Immunological and genetic determinants of pulmonary outcome in school aged children

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Prof. Dr. Martin Spiess

For my three beloved ladies

Content

Abstract of the PhD

Background: The prevalence of respiratory disorders in children has steadily increased over the past decades to such an extent that asthma is now the most common chronic disease of childhood. Childhood asthma resembles a complex syndrome rather than a single disease, and includes many wheeze phenotypes, making its diagnosis challenging. Most likely, it is not a single risk factor that determines whether a child develops asthma, but several risk factors (e.g. environmental, immunological, genetic, onset of respiratory symptoms) that each make small contributions to the development of the disease. Already infancy, lung function tests are available to assess airway disease. These tests are predominantly used in patients with Cystic Fibrosis (CF), for whom preservation of normal lung function is crucial. Despite recent advances in lung function testing, several methodological issues remain unanswered. Higher quality tests are required in order to effectively study the various risk factors involved in the development of complex airway diseases

Aim: The first aim was to describe methodological issues during infant lung function testing in order to improve their quality. The second aim was to study different risk factors for asthma development, and to investigate their association with respiratory diseases during childhood.

Methods: The study was conducted within the prospective Basel-Bern infant lung development (BILD) cohort, a population-based cohort of unselected infants of Central-European origin. The survey collects prenatal data via standardized interviews and cord blood samples for the assessment of immunological and genetic information. During the first year of life, research nurses call the parents weekly to assess the occurrence of respiratory symptoms. Pulmonary function tests, as well as measurement of fractional exhaled nitric oxide (FeNO) to assess airway inflammation, are completed at 5 weeks of age, and again at 6 years of age during follow-up.

Results: We provided specific recommendations on how to improve outcomes from infant lung measurements. Furthermore, we measured airway obstruction using the interrupter technique (Rint) in unsedated infants shortly after birth, and were able to show that measurements were

feasible but had a high variability. We compared Rint between term and preterm infants, and found that Rint was higher, and variability of Rint lower, in term-born infants. We assessed FeNO in healthy newborn infants, and in infants with CF. FeNO at birth had no predictive value for asthma development at school age. In CF patients, FeNO at birth was lower compared to matched healthy controls.

We could also show that polymorphisms in the chitinase 3-like 1 (*CHI3L1)* gene encoding YKl-40 were associated with asthma at 6 years. There was some indication that increased YKL-40 levels at birth may also be involved in the development of airway disease. We also developed a novel method to characterize the time series of prospectively assessed respiratory symptom scores during infancy. This method assesses symptom dynamics in an observer-independent manner. Using this method, we were able to identify a high-risk phenotype, which was predominantly male, and contained more infants exposed to maternal asthma, and environmental tobacco smoke. This phenotype was also at increased risk for asthma and atopy at school age.

Conclusions: Infant lung function is useful to study airway disease at an early age, and outcomes can be improved by applying minimal changes in analyses algorithms. Assessment of airway obstruction in infants is feasible, but measurements require careful interpretation due to the high variability. We found some indication that FeNO levels early in life are determined by environmental factors and the child's genetic profile. In CF patients, FeNO after birth was associated with the severity of the genetic mutation. In healthy infants, FeNO levels early in life seem to be influenced by environmental exposures.

Our findings contribute additional, relevant knowledge on asthma risk factors and their association with respiratory symptoms from birth through school age. We found associations between genetics and the immunological status at birth with asthma at school age. The development of asthma may also depend on respiratory symptoms early in life. We could show that the pattern of symptom deterioration and recovery during the first year of life determines whether or not a child has persistent wheezing until school age.

1 Introduction

The prevalence of respiratory disorders in children has steadily risen over the last several years [1]. Asthma is currently the most common chronic respiratory disease in childhood, and because of its high prevalence, it is a major health issue. Asthma is an inflammatory disease of the small airways with the most common symptom in children being cough and wheeze. In more severe cases, breathlessness, chest tightness or pressure and even chest pain, are reported [2].

Asthma is a complex syndrome with many different wheezing phenotypes. The classic phenotypes of transient, persistent and late-onset wheeze in childhood were initially described in the Tucson Children's Respiratory Study [2], and were found to be associated with distinct patterns of lung function changes [3]. While these phenotypes were frequently used in epidemiological research, recent studies have attempted to further characterize phenotypes of preschool wheeze using modern mathematical techniques and found similar phenotypes [4-7]. Furthermore, the distribution of risk factors has been shown to differ for the various wheeze phenotypes [5, 8], which underlies the complexity of the interaction between risk factors and respiratory diseases.

The varying presentation and disease courses of these phenotypes often make it difficult to distinguish health from disease in early childhood. Lung function tests are currently available to assess airway function in young children, and even infants [9, 10]. These tests can help improve our understanding of the multiple determinants influencing lung development during the critical time window of early childhood.

1.1 Complexity of respiratory disease in childhood

Many factors are likely to contribute to the development of respiratory diseases. There are host factors (e.g. maternal atopy, sex) [8, 11], which may predispose children to respiratory symptoms, and environmental factors (tobacco smoke exposure, air pollution), which may further influence the severity and recovery patterns of respiratory symptoms [8, 12]. Prospective

studies have shown that life style factors, such as overweight during early childhood, preceded of the later development of asthma symptoms [13]. Although some risk factors maybe more relevant for later respiratory disease than others [14], it is most likely a complex interplay between various factors that determine whether or not a child will develop persistent symptoms. Furthermore, given that there are many risk factors known to increase the risk for asthma, it can be speculated that each risk factor has only a small impact on asthma development. Several studies support this hypothesis. For example, several prospective studies repeatedly found that air pollution exposure early in life was associated with reduced lung function at school age [15-18] and adolescence [19]. However, effect sizes of increased air pollution levels were small: e.g. a decrease of 4.8% in forced expiratory volume in 1 second (FEV_1) per 10-$\mu g/m^3$ increase in traffic-particulate matter with aerodynamic diameter <10 µm (PM_{10}) was reported [15], and a decrease of 3.2% in peak expiratory flow (PEF) per $\mu g/m^3$ increase in nitrogen dioxide (NO_2) [16], respectively.

It is difficult to assess the small effects of different risk factors on lung development, which can only be achieved by high quality lung function tests. Especially in infants, achieving this goal is extremely challenging, since some outcomes have high variability [9, 20, 21]. In other words, infant lung function tests have an unfavorable signal-to-noise ratio, as the power of the signal (the outcome of interest) is limited by the background noise (e.i. variability of the outcome). Improving the quality of infant lung function outcomes is therefore needed to better characterize airway disease and understand the impact of risk factors early in life.

1.2 Assessing lung function in childhood

Lung function measurements provide objective information on airway function, and are a fundamental measure in the diagnosis and management of respiratory diseases. They are widely applied in epidemiological studies to investigate the impact of risk factors, as well as interventional studies to assess treatment response. Classic lung function tests, such as

spirometry, measure expiratory flow in the large airways [22]. The breathing maneuvers during spirometry, however, require cooperation, making this technique inapplicable in younger children.

There are lung function tests available that require only minimal cooperation, making them more attractive in the pediatric setting. For example, airway obstruction can be measured during tidal breathing using the interrupter technique (Rint) [23]. Multiple breath washout (MBW) is also a tidal breathing lung function test. MBW allows for the detection of early lung disease, even before pathologies can be detected by spirometry [24]. While these tests are attractive, as they can be performed in infants during natural sleep, several methodological aspects of these lung function tests require careful interpretation.

This PhD thesis used results from all of the aforementioned lung function tests. A description of the applied tests is provided in the general methods section.

1.3 Methodological challenges of lung function tests in childhood

Methodological challenges to assess airway function in young children and infants relate, e.g. to complex analyses algorithms, and to the high variability of results. For example, studies systematically assessing Rint in infants and children reported poor repeatability [21, 25] and high variability of measurements [26-28]. Often discussed is the impact of dead space on test results. The total apparatus dead space is the volume through which the infants must breathe while attached to the equipment. The ERS/ATS task force recommended a maximum of 2 ml/Kg in newborn infants [29], but many studies do not achieve this recommendation [21]. An unfavorably large dead space may have a physiological effect, and contribute to variability of measurements.

