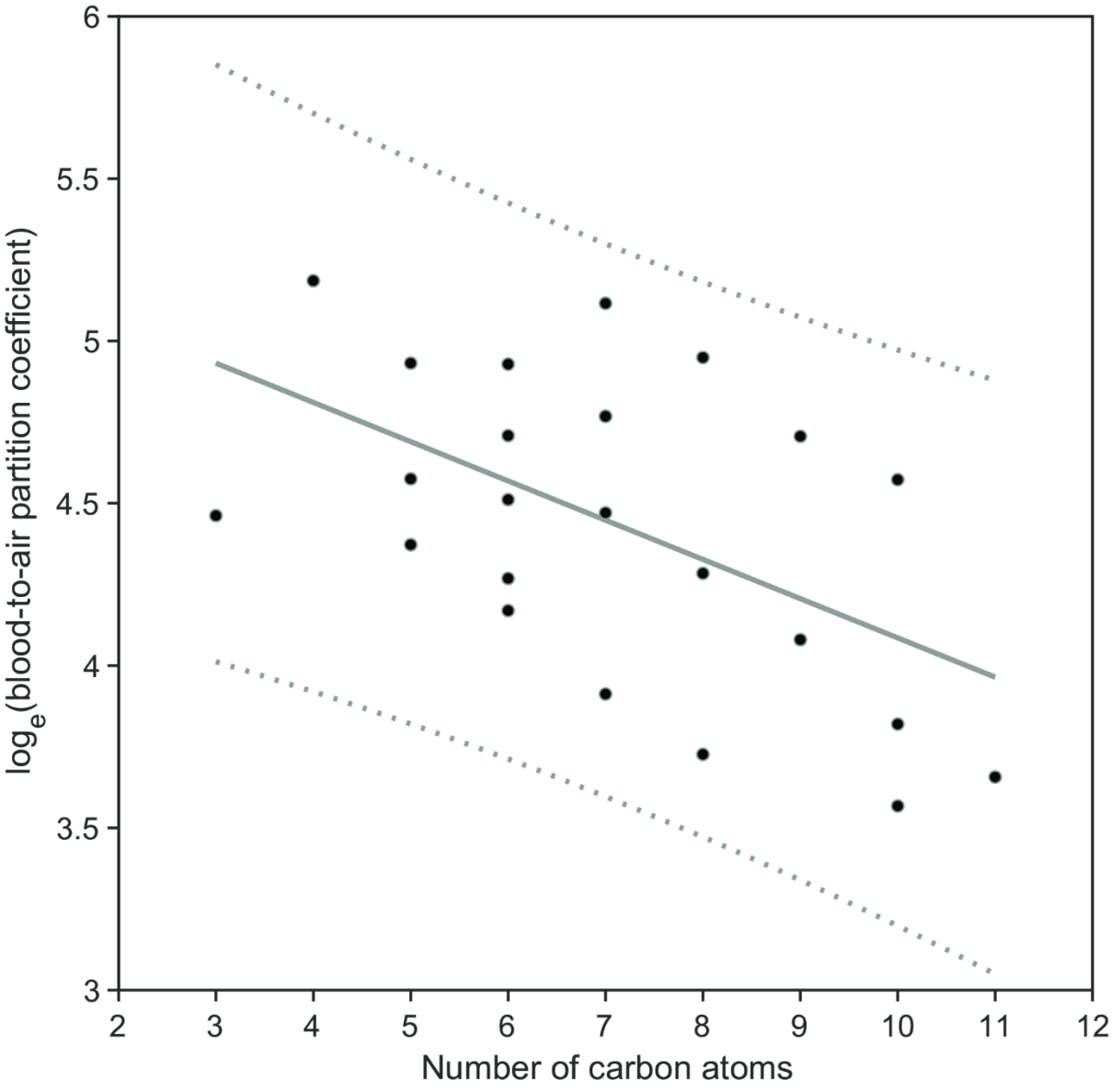

Fig. S9 Dependency of blood-to-air partition coefficient on chain length for a series of aldehydes. Data extracted from Kramer *et al.* [1]