MBW tests allow for the assessment of the functional residual capacity (FRC), as well as, ventilation homogeneity of the small airways. However, relevant methodological aspects should be noted. For example, studies have shown that ventilation homogeneity, as assessed by MBW,

depends on software settings and device setups [30, 31]. Several studies have assessed FRC in newborn infants and reported large differences. Our research group previously published reference values for FRC from an European population [27]. A recent birth cohort study in Africa [32] performed lung function tests at the same time point at infancy, but reported significantly different FRC values. Differences in software settings between studies may be one reason for the observed differences in FRC. Furthermore, differences in population characteristics may have also contributed to differing FRC values, as numerous reports documented differences in lung function between ethnic groups [33]. Besides ethnic differences, different exposures between study populations might also have contributed to differences in lung volumes. While it is not easy to discern whether the differences in FRC are due to ethnicity, environmental factors, or a combination of both, this complex interaction should be considered when interpreting study results or determining associations with environmental factors.

1.4 Methodological challenges to assess airway inflammation in childhood

The varying presentation of asthma symptoms and airway obstruction, especially in young children, makes it difficult to distinguish health from disease. Methods to assess airway inflammation are already applicable in infancy. The fraction of exhaled nitric oxide (FeNO) is a non-invasive biomarker used to assess airway inflammation, and can be measured already in infancy [34-36]. The biomarker is routinely used in asthmatic children to monitor treatment response. Recent studies assessed whether or not an early assessment of FeNO might be predictive for later asthma development. There was some indication that increased FeNO levels during early childhood were associated with a later diagnosis of asthma [36, 37]. While elevated FeNO seems to be a sensitive biomarker in the early march of asthma development [38], it is important to remember that airway inflammation and FeNO levels fluctuate in asthma [39, 40]. The dynamic nature of FeNO may, therefore, limit its application for later disease prediction.

1.5 Impact of immune regulation and the environment on respiratory disease

Immune regulation during the prenatal period and early infancy is known to be important for later lung development [41]. The direct and detailed assessment of immune function is routinely performed by obtaining blood samples. However, especially in children, it is better to assess inflammatory processes non-invasively. Assessment of airway inflammation is already feasible in infants, by way of FeNO measurements [27, 35, 42]. FeNO is one of the few biomarkers which made it from bench to bedside, and is now in routine clinical use in order to detect eosinophilic airway inflammation and to monitor corticosteroid treatment response in asthmatic patients [43]. FeNO measured during early childhood is useful to predict subsequent respiratory morbidity [36, 37]. This association should, however, be interpreted within the context of environmental factors since e.g. air pollution [44] and maternal smoking [45, 46] impact upon FeNO levels. Furthermore, some studies reported a direct induction of the inducible nitric oxide synthase (iNOS) after environmental exposure to viruses [47] and allergens [48], underlining the relevance of the environmental impact on FeNO levels.

Recent studies have suggested new methods to non-invasively assess airway inflammation within the lungs, by collecting exhaled breath condensate [49-51]. This method has great potential, since it enables measurements of a variety of cytokines (e.g. interferon-gamma, interleukins) [50]. However, this technique is still in development and not yet available for clinical use. Aside from studies needed to investigate methodological issues of this novel technique, the impact of environmental factors e.g. tobacco smoke exposure, and air pollution, remain largely unknown [52, 53].

There are a variety of other immune markers to assess the risk for the development of respiratory diseases [41]. For example, the assessment of eosinophilia is useful to identify specific asthma phenotypes [54]. Further, assessment of eosinophilia in blood is part of the asthma predictive index (API), a well validated tool to assess the risk of a child to develop asthma [55]. With the assumption that asthma has several phenotypes, and that there are potentially several

immunological processes involved in their development, recent studies searched for new biomarkers to describe these phenotypes. These novel biomarkers may present the opportunity to improve the diagnosis of asthma sub-phenotypes, and may assist in the prediction of outcomes and optimal treatment options [41].

Much attention has recently been given to chitinase-like proteins (C-CLP), which have been found to cross talk with the innate and adaptive immune system. The prototype of C-CLP in the human body is YKL-40, which has been found to be elevated in a variety of inflammatory diseases including asthma [56]. YKL-40 was shown to be increased in adult asthmatics [57, 58] and in children with severe asthma [59]. A specific pathophysiological importance of YKL-40 for lung diseases has been discussed, since YKL-40 may be involved in the development of fibrosis and remodeling of the lung [60]. YKL-40 was shown to be increased in adult asthmatics [57, 58] and in children with severe asthma [59]. Genetic studies revealed that variation in the gene encoding YKL-40, chitinase 3-like (*CHI3L1)*, contributes to the pathogenesis of asthma [61]. Genetic variation in *CHI3L1* was associated with pathological lung function values in adults [61], and correlated with poor asthma control and inflammatory markers in severe asthmatic children [59]. Whether or not genetic variation of *CHI3L1* or YKL-40 levels can serve as predictors for asthma development in unselected, healthy infants is unknown.

Early immune regulation is modified by exposure to environmental factors. Depending on the exposure, they can increase- or reduce the risk for respiratory diseases. Exposure to outdoor air pollutants, such as PM_{10}, NO_2, ozone (O_3), diesel, and tobacco smoke are known to impact upon immune regulation [62, 63], and are associated with subsequent respiratory diseases and reduced lung function [64, 65]. On the other hand, some factors are described as protective against asthma. An inverse association between living on a farm with the development of IgE-mediated allergic diseases is well described [66]. Further, greater microbial diversity was protective for asthma development [66-68], and specific species, e.g. *S. pneumoniae*, *H. influenzae*, seem to increase the risk [69]. Further understanding on the interaction between environmental factors

and genetics, as well as their impact on early immune development, may help to identify infants at risk for childhood asthma.

1.6 Impact of genes and the environment on respiratory disease

Twin studies demonstrated the high heritability of asthma, and further analyses have suggested that this heritability might be due to few genes with moderate effects [70]. This was suggested from findings in which data were derived from studies using the positional cloning approach and candidate gene studies. These two approaches, however, were limited by the *a priori* selection of candidate genes. The completion of the human genome sequence project offered the possibility to identify novel genetic effects or pathways relevant for subsequent respiratory disease [70]. Already a decade ago, over 100 genes were studied with about 25 of those genes found to have the potential of being true susceptibility genes. These genes were replicated in several populations [71] and further genes were added to the list of susceptibility genes reported for asthma and associated traits [71].

Nowadays, there are affordable toolkits available to study the genetic basis of complex diseases. Sequencing the entire human genome together with technological development has opened up the possibility to perform genome wide association studies (GWAS) in large populations [70, 71]. The first GWAS in childhood asthma discovered a major signal from chromosome 17q21, harboring the Orosomucoid like 3 (*ORMDL3)* gene [72]. *ORMDL3* is a member of a novel class of genes that encodes transmembrane proteins anchored in the endoplasmic reticulum. Although this gene has been extensively studied, its function in asthma is still only poorly understood [72, 73]. While the GWAS approach led to the discovery of novel disease loci, it is interesting to note that a number of candidate genes previously described could not be replicated in genome-wide significance levels [74], possibly due to the fact that previous associations were false positive. On the other hand, it could also be that chips used for GWAS did not sufficiently cover many asthma candidate genes [74]. Nevertheless, the numerous studies on genetics in asthma so far

have provided a well validated list of susceptibility genes, helping to define important biologic pathways [75].

Recently, there has been increased interest in epigenetic-regulation, i.e. heritable changes in gene expression without any change in the DNA. Epigenetic mechanisms may be influenced by external, environmental factors, and recent studies suggest that complex gene-environment interactions may lead to the development of pediatric airway diseases [76]. This underlies the significance of the environment for respiratory morbidity in childhood. While the risk for asthma maybe partly inherited, environmental factors act together with susceptibility genes during special windows of opportunity in time. The effect of this interaction may result in impaired lung development, tissue remodeling and persistent dysregulation of the airway tone [77]. Genetic and environmental factors may negatively affect lung growth independently, or by an interplay. Early lung development, in particular, is suggested to be a very sensitive time period for these stimuli [64, 78] with long term effects. Traffic related air pollution is considered a relevant environmental factor resulting in increased frequency of asthma exacerbations and impaired lung development in infants [79], children [17], and adults [19]. For younger children and infants, there is evidence that environmental tobacco smoke impact upon lung development during pregnancy and early life [80].

1.7 Aims of the PhD

The first aim of this thesis was to strengthen the quality of infant lung function tests. The second aim was to study different asthma risk factors (biomarkers, genetics, respiratory symptoms early in life), and to investigate their association with respiratory disease during childhood. These aims were investigated mainly within the context of the ongoing BILD cohort study, for which I organized and collected data. Furthermore, I worked in the affiliated wet lab of the University Children's Hospital Basel under the supervision of Prof. Philipp Latzin (former group leader of the Pediatric Pneumology research group, funded by the Fondation Botnar), and Dr. Loretta Müller.

The following specific aims will be addressed in this work:

1) To address the complex mechanistic interaction of factors influencing the development of airway disease in children.
2) To comprehensively describe the currently available lung function tests which serve to diagnose airway disease and function at an early age. Next, the aim was to provide recommendations of how to improve quality of the outcomes.
3) To further understand airway function in infancy, the aim was to compare airway resistance measurements by Rint assessed at 42 weeks' postmenstrual age between term and preterm infants. In addition to this, we assessed variability of Rint in both study groups. We hypothesized that: i) preterm infants have increased airway resistance compared to term infants in infancy and ii) measurements have high variability in both study groups.
4) To study the association between FeNO measured within a white, Central European population shortly after birth [78], and diagnoses of asthma and atopy at school age. Further, we compared FeNO levels after birth between healthy infants of the BILD cohort and FeNO levels from patients with CF enrolled in the Swiss Cystic Fibrosis Infant Lung Development birth cohort [81].

5) To analyze the effect of polymorphisms in genes known to be relevant for asthma development. We focused on a biomarker suggested to be useful in the diagnosis of asthma [56], called YKL-40. Genetic studies in adults and infants at an increased risk for asthma development revealed that variation in the gene encoding YKL-40, *CHI3L1*, contributes to the pathogenesis of asthma. However, if *CHI3L1* polymorphisms are associated with respiratory morbidity in unselected, healthy infants is unknown.
6) To characterize the symptom dynamics of respiratory symptoms during infancy. For this, we used weekly assessed respiratory symptom scores during infancy to develop a method which characterizes this time series. Next, we tested whether specific dynamic symptom patters predicted wheezing and atopy at school age. Lastly, we investigated whether different environmental factors were associated with specific dynamic symptom patterns.
7) Several epidemiological studies indicate that exposure to air pollution is associated with respiratory disease [64, 82, 83]. Well studied are the effects of short-term exposure to high levels of air pollution on an individual level [84], and some epidemiological studies have investigated long-term effects [17, 85]. However, the effects of short-term exposure on immune cell function have not been studied in detail. Therefore, our aim was to assess the influence of short-term gasoline exhaust exposure (freshly emitted from and passenger car) on immune cells within an experimental *in vitro* setting.

2 Methods

2.1 The Basel-Bern infant lung development cohort

In 1999, the Basel-Bern infant lung development (BILD) cohort was established in Bern, and later in Basel (2011), to study and better understand early lung development. The study aims to investigate physiological properties of the respiratory system, and environmental and genetic risk factors, affecting lung development in healthy individuals from infancy through childhood in relation to wheeze and asthma.

Infants were recruited before birth and standardized interviews and questionnaires were used to assess risk factors for respiratory morbidity during childhood. At birth, cord blood samples for the assessment of immune cells and genetic information were collected. Additionally, the infant's urine was collected at several time points during infancy for analyses of metabolic markers. During infancy, the respiratory health of the infants was monitored in detail. This includes conducting a non-invasive lung function test at 42 weeks' postmenstrual age, as well as weekly calls during the first year of life by study nurses to assess the child's health and occurrence of respiratory symptoms. At six years of age, the study participants are invited for a follow-up, including lung function tests and assessment of asthma and allergic diseases. An overview of the study outline is given in Figure 1. The study has been previously described in greater detail [78]. The Ethics Committees of Bern and Basel, Switzerland approved the study. Written informed consent was obtained from parents before enrolment.

2.2 Respiratory symptoms during infancy

Respiratory symptoms during the first year were assessed by weekly telephone interviews done by research nurses using a standardized symptom score that groups symptoms into four levels according to severity, with a high sensitivity for lower respiratory tract symptoms [86, 87].

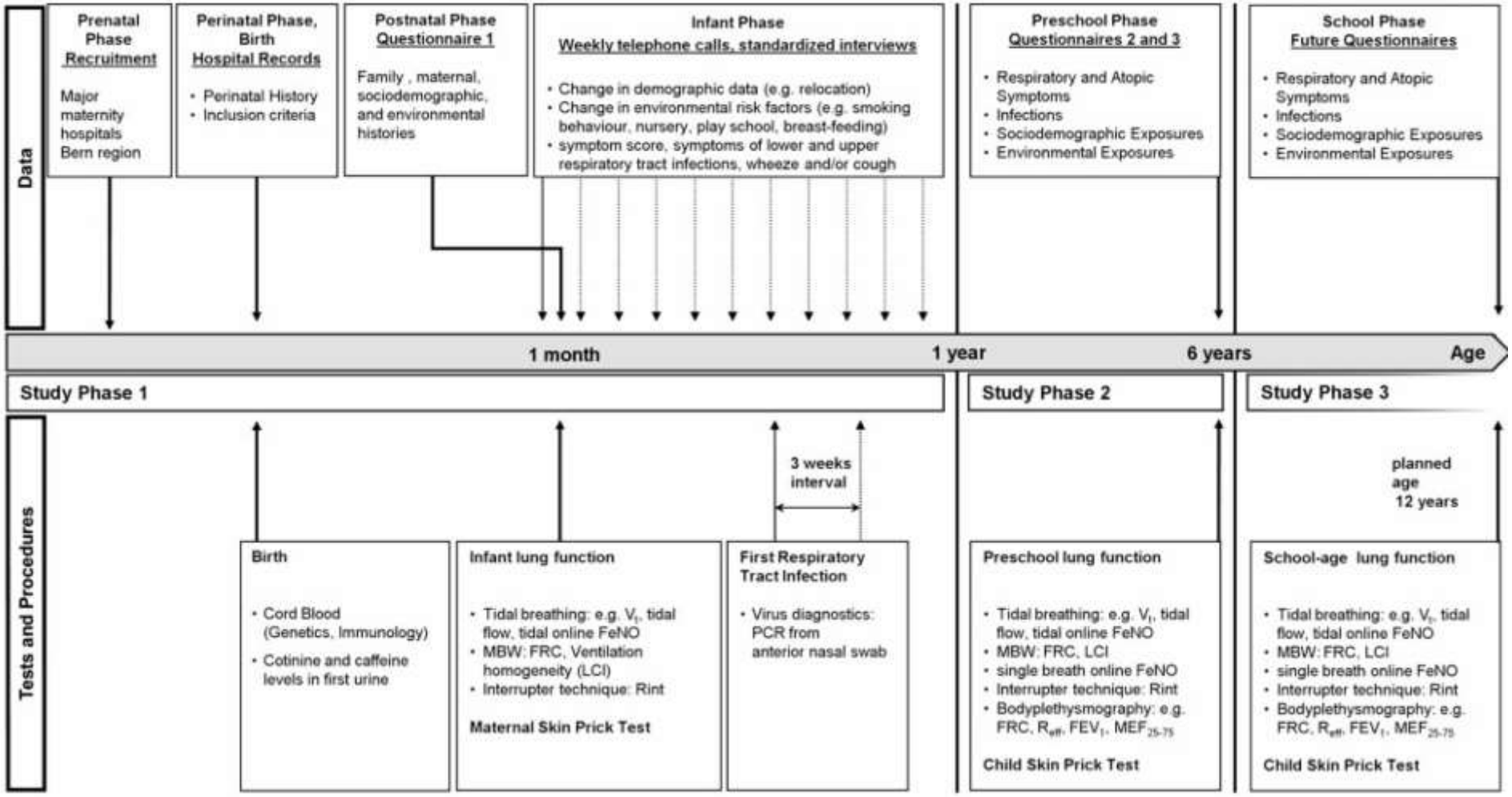

Figure The BILD cohort: time-flow of recorded data, as well as of tests and procedures performed during the follow-up. Vt: tidal volume; FeNO: fraction of exhaled nitric oxide; MBW: multiple breath washout; FRC: functional residual capacity; LCI: lung clearance index; PCR: polymerase chain reaction; Rint: airway resistance by interrupter; Reff: effective airway resistance (measured by bodyplethysmography); FEV_1: forced expiratory volume during the first second of expiration; MEF_{25-75}: mid-expiratory flow [78].

2.3 Cord blood sampling

Shortly after birth, cord blood was sampled and stored at -80°C for the assessment of genetic information and immunological markers. Genome-wide single nucleotide polymorphism genotyping was conducted in collaboration with asthmagene.de (University of Regensburg, Germany). Cord blood YKL-40 was measured in duplicates by an enzyme-linked immunosorbent assay (ELISA) (R&D Systems, USA).