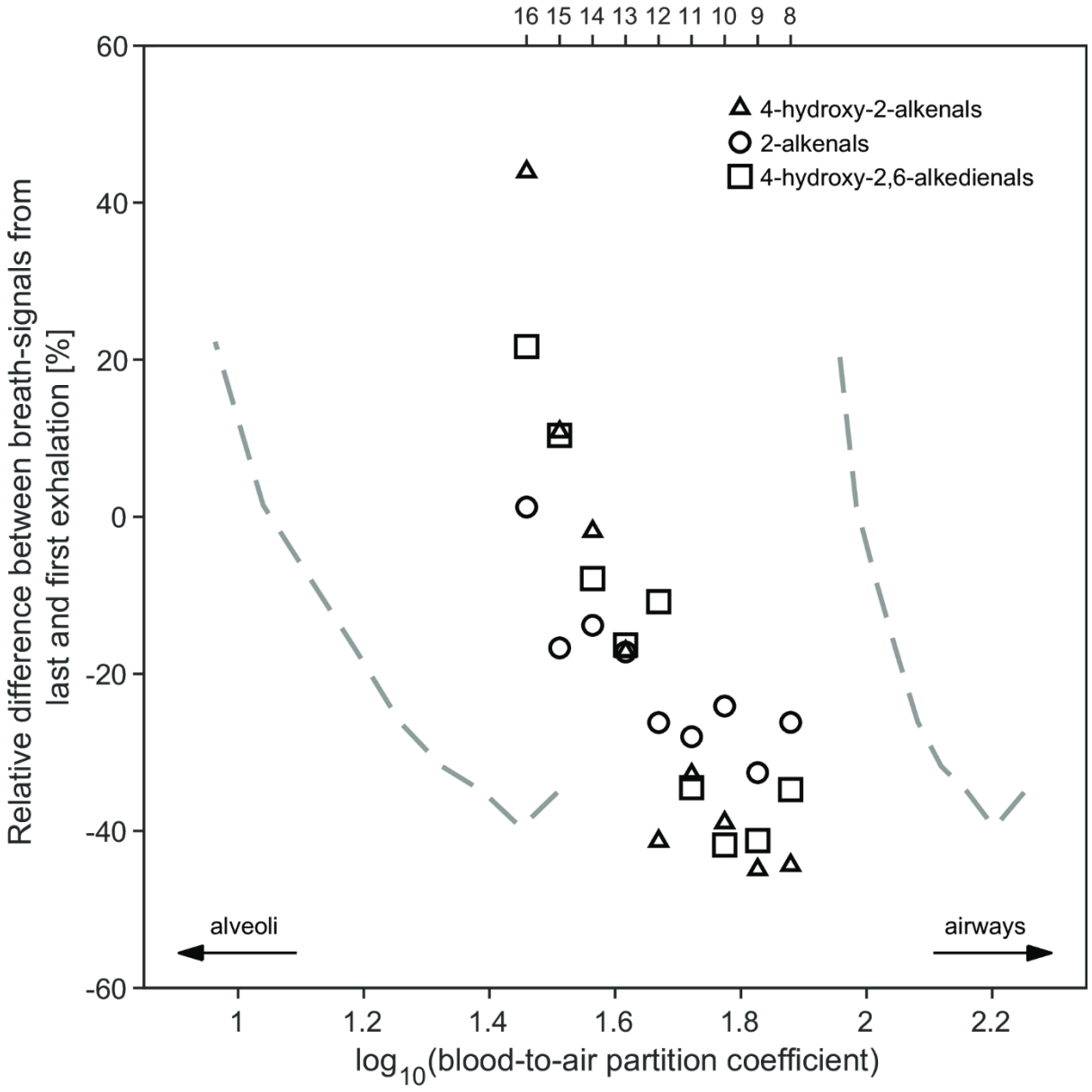

Fig. S10 Exhalation profile of breath metabolites indirectly depends on their blood-to-air partition coefficient. Same as Fig. 3, but x-axis is on $\log_{10}$ scale to show the complete range of 95% CIs

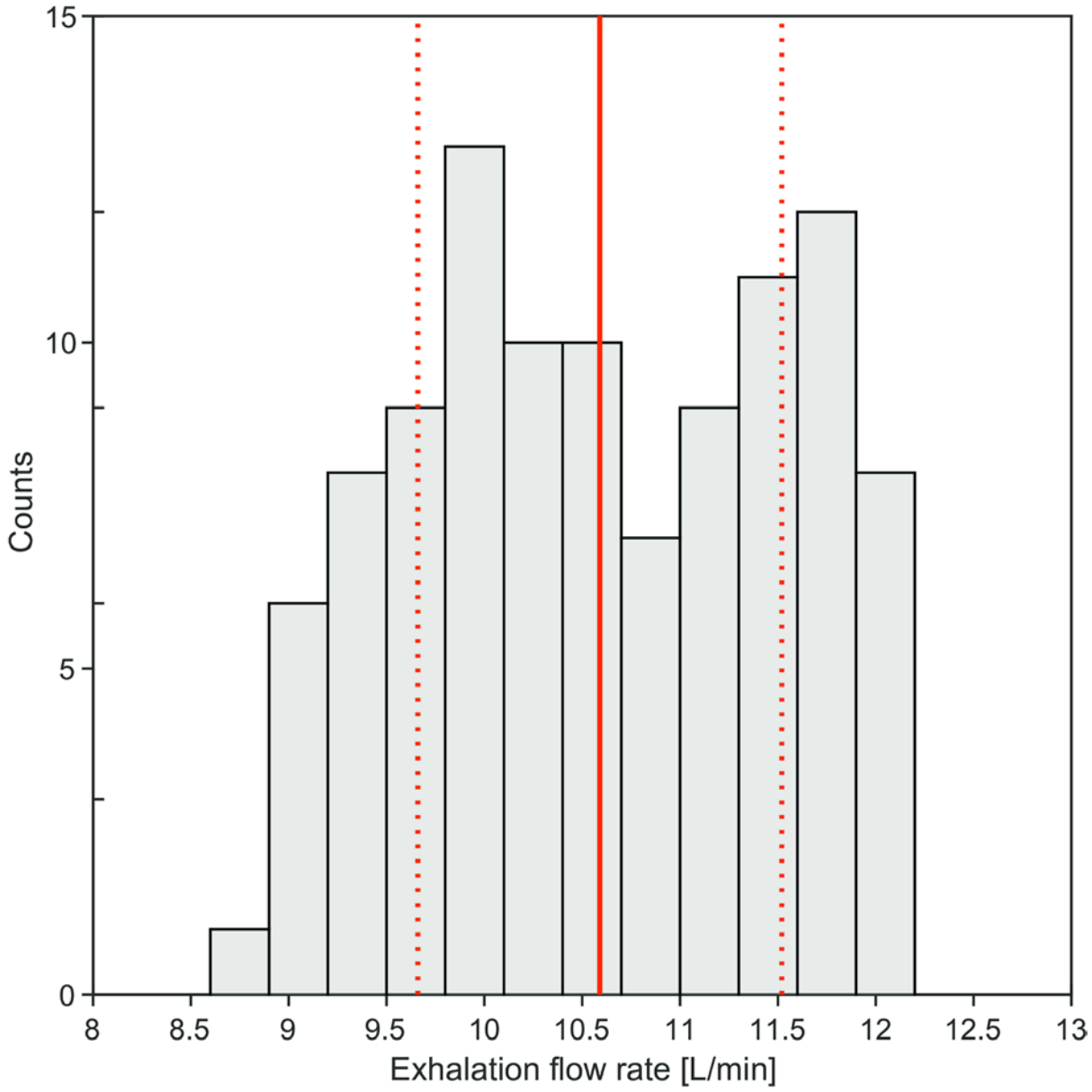

Fig. S11 Distribution of the exhalation flow rates (L/min) from all the experiments of this study. Solid red vertical line represents the mean and dotted red vertical lines represent SD