2.4 Lung function

Interrupter technique

At 5 weeks of age, Rint was measured according to ATS/ERS guidelines for preschool children [10] and recommendations for infants [29, 88] during behaviorally defined, unsedated non-REM sleep in supine position using a rigid face mask (Teleflex medical AG, Ruesch, Silicone, size 2, Belp Switzerland) lined with putty to ensure a leak-free seal and to reduce dead space. Interruptions were manually triggered (Exhalyzer D, Eco Medics AG, Duernten Switzerland) every 3-6 breaths. Rint was calculated using the linear back-extrapolation method, applied to the trace of pressure at airway opening between 30 and 70 ms post-interruption [26]. Rint was recorded and processed [26, 89] using WBreath (Version 3.28.0.0, ndd Medizintechnik AG, Zurich, Switzerland). Before data analysis, values were corrected to body temperature pressure saturation (BTPS) and zero-offset of flow baseline was performed, as described previously [90].

Multiple breath washout

MBW measurements were performed in infants at 5 weeks of age according to current ERS/ATS standards [9], as previously reported [91]. In brief, unsedated infants were measured while breathing through an infant facemask (Homedica AG, Baar, Switzerland), during natural sleep, in a supine position with the head midline through an ultrasonic flowmeter (Exhalyzer D, Eco Medics AG, Duernten, Switzerland) using 4% SF_6, as previously described [91, 92].

Spirometry

During follow up at six years of age, spirometry was performed to measure forced expiratory volume in 1 second (FEV_1), forced vital capacity (FVC), and forced expiratory flow at 25-75% of FVC ($FEF_{25-75\%}$), according to ATS standards [22]. Data were expressed as z-scores using normative data from the Global Lung Function Initiative [33]. Spirometry was done using the MasterLab setup (Jaeger, Wuerzburg, Germany).

2.5 Exhaled nitric oxide

FeNO measurements in newborns

FeNO measurements were performed at 5 weeks of age from multiple breaths during natural sleep, as previously described [29, 35], with a rapid response chemiluminescence analyser (CLD 77; EcoMedics, Duernten, Switzerland; Analysis software: WBreath Version 3.28.0.0, ndd Medizintechnik AG, Zurich, Switzerland. Measurements were done in unsedated infants during natural sleep.

FeNO measurements in school aged children

During follow up at six years of age, FeNO was measured by the single-breath method with a rapid-response chemoluminescence analyzer (CLD 88 sp; EcoMedics, Duernten, Switzerland). Flow was recorded using an ultrasonic flow meter (Spiroson; EcoMedics) according to current guidelines [93].

2.6 Clinical outcomes at school age

Respiratory health was assessed using questions from the International Study of Asthma and Allergies in Childhood (ISAAC) [94]. Asthma was diagnosed if one on the following was present in the previous year: (1) physician diagnosis of asthma or (2) episodic wheeze. Atopy was defined by allergic rhinitis, allergic asthma, or atopic dermatitis. A skin-prick test was done for the following allergens: (Dog dander, cat dander, *Dermatophagoides pteronyssinus*, mixed tree pollens, mixed grass pollens, *Alternaria tenuis*, positive control (histamine), negative control

(NaCl), Allergomed, Switzerland) positive in case of hives bigger than histamine in any of the tested allergens.

2.7 Assessment of air pollution *in vitro*

A coculture model composed of bronchial epithelial cells (ECs) and natural killer cells (NKs) mimicking the human airways was used to compare toxic effects between pure gasoline and ethanol-gasoline-blend exhaust emitted from a flexfuel gasoline car. The effects of exhaust on the cells were assessed by quantitative real-time-polymerase chain reaction, flow cytometry analysis, and oxidative stress assay.

3 Results

3.1 Complexity of airway disease

Asthma and obesity in children: current evidence and potential systems biology approaches

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REVIEW ARTICLE

Asthma and obesity in children: current evidence and potential systems biology approaches

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Abstract

Both obesity and asthma are highly prevalent, complex diseases modified by multiple factors. Genetic, developmental, lung mechanical, immunological and behavioural factors have all been suggested as playing a causal role between the two entities; however, their complex mechanistic interactions are still poorly understood and evidence of causality in children remains scant. Equally lacking is evidence of effective treatment strategies, despite the fact that imbalances at vulnerable phases in childhood can impact long-term health. This review is targeted at both clinicians frequently faced with the dilemma of how to investigate and treat the obese asthmatic child and researchers interested in the topic. Highlighting the breadth of the spectrum of factors involved, this review collates evidence regarding the investigation and treatment of asthma in obese children, particularly in comparison with current approaches in 'difficult-to-treat' childhood asthma. Finally, the authors propose hypotheses for future research from a systems-based perspective.

In children, both asthma and obesity are complex diseases (1) related to gene–environment interactions (2) and various lifestyle factors. In the Western world, the prevalence of obesity is increasing and varies between 4% and 14% in schoolchildren in developed nations (3). There is no global standard for classifying obesity in children. One classification by the World Health Organization defines obesity as three standard deviations above the median body mass index (BMI) (3). Worldwide, there are more than 300 million people affected by asthma, but incidence rates of asthma in children can differ significantly between regions and countries (4). Several reviews clearly provide evidence for an association between obesity and asthma in both adults and children (5, 6). While some earlier cross-sectional and case–control studies have been partly inconsistent, recent studies consistently reported an increase in the prevalence of asthma symptoms in relation to overweight in children (7). These findings are supported by a meta-analysis where a dose–response relationship between body weight and incident asthma was observed (8). Most importantly, the majority of prospective studies have shown that overweight preceded later occurrence of asthma symptoms (reviewed in 9). This makes reverse causation rather unlikely. Shortcomings in some of the studies are the problems in identifying asthma and obesity phenotypes; the lack of control for confounding factors such as diet and physical activity; and the difficulty in drawing causal relationships.

This comprehensive review attempts to highlight the *breadth of the spectrum* of mechanisms and factors involved in the relationship between obesity and asthma, and to consider their specific roles and importance during particular phases of childhood development.

Clinical manifestations

The classical symptoms of asthma include wheezing, dyspnoea and cough. Children with asthma and obese children both often present with exercise intolerance and related physical inactivity or sedentary behaviour. These entities are often difficult to disentangle, and diagnostic investigations may aid in distinguishing asthma-related exercise intolerance from poor training condition (10).

Obesity and asthma also *share common comorbidities* (Table 1) that complicate and perpetuate the interactions

between them. Both asthma and obesity are related to gastro-oesophageal reflux and sleep disturbances (11, 12). Orthopaedic problems are often under-recognized in obese asthmatic children, yet they are still relevant in the context of impaired physical activity (1). As in any form of difficult-to-treat asthma, the early recognition and treatment of comorbidities is a key element for successful management of the disease (13).

Pathophysiology

It is extremely difficult to separate the effects of various genetic, physiological and environmental factors involved in the association between asthma and obesity. As a result, there is no evidence in the literature that any one particular mechanism dominates a causal relationship between obesity and subsequent asthma. However, some of these mechanisms are more relevant in the context of foetal programming and others in early and later post-natal life (14, 15). There seem to be critically sensitive periods during development at which certain exposures or stimuli may have long-term consequences. In Figure 1, we have attempted to represent the vulnerable phases for various mechanisms and risk factors. We will use this general structure as a model to interpret the literature.

Genetic factors

As reviewed in Tantisira and Weiss (6), early linkage studies have suggested shared *genetic determinants* for asthma and overweight. Those genes were the β_2-adrenergic receptor on chromosome 5q, the gene encoding for TNFα (16), the glucocorticoid receptor beta and the leptin gene (17). These hypothesis-driven analyses of candidate genes have not been confirmed in a recent systematic genomewide association study (GWAS) where in particular the role of the gene *DENND1B*, suspected to be associated both with asthma and with obesity, was discussed (18). In the most recent analyses,

Table 1 Physical consequences and comorbidities of childhood and adolescent obesity and asthma