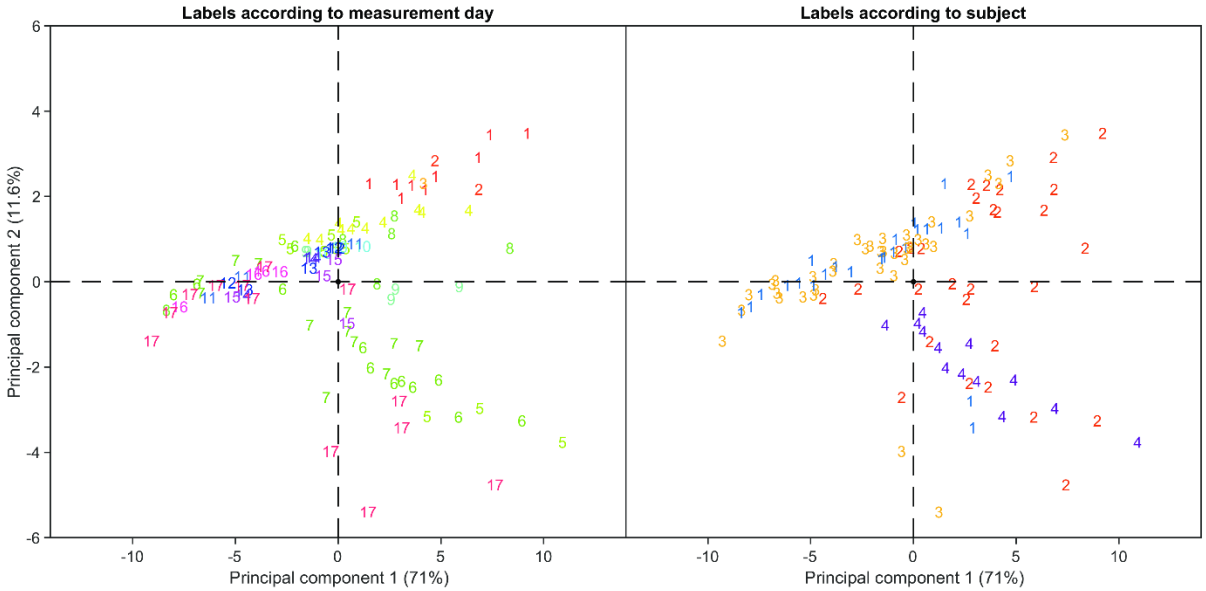

Fig. S12 Variance observed for the 27 aldehydes studied in this work is better explained by the subject number rather than measurement day (see also Fig. 5 for more details)

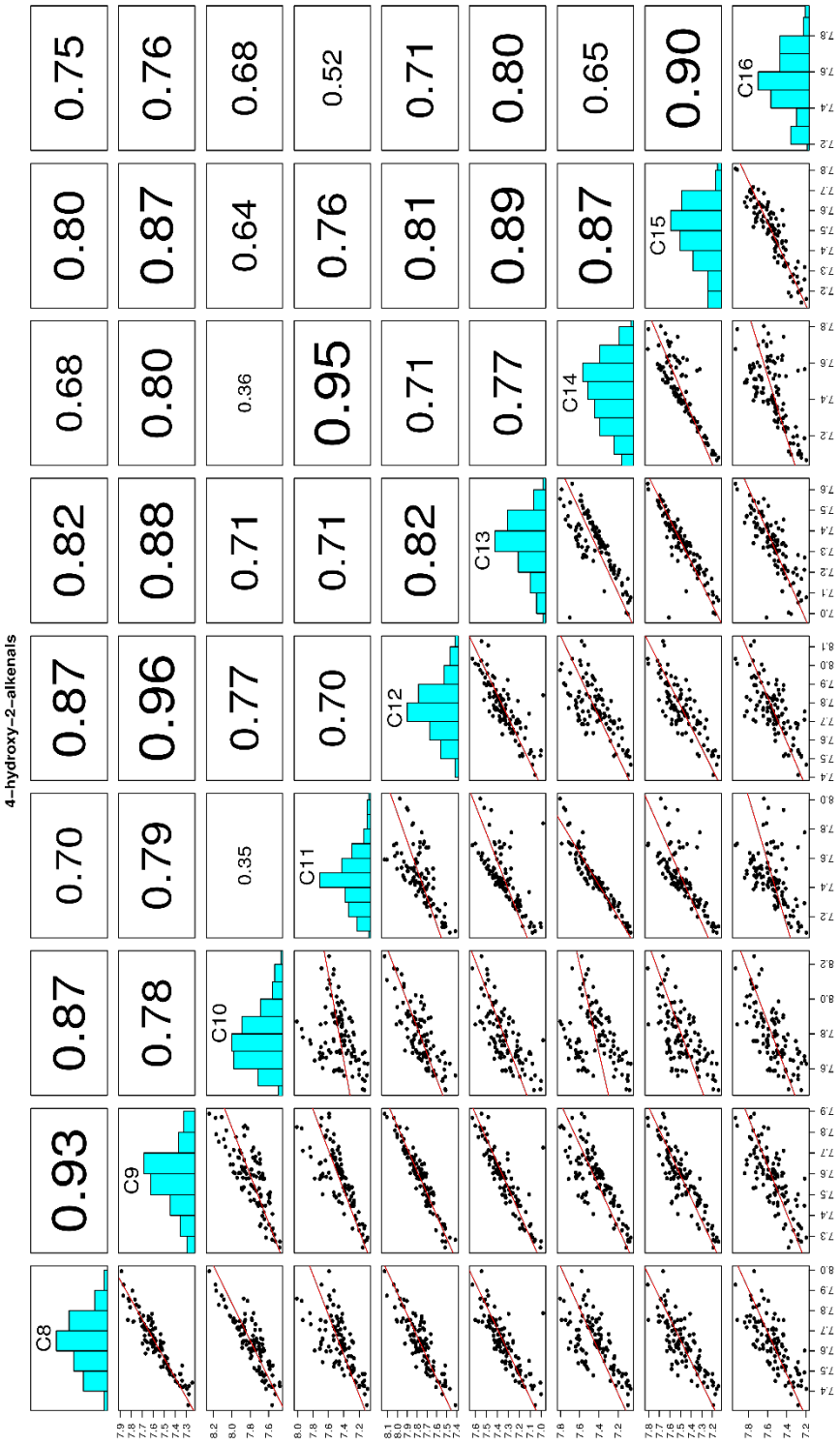

Fig. S13 Correlation matrix for the breath-signals (log_{10} scaled) of 4-hydroxy-2-alkenals class aldehydes from 104 experiments. The solely positive correlation among all molecules indicates a common mechanism by which they are generated. Diagonal shows distribution of breath-signals from 104 experiments for C_8-C_{16} aldehydes members of class 4-hydroxy-2-alkenals. Scatter plots between members are shown below the diagonal and Pearson correlation coefficients between members are shown above the diagonal