Comorbidity	Obesity	Asthma
Pulmonary	Sleep apnoea	Sleep apnoea and disturbances
	Pickwickian syndrome	Lung function decline
	Exercise intolerance	Exercise intolerance
		Airway remodelling
Orthopaedic	Slipped capital epiphyses	Thorax remodelling
	Tibia vara (Blount)	
	Tibia torsion	
	Flat feet	
	Ankle sprains	
	Increased fracture risk	
Neurological	Idiopathic intracranial hypertension (e.g. pseudotumor cerebri)	
Gastroenterological	Gastro-oesophageal reflux	Gastro-oesophageal reflux
	Cholelithiasis	
	Liver steatosis/nonalcoholic liver disease	
Endocrine	Insulin resistance/impaired glucose tolerance/type 2 diabetes	
	Menstrual abnormalities	
	Polycystic ovary syndrome	
	Hypercorticism	Drug-induced hypercorticism
Cardiovascular	Arterial hypertension	Pulmonary hypertension in severe asthma
	Dyslipidaemia	
	Fatty streaks	
	Left ventricular hypertrophy	
Inflammatory	Systemic inflammation/raised CRP	Chronic airway inflammation
		Atopy
Psychosocial	Poor therapy adherence	Poor therapy adherence
Behavioural	Physical inactivity with exercise intolerance	Physical inactivity with exercise intolerance
	Increased electronic media use and sedentary behaviour	Increased electronic media use and sedentary behaviour
	Psychosocial stigmatization	Psychosocial stigmatization
	Familial overprotection	Familial overprotection
	Low self-esteem, depression	Low self-esteem, depression
	Social isolation	
	Malnutrition	
	Addictive behaviour	

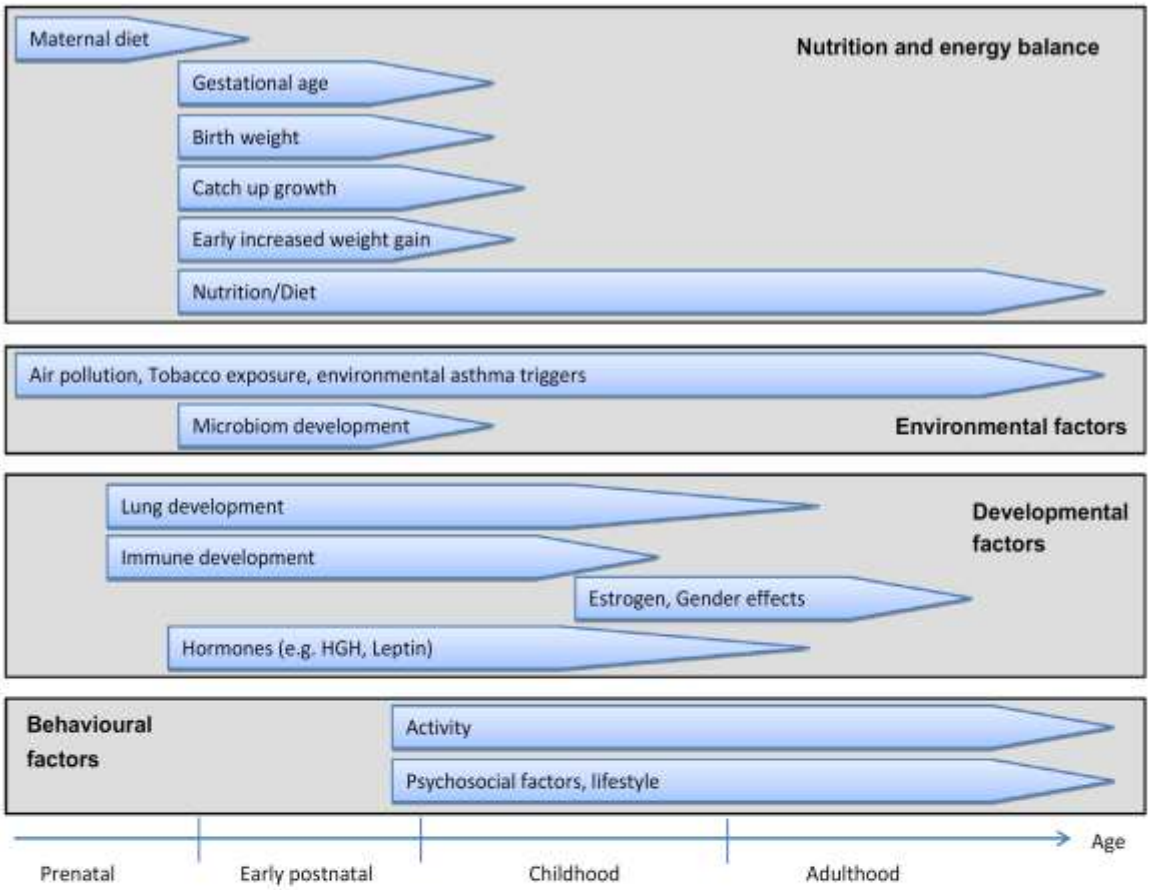

Figure 1 Nutrition- and lifestyle-related mechanisms leading to increased risk for subsequent asthma in children. Each mechanism is illustrated in the context of the phase of childhood development, where it may have its dominant role (often referred to as a 'window of opportunity').

susceptibility signals for genetic pleiotropy of asthma and obesity have been found; however, it is not clear whether these signals are due to neighbouring genes (19). In sum, further studies need to be undertaken for clear evidence to emerge.

Nutrition and energy balance

Maternal nutrition

In obese children, strong evidence exists suggesting that maternal nutrition during pregnancy containing folate, methionine and vitamin B12 can lead to epigenetic changes which include methylation and alterations to histone proteins that affect specific gene transcription (20). In a pilot study, DNA methylation in peripheral mononuclear cells from children with obesity-associated asthma were distinct and indicative of activation of innate and adaptive immune responses, including the release of pro-inflammatory substances from macrophages as well as T-cell differentiation, both important for the pathogenesis of asthma (21). Undernutrition during prenatal development is linked to higher post-natal fat consumption (22), and glycemia during pregnancy has also been shown to be important for later obesity (14). It has been suggested recently that an increased Mediterranean diet with its antioxidants and anti-inflammatory compounds during pregnancy may be protective for the development of wheeze and atopy at the age of 6.5 years (23). Furthermore, increased consumption of vitamin D, dairy products and calcium during pregnancy was related to a decreased risk of infantile wheeze (24), while fatty acids increased the risk of childhood asthma (25). However, the exact relevance of maternal nutrition for the development of asthma in later life still remains unclear (26) (reviewed in 27).

Birthweight

One similarity of the two multifactorial diseases is the U-shaped risk association with birthweight, with both low and high birthweight increasing the risk for subsequent development of overweight (28) and asthma (29). Even though this correlation is hypothesized by other authors as

well, the causative mechanistic network of this phenomenon remains unclear (30). A low birthweight is discussed as an independent risk factor for the asthma–obesity association, but how much it is influenced by other factors, for example gestational age, is unknown (reviewed in 31).

Weight gain

The effect of timing and dose of overweight and weight gain on asthma development has only recently been recognized. Using data from prospective studies, it was possible to show that the age of onset and the duration of overweight during childhood had different effects upon later asthma development (32). A longitudinal study was able to show that being overweight at the age of 1 year was associated with a decreased risk of asthma and better lung function at ages 6 and 8 years, while being overweight at the age of 5 years, but not at the age of 1 year, was associated with an increased risk of asthma at the age of 6 years (33). Data on early weight gain are more controversial with some studies showing that early life weight gain was associated with later asthma as well as obesity (7), while others showed that this association was instead dependent on later weight changes (33).

Composition of infant and childhood nutrition

Regarding asthma development, lung functional growth and development of obesity, the role of breastfeeding is still controversial (34). As the practice of breastfeeding is very heterogeneous worldwide, and definitions of asthma and obesity in early life are not yet internationally standardized, it is difficult to draw conclusions from the large body of existing studies. A recent meta-analysis summarized the existing data on breastfeeding and asthma and found a strong protective effect for children between the ages of 0 and 2 years (35). As the child develops, numerous factors including infections and allergen exposure modify respiratory morbidity, and hence, this risk reduction was not visible in school-aged children (35). A recent review found a protective effect of breastfeeding on childhood overweight and obesity in most studies, but argued that confounding factors prevent any definitive conclusions (36). Although breastfeeding has multiple benefits on a child's health, the complicated interactions between breastfeeding, genetic background, gender, viral infection and other risk factors do not allow concrete conclusions to be drawn on the optimal duration of breastfeeding (34). Vitamins A, C, D and E, beta carotene, minerals (zinc, Mg), flavones, flavonoids, pyridoxine and nutrients, such as omega-3 polyunsaturated fatty acids and omega-6 and trans-fatty acids, have been associated with antioxidative activity and with several aspects of asthma and lung development (reviewed in 37). The relevance of childhood nutrition for the development of allergic diseases is highlighted in several recent studies. An increased consumption of fast food is suspected to be a risk factor for developing asthma, whereas a Mediterranean diet is discussed as protective for asthma symptoms in children (38, 39). Latest research suspects the microbiome to be relevant to both diseases (15, 40).