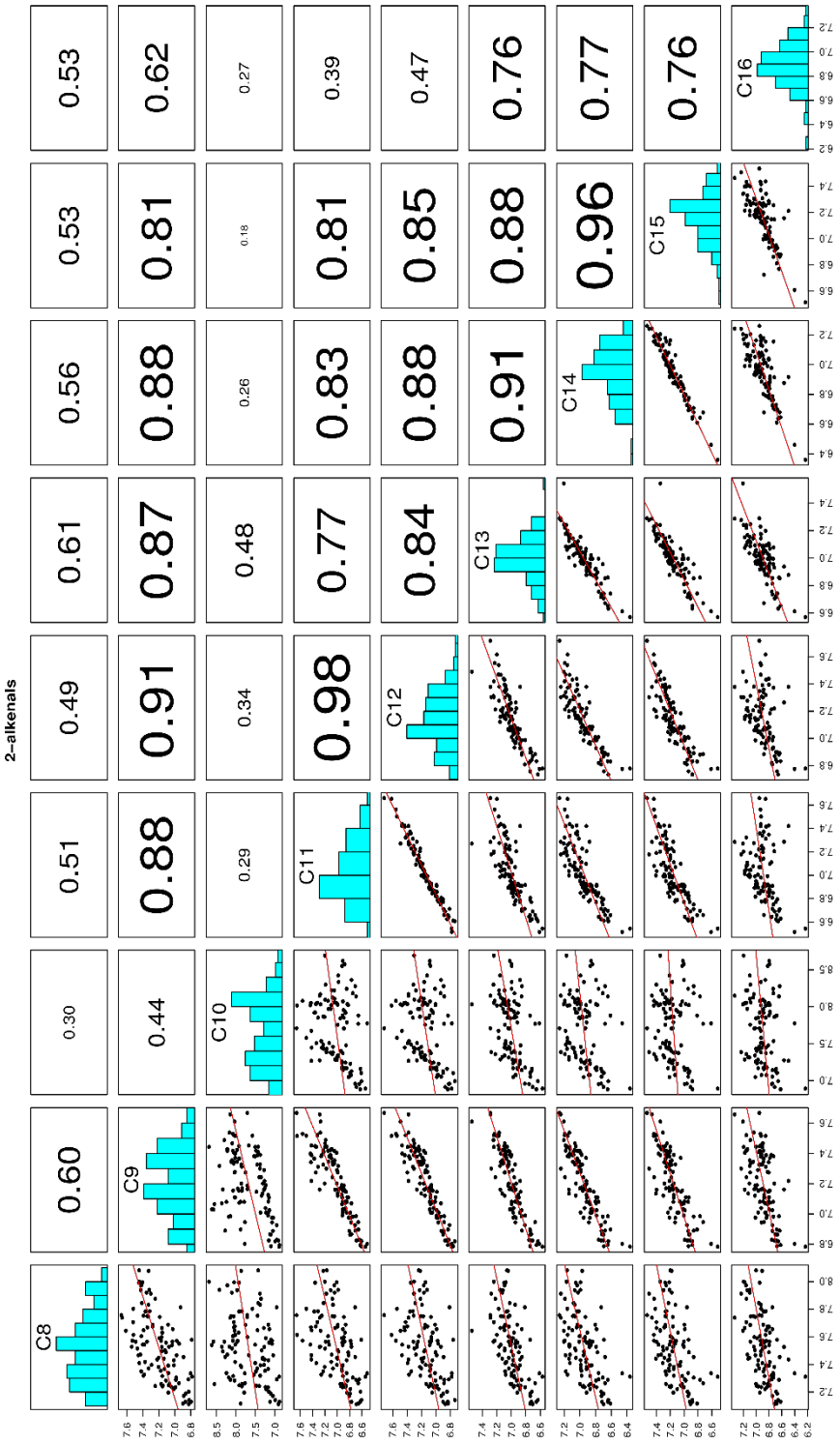

Fig. S14 Correlation matrix for the breath-signals (log_{10} scaled) of 2-alkenals class aldehydes from 104 experiments. The solely positive correlation among all molecules indicates a common mechanism by which they are generated. Diagonal shows distribution of breath-signals from 104 experiments for C_8-C_{16} aldehydes members of class 2-alkenals. Scatter plots between members are shown below the diagonal and Pearson correlation coefficients between members are shown above the diagonal

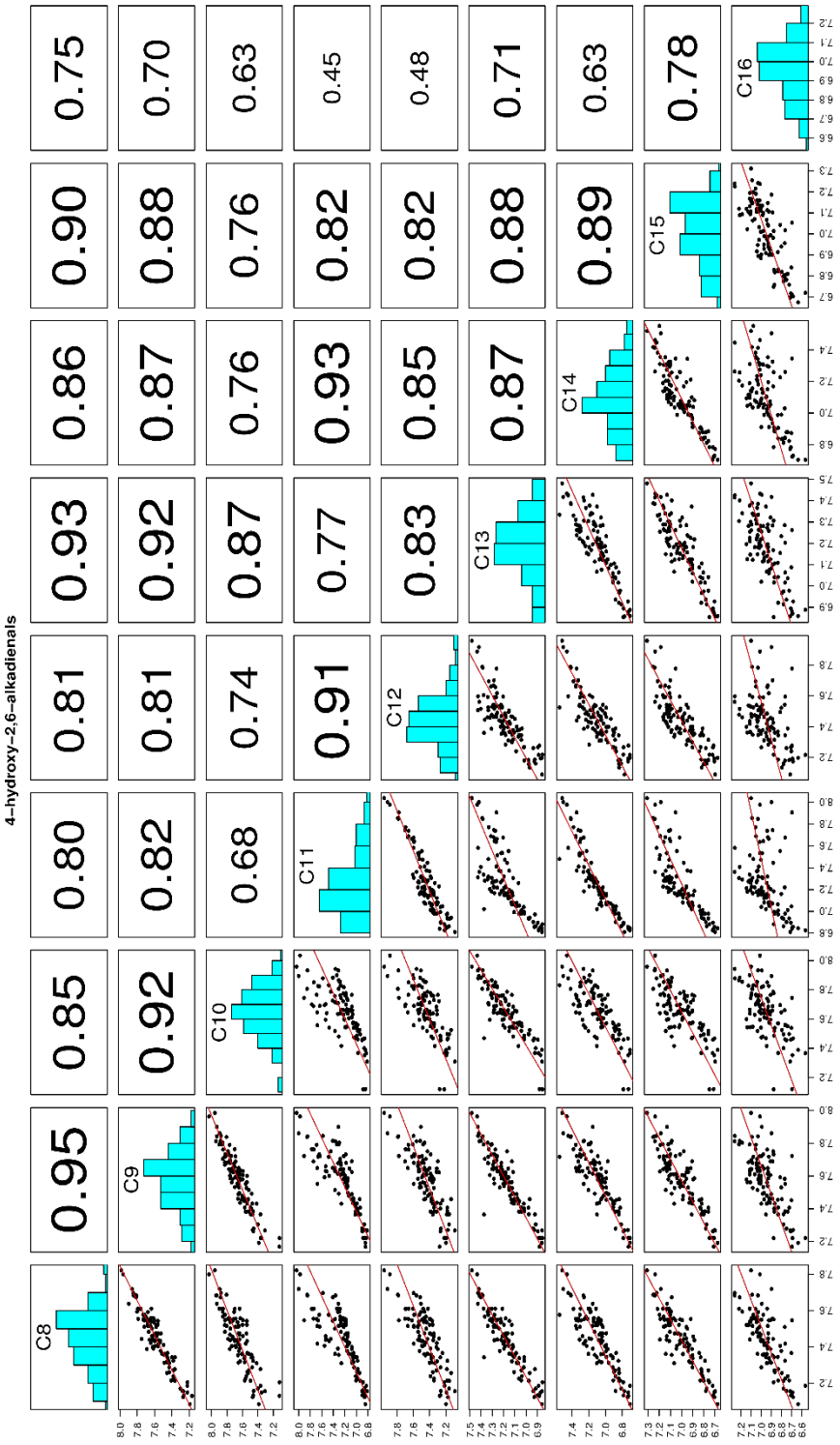

Fig. S15 Correlation matrix for the breath-signals (log_{10} scaled) of 4-hydroxy-2,6-alkadienals class aldehydes from 104 experiments. The solely positive correlation among all molecules indicates a common mechanism by which they are generated. Diagonal shows distribution of breath-signals from 104 experiments for C_8-C_{16} aldehydes members of class 4-hydroxy-2,6-alkadienals. Scatter plots between members are shown below the diagonal and Pearson correlation coefficients between members are shown above the diagonal

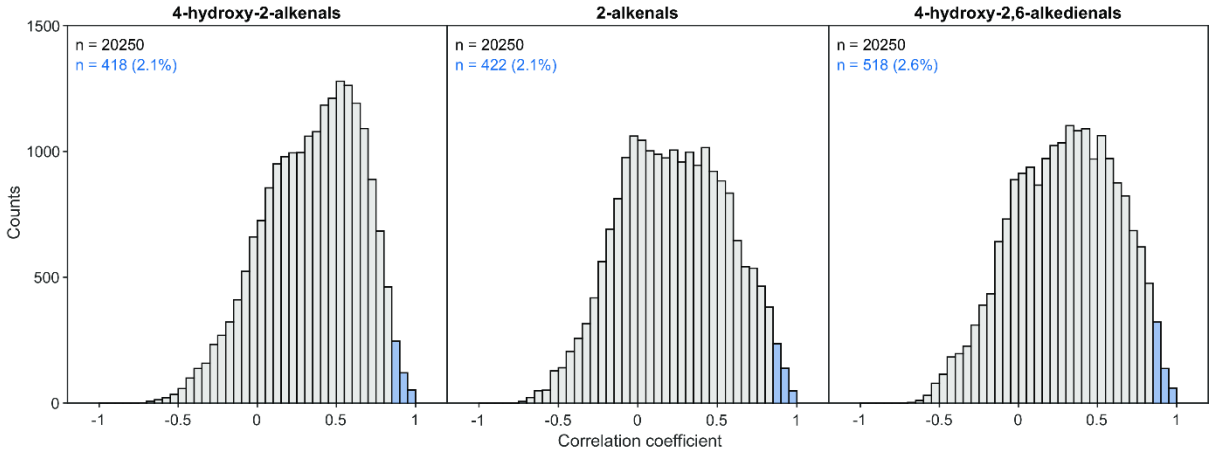

Fig. S16 Selected aldehydes from three classes correlates strongly with each other but not with the rest of the detected features. Data shown are the Spearman correlation coefficient of aldehydes from each class with all quantified features (leading to total 20,250 pairwise correlation values). Bars highlighted in blue shows the fraction of correlation higher than 0.85

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1. Kramer C, Mochalski P, Unterkofler K, Agapiou A, Ruzsanyi V, Liedl KR. Prediction of blood:air and fat:air partition coefficients of volatile organic compounds for the interpretation of data in breath gas analysis. J Breath Res. 2016;10(1):Artn 017103. https://doi.org/10.1088/1752-7155/10/1/017103.