Hormones and gender effects

Another example of the importance of timing and development in the relationship between asthma and obesity is the effect of hormones. Insulin resistance is known to be associated both with obesity and with type 2 diabetes and has recently been identified as a risk factor for later development of asthma (41). Effects of leptin are known from very early on: cord blood leptin is associated with subsequent development of both obesity (42) and asthmatic symptoms in early life (43). Leptin has not only been shown to strongly regulate weight gain (42), but also to influence lung development (44) and immune regulation (45). However, the role of leptin in asthma development is still uncertain (46). In later childhood, changes in oestrogen levels over time are thought to explain the differences of the overweight–asthma association between females and males (reviewed in 47). While in adults, overweight and asthma are clearly more strongly related in females (reviewed in 47 and 48), data on children are less clear. A large retrospective study in children identified fat mass to be positively linked to asthma in both preschoolers and school-age children with no difference in association between genders (49). However, an association between asthma and obesity was found in girls (50), conflicting with another systematic review of six studies where a significantly larger effect of overweight on asthma occurrence in boys than in girls (51) was reported.

Lung mechanics, functional development and exercise physiology

Lung function dramatically changes during development, and thus, it is very likely that the effects of a high body mass on lung mechanics vary at different phases of lung development. In young infants and toddlers, airway resistance, thorax compliance, airway wall compliance and flow limitation are typically higher than in older children. In infancy, functional residual capacity (FRC) approaches the lung's 'closing volume' (52), where alveolar regions and small airways are closed, and gas exchange surface is decreased. Theoretically, all these effects become even more critical in the presence of restricted thorax excursion as, for example, with obesity (32). Obesity is associated with low FRC (53) and will increase the expiratory flow limitation during tidal breathing in severely obese patients (54). Obesity also restricts tidal volume changes (55), particularly during exercise (56). Furthermore, restricted cyclic tidal volume will also directly affect smooth muscle function. As a consequence, muscular latch cross-bridges between actin and myosin adapt to a slower cycling state (57). As a result, airway smooth muscles become constricted and difficult to stretch, which is thought to subsequently lead to persistent airway obstruction and bronchial hyperactivity (57).

While the effect of obesity on lung mechanics and lung function seems clear already in childhood (58), the association between overweight and airway hyperreactivity (AHR) has recently been questioned (59). One hypothesis is that obese subjects do exhibit AHR, but only during exercise, due to

reduced lung volumes and faster limitation of expiratory flow. Why they did not show any signs of AHR on formal pharmacological hyperreactivity testing, as reviewed in (60), remains unclear. A possible explanation might be that full lung expansion is associated with AHR (61) and airway obstruction (62) especially during exercise, even unrelated to the existence of asthma.

Exercise per se might have the potential to mitigate chronic inflammation, which may be relevant in the context of asthma as an inflammatory condition (63). Potential mechanisms are the reduction in visceral fat mass and/or epigenetic changes and their related reduction of pro-inflammatory reactions (64). Indirect evidence comes from observational studies that demonstrated that increased physical fitness was associated with decreased risk for asthma (48, 65) and lower bronchial reactivity (65).

Immunology and immune development

As recently reviewed (6, 47), not only mechanical but also complex inflammatory and immunological interactions may occur in both asthma and obesity (66). In obesity-related metabolic syndrome, several pro-inflammatory cytokines (TNFα, IL-6, IL-12, IL-17, IL-1β) as well as inflammatory proteins (CrP) are released. High levels of leptin in obesity facilitate the TNFα, IL-6 and IL-12 release of LPS-stimulated macrophages and may, through inflammatory processes, interfere with lung development (44). In asthma, IL-6 increase is associated with IL-1, IL-4, TNFα and histamine release, IgE modulation and profibrotic activity in airway remodelling. Additionally, IL-1β is associated with IL-5 induction from CD4+ cells. TNFα is an important mediator of IL-4 in allergen-induced T cells and of IL-5 from bronchial epithelial cells. A recent study in mice identified an IL-17 increase to be associated with AHR and smooth muscle contraction from cholinergic stimuli (67). Despite these inflammatory mechanisms described, there is inconsistent epidemiological evidence of an association between obesity and atopy. The NHANES III study suggested a relationship between positive skin prick tests and BMI (68), as obesity in early life correlated with higher incidence and severity of atopic dermatitis (69). However, another study found this association only in teenage girls, but not boys (70). Furthermore, several studies have shown a clear relation between nonatopic asthma and body weight, suggesting that nonimmunologic pathways may also play a role (71–73). In summary, the multifactorial pathogeneses of asthma and obesity are still poorly understood, and a common causative biological or immunological link is still highly questionable (74).

Lifestyle and behavioural factors

It is difficult to disentangle the exact interaction of lifestyle factors with asthma and obesity. Both asthmatic and obese children can present with low levels of physical activity. While lack of exercise is one lifestyle aspect worth considering, there is another that is similar but distinct and becoming increasingly common: sedentary behaviour (e.g. watching television or playing video games). A recent systematic review in children hypothesized that levels of physical activity and sedentary behaviour can be separate risk factors for negative health outcomes (75). Other cross-sectional epidemiological studies suggest that TV viewing (as a surrogate of physical inactivity) and asthma were independently associated with obesity (76) and that children with wheezing disorders demonstrate lower levels of physical activity (77). Prospective studies that are better suited to demonstrate a true interaction are scarce in children. Rasmussen et al. (65) followed 9-year-old children over 10 years and found physical fitness at inclusion (but not physical activity during the study period) to be associated with reduced risk of asthma in adolescence. Whether low levels of physical activity lead to asthma and/or obesity or vice versa is an example of the classic chicken and egg scenario: it is not yet established which comes first and why (for further discussion, see 'Outlook: potential insights from a disease network approach').

Psychosocial factors

Important psychosocial contributors to obesity include external stressors such as being teased, bullied, discriminated or neglected that trigger emotional eating (78) and personal psychosocial problems such as diminished self-esteem, behavioural problems (78), dissatisfaction with body image or a living situation where consistency, limit setting and supervision are lacking (79). These psychological traits can perpetuate the disease process and end in a vicious circle: the children are unable to follow healthy nutritional plans because of either their emotional eating behaviours or the fear of bullying that makes them physically less active and more likely to stay indoors, resulting in further weight gain. While asthma may not have a direct psychological impact, several studies have demonstrated that children with severe asthma tend to adopt a sedentary lifestyle, which can in turn promote obesity, leading to the above-mentioned vicious circle (80). Interestingly, a recent study in asthmatic children exposed to chronic stress identified a psychological imbalance to interfere with processes of the immune system. Stress-exposed asthmatics showed increased IL-5, IL-13 and eosinophil counts, supporting the assumption that stress induces inflammatory processes (81).

Therapy

Effects of therapeutic interventions in children

Effects of weight reduction, dietary measures

In adults, weight loss seems to affect a variety of asthma outcome measures and lung function (reviewed in 82). However, a Cochrane review suggested that the existing studies should be interpreted cautiously, mainly due to methodological constraints (10). Weight reduction through surgical interventions led to consistent improvement of asthma control in nearly all studies (83), but to date it is not possible to differentiate cardiorespiratory effects due to weight loss from true asthma-

specific improvements. Effects may also be different for those with or without allergic asthma. More improvement could be found in the group with nonatopic (late-onset) asthma – that is more likely to be *caused* by obesity – than in the allergic (early-onset) asthma, that is more likely to be *complicated* by obesity (83). A randomized controlled trial investigating the efficacy of dietary energy restriction in obese children with asthma suggested improvements in lung function and asthma control in the intervention group (84).

Effects of exercise programmes

The WHO and many nations agree that children and adolescents should spend a minimum of 60 min daily in at least moderately intense physical activity, as it plays a critical role in adequate growth and development, serves as therapy for certain chronic diseases and minimizes several adult bone and cardiometabolic diseases with paediatric origins (85). In adults, it has recently been shown that vigorous physical activity was positively associated with asthma symptoms (86). Preventive steps at individual and school levels have demonstrated that increased exercise improves fitness and the cardiometabolic profile (87) in normal and overweight children. Systematic intervention studies tackling the effect of exercise programmes on respiratory symptoms in the obese asthmatic child are still lacking.

Effects of inhalation therapy

It has been known for several years that obese asthmatics show altered responses to standard inhalation therapy (88). Initial studies showed that obese adult asthmatics have worse symptom control and higher reliever use compared with nonobese asthmatics (89). Overweight children with asthma have a decreased response to inhaled steroids clinically and by lung function measures (90) and increased use of reliever medication and oral steroids (91).

It is unclear whether the resistance to inhaled steroids is related to metabolic effects of obesity, the nonatopic phenotype of obese asthmatics (92, 93), increased systemic inflammation with relatively little airway inflammation, genetic variation in the glucocorticoid receptor, hormonal effects or difficulties in compliance. Interestingly, obesity-associated cytokines such as TNFα resulted in accumulation of the inactive form of the glucocorticoid receptor, a mechanism that may explain steroid resistance (94). Until the exact reasons have been clarified, obese children should be treated for their asthma as nonobese children according to guidelines.

Effects of psychological counselling

The combination of discrimination in bullying, teasing, neglect and personal psychosocial problems, such as dissatisfaction with body image and diminished self-esteem of the obese (asthmatic) child, is a harmful and most relevant social problem that needs to be addressed during treatment (95). Children who are overweight benefit from psychological and behavioural counselling as shown by a recent intervention study that resulted in the improvement of psychological symptoms (96). Psychological intervention trails in adults have been shown not only to improve quality of life, but also to promote physical activity (97). Unfortunately, data from psychological counselling trials in asthmatic children are still lacking.

The daily practice: Investigating and treating children with obesity and asthma

There are international standards on how to investigate and treat obesity in childhood (1), and there are also international standards to investigate and treat asthma (98), even in severe and difficult cases (summarized in 13). But there is only limited conceptual evidence available in terms of how asthma in the presence of obesity should be investigated and treated (99). In daily clinical practice, many of the clinicians use a diagnostic and therapeutic approach, which is based on the treatment concepts of 'difficult-to-treat' or 'problematic' asthma (13, 100). In Figure 2, we propose a treatment concept for the obese asthmatic child based on this latter approach, but here we would like to emphasize *how little evidence* is actually available.

Diagnostics: Baseline assessment

Several reviews suggest that prior to treatment, baseline assessment of asthma control (98), obesity (Table 2) (1) and diet, exercise limitation (101), comorbidities, lifestyle factors, therapy adherence and psychosocial context (102) should be performed and exclusion of other differential diagnoses should be made. Before treatment programmes start, these factors, and particularly asthma treatment, should be optimized (98, 101). The question as to whether this can be best achieved in an ambulatory or hospital setting is still being debated (103).

The reasons behind exercise limitations and respiratory symptoms might be multifactorial, and baseline diagnostics may help to optimize therapy. Baseline asthma diagnostics including history, adherence to therapy, clinical examination, lung function, and, in specific cases, X-ray and allergy testing according to international guidelines need to be performed (98). Exercise-related respiratory symptoms may reflect uncontrolled underlying asthma that should be treated with appropriate controller medications, as many children do not use short-acting bronchodilators before exercise as recommended in national guidelines. Alternatively, exercise limitation may just be related to lifestyle-related physical inactivity and subsequent low fitness. Careful history, baseline lung function and exercise testing may help to elucidate the situation (101). True exercise-induced asthma (EIA) presents with cough, wheeze, chest tightness or pain, difficulties in breathing or any combination of these symptoms during or shortly after exercise, usually with a resolution within 30–90 min (101). If EIA in the asthmatic, obese child is suggested, a simple therapeutic trial may be conducted. Only if symptoms are not controlled or the single exercise-related complaint is dyspnoea, which is more likely to be related to other causes, for example deconditioning or vocal cord dysfunction, is further testing warranted. Guidelines for EIA and exercise testing in childhood are available (101).

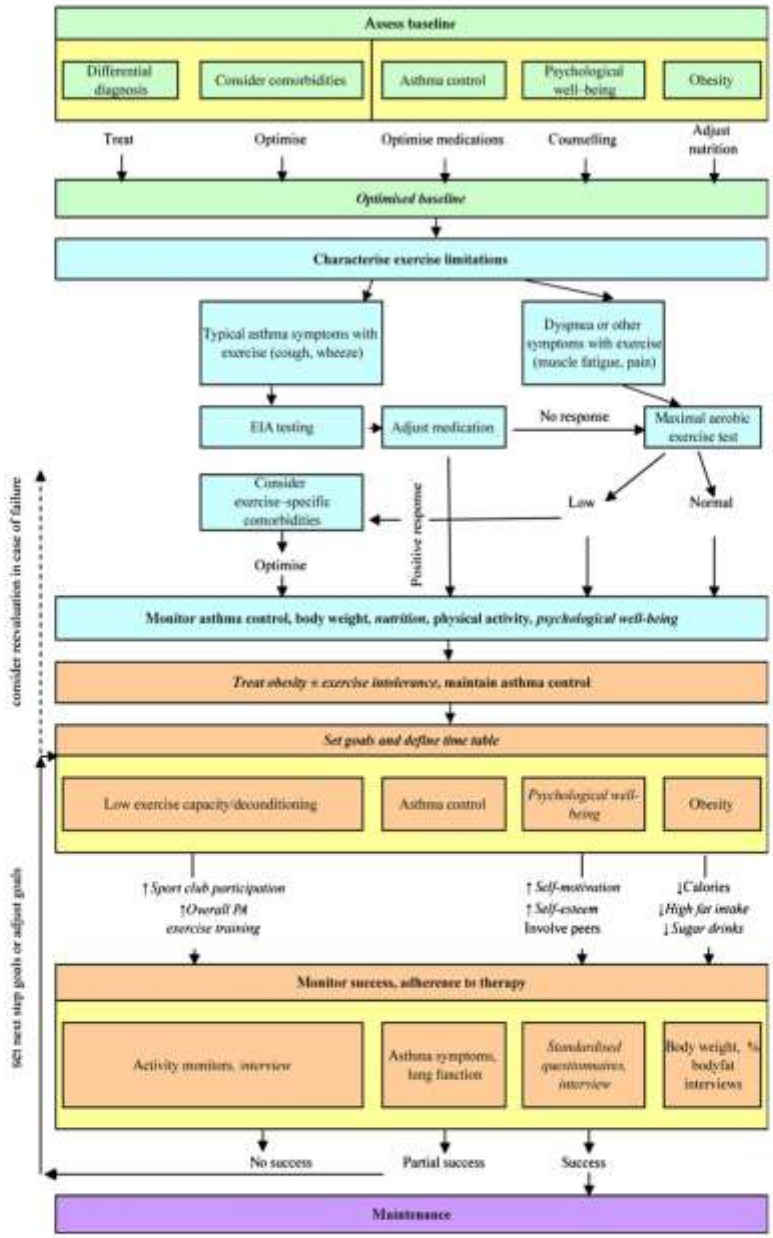

Figure 2 Based on the general schemata of difficult-to-treat childhood asthma, we illustrate *how little evidence* is available related to diagnostics and treatment of asthma in the presence of obesity. It has been suggested that diagnostics and treatment should be performed in iterative and small steps, and in a multidimensional manner, including asthma treatment and control, exercise training, dietary and lifestyle measures and weight control as well as psychosocial counselling. Overall, there is only minimal evidence for such an approach. Steps in the schemata *where more evidence is required* are printed in italics.

Treatment and training phase

In children, evidence is still lacking regarding a specific treatment regimen of asthma in the presence of obesity, although in daily practice every clinician is faced with this problem and asked for possible treatment recommendations. We present some general principles derived from adult medicine; however, we use this framework only to indicate the specific gaps in knowledge in children. A multidimensional treatment approach is suggested to be most successful (104) and should be performed in small iterative steps (105), followed by a multidimensional monitoring and re-evaluation phase. Such therapeutical concepts are based on the stages of behavioural

Table 2 Diagnostics in childhood obesity and asthma

	Obesity	Asthma
History	Personal and family history	Personal and family history
	Start, development of obesity	Start, development of asthma
	Triggers: eating habits, physical activity and sport participation, media use ±snacking, psychosocial history	Risk factors: atopy, sex, prematurity, exposure to viral infections, siblings
		Environmental history, smoking, pollution, allergens
	Comorbidities	Comorbidities
	Medication adherence	Medication adherence
Clinical examination	Weight, height, BMI percentiles	Cardio-pulmonary exam
	Waist and hip circumference	ENT status
	Blood pressure	
Laboratory exam – baseline	Fasting blood glucose	Lung function
	Total, HDL/LDL cholesterol	FeNO
	Triglycerides	Allergy testing
	ALAT	Exercise testing
	TSH	
Further specific diagnostics		
If BMI > 90 and age > 10 years and positive family history for type 2 diabetes, acanthosis nigricans, polycystic ovary syndrome, Mexican, African or Asian background	Oral glucose tolerance test	
Sleep disturbance	Sleep apnoea screening	Sleep apnoea screening
If indicated		Radiological examinations
Puberty or menstrual cycle disturbances	Endocrine investigations	
Protein loss in urine	Nephrological work up	
	Ultrasonography of the liver	
Orthopaedic problems	Radiological exams	

change theory by Prochaska (102), who described a process of stepwise behavioural change from precontemplation to maintenance. The proposed concept implies that the clinician should help patients to move along these stages rather than prescribing a behavioural change when the patient is not ready. Motivational interviewing with encouragement of goal setting, monitoring of behaviours targeted for change and use of positive reinforcement seem to be most promising (106), allowing clinicians to better tailor interventions to the behavioural and psychological needs of their patients.