8 Curriculum vitae

Personal Data

Date of Birth	29.03.1992
Nationality	Switzerland
Marital status	married, 1 child

Education / Academic

10.2017 – 09.2021	MD-PhD Dissertation in the BILD cohort at the University-Children's Hospital Basel (UKBB): "*Impact of environmental factors on preterm and term-born infants and children*", supervised by Prof. Dr. Urs Frey
10.2018 – 04.2019	Resident in pediatrics at the UKBB
09.2011 – 09.2017	Studies of human medicine, University of Basel

Qualifications

01.2018 – 09.2021	CBMC (Cord blood mononuclear cell) isolation in the
09.2018	WetLab Good Clinical Practise (GCP) advanced, USB
05.2018	Start4Neo Basic Skills Course, UKBB
03.2016	GCP basics, USB

Languages

German	Native
English and French	Fluent, written and spoken

Spanish	Good knowledge

Memberships

Swiss Society for Pneumology; German Society of Paediatric Pneumology; European Respiratory Society

Research

Prizes and Awards

03.2021	Participant of the Antelope Program from the University of Basel
01.2021	Best oral presentation clinical research day USB
10.2020	GetOnTrack scholar from the University of Basel
10.2020	Best MD-Thesis 2018/2019 from the University of Basel
08.2020	Flexibility Grant scholar from the Swiss National Science Foundation
06.2019	Publication "*Exposure to moderate air pollution on lung function at school-age: a birth cohort study"* was awarded with the talent prize SGP 2019
01.2019	Best poster clinical research day USB
10.2018	Best oral presentation research day UKBB
07.2017	Scholar of the Goldschmidt-Jacobson-Stiftung 2017-2018

Reviews

Pediatric Pulmonology; ERJ Open; Environment International;
Pediatric Allergy, Immunology and Pulmonology

Publications

- **Decrue F**, Singh KD, Gisler A, Awchi M, Zeng J, Usemann J, Frey U and Sinues P. *Combination of exhaled metabolites analysis with parallel lung function and FeNO measurements in infants.* Submitted 2021 May.
- **Decrue F**, Gorlanova O, Salem Y, Vienneau D, de Hoogh K, Gisler A, Usemann J, Korten I, Nahum U, Sinues P, Schulzke S, Fuchs O, Latzin P, Röösli M and Frey U. *Increased impact of air pollution on infant lung function in term vs. preterm infants; the BILD study.* In Revision AJRCCM 2021. **IF 17.5**
- **Decrue F**, Gorlanova O, Usemann J, Frey U. *Lung functional development and asthma trajectories.* Semin Immunopathol. 2020 Feb. **IF 7.5**
- Usemann J*, **Decrue F*,** Korten I, Proietti E, Gorlanova O, Vienneau D, Fuchs O, Latzin P, Röösli M, Frey U, BILD study group. *Exposure to moderate air pollution on lung function at school-age: a birth cohort study.* Environ Int. 2019 May. **IF 7.6**
- Signh KD*, Tancev G*, **Decrue F**, Usemann J, Appenzeller R, Barreiro P, Jaumà G, Macia Santiago M, Vidal de Miguel G, Frey U, Sinues P. *Standardization procedures for real-time breath analysis by secondary electrospray ionization high-resolution mass spectrometry*. Anal Bioanal Chem. 2019 Apr 15. **IF 3.3**
- Gorlanova O, Appenzeller R, Mahmoud YS, Ramsey KA, Usemann J, **Decrue F**, Kuehni CE, Röösli M, Latzin P, Fuchs O, Soti A, Frey U, On Behalf of the BILD Study Group. *Effect of breastfeeding duration on lung function, respiratory symptoms and allergic diseases in school-age children.* Pediatr Pulmonol. 2020 Jun. **IF 2.5**
- Soti AL, Usemann J, Schaub B, Frey U, Latzin P, Fuchs O; BILD Study group. *Can biomarkers in umbilical cord blood predict atopic disease at school age?* Pediatr Res. 2019 Dec 6. **IF 2.9**