Any multidimensional treatment approach includes asthma therapy, exercise training, dietary measures (reviewed in 38) and psychosocial counselling (107). The approach should be iterative with clear reachable goals and an appropriate monitoring concept (100). The iterative characteristic of such a therapeutic approach is important as there is a large individual heterogeneity in the treatment response (1). It also enables a personally tailored optimization and continuous adaptation of the therapy concept to the progress of the patient.

There are no published recommendations concerning how weight reduction programmes should be structured in children other than using realistic steps and focusing on healthy lifestyle change rather than on weight reduction (108). However, general principles recommend a diet rich in vegetables and fruit, limiting sugar-sweetened beverages, consuming regular breakfasts, allowing the child to self-regulate his or her meals and avoiding overly restrictive feeding behaviours, and involving the whole family in lifestyle changes that should also include an increase in physical activity and a reduction in sedentary behaviour by small incremental steps (108). Attempts should be made to make such lifestyle changes socially and emotionally attractive, with the use of peer groups and motivational interviewing to increase motivation and self-esteem (106).

Similarly, there are no published recommendations pertaining to whether training programmes have an impact on the decrease of asthma symptoms in children; nevertheless, they are a mandatory precondition for successful weight control and maintenance. There are various types of optimal training programmes proposed, and virtually no evidence in the literature favours one particular method (reviewed in 10). A few general principles are summarized in Table 3.

Monitoring and re-evaluation

Very similar to treatment strategies in problematic and difficult asthma in children (13, 100), monitoring of success and appropriate adaptation of therapy is central. It is suggested that also in obesity-related asthma, monitoring targets should be multidimensional, and therapy adherence should

Table 3 Proposed principles of exercise and physical activity for the obese, asthmatic child

Preprogramme
Establish baseline, optimize asthma therapy, exclude or treat comorbidities
Define a realistic plan and steps
General
Fun, small steps, socially and emotionally attractive
Include peers as ideal social supporters
Emphasize benefits of becoming physically active and consequences of physical inactivity
Use motivational interviewing
Pre-exercise warm-up (at 60–80% HRmax) and short-acting β-mim provide partial attenuation of exercise-induced asthma
Prevent outdoor exercise with critical levels of air pollution or very cold air (<−15°C)
Small steps: start with intermittent exercise of 5 min duration
Increase by 10% per week in duration or intensity
Final aim of 60–120 min of moderate-to-vigorous PA, but little is more than nothing
Adapt the environment
For younger children, outdoor activity is a major determinant of overall PA
For older children and adolescents, peers are the most important in life and should be included
Be active as a family; parents are role models
Specific physical activities
Strength-dominated sports are attractive as the obese child usually has a higher amount of muscle mass
Swimming is preventative for asthma, hides the body and is possibly due to a certain fat-related buoyancy attractive
Gliding sports prevent side effects from the excessive weight
Every sport/physical activity with no restriction

be the focus of the monitoring process. Monitoring of dietary efforts (108), exercise (109) and inhalation therapy (100) should be carried out using appropriate measures such as age-related BMI, body fat content, waist circumference, exercise tolerance (110), lung function (100) and standardized symptom scores. These monitoring outcomes should be compared to the initially set reachable goals and should be used to demonstrate stepwise progress as an important motivating factor (102) to pursue the programme. In case of treatment failure, concomitant factors should be reconsidered (Fig. 4).

Outlook: potential insights from a disease network approach

From large epidemiological observational studies, *statistical interaction terms* are found between mechanisms related to obesity and mechanisms related to asthma. Similarly, intervention studies changing the state of obesity affect mechanisms in asthma. These effects are typically relatively small but nonlinear and modified by numerous other mechanisms. Such behaviour is typically seen in biological networks with *weak functional interaction* between the different subsystems. In the current model, for example, genetics, inflammation, immunology, lung mechanics, clinical symptoms and psychosocial behaviour can be considered to be such subsystems. Given that the relationship between asthma and obesity is highly complex, a systems-based approach might have the potential to offer additional insight into the key gaps in our understanding to date. We would like to raise the following questions and hypotheses to stimulate thinking of future systems-based approaches:

(1) Is it theoretically possible that two complex disease systems sharing similar lifestyle factors or similar pathophysiological mechanisms interact, even if there is no single direct causal relationship?

It is difficult to disentangle the impact of the individual factors linking obesity with asthma. We thus hypothesize from a systems-based approach that there are small interactions that lead to an overall enhanced system effect that is difficult to predict. These interacting effects can happen on a genetic level, cytokine level, cellular level, organ level or even on a patient level including psychosocial behaviour. Even multilevel interactions are possible. These types of phenomena are known in the dynamic behaviour of coupled networks (111).

(2) Is it possible that in such a system, the evolvement of the disease process in small steps is determined by perpetuating and even potentiating interactions of multiple factors, even if each of them is only contributing a small amount?

We hypothesize that the multitude of these small interactions may lead to a gradual deterioration from a healthy state of equilibrium to a state of poor asthma control, obesity and physical inactivity (Fig. 3). To illustrate this disease progression, we can consider the complex interaction of obesity and asthma symptoms with subsequent physical inactivity resulting in weight gain and additional respiratory symptoms and exercise limitations. The interdependence of this downward spiral of behaviour is depicted with red arrows, as well as the reverse progression from disease to healthy state with a blue line (Fig. 3). Previously, we suggested that asthma, obesity and physical (in)activity beg the question of the chicken and the egg, but from a systems theory approach, we might argue that such a question is less relevant. It is important to intervene to break the vicious cycle. To reverse the direction of the vicious cycle, therapy should be aimed at promoting a positive correlation between the two diseases.

(3) Is it possible that growth and development is interacting with disease progression?

A current topic of interest in biology is the dynamic behaviour, stability and interactions of loosely coupled networks (111). We hypothesize a coupling between the complex system of obesity and the complex system of asthma that may explain some of the complex risk interactions between weight gain during development and risk for subsequent wheezing disorders. Similar couplings are seen in complex network systems, which are in optimal function in a dynamic, equilibrium state; deviations from the equilibrium may be deleterious. In Figure 4, we suggest that the deviation from

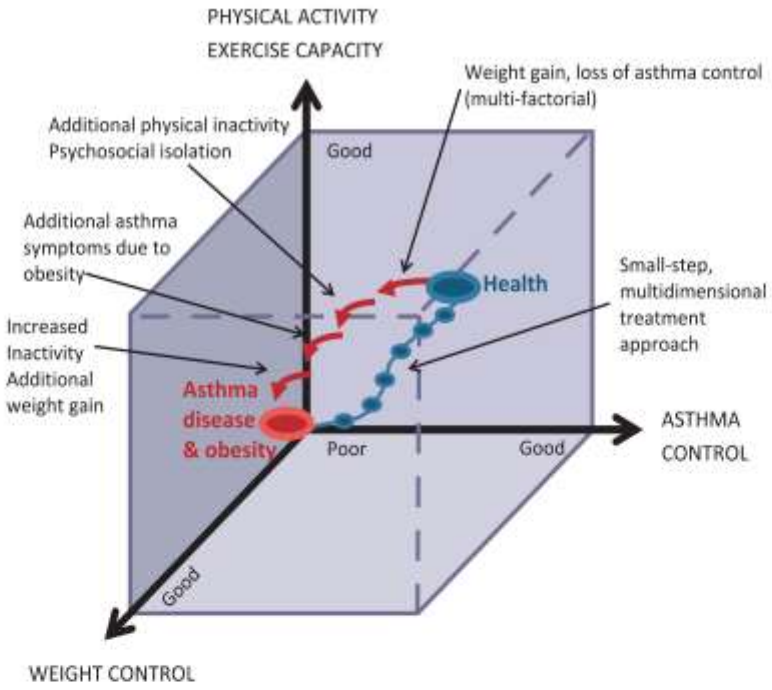

Figure 3 Hypothetical model (of question 2, 4) to illustrate the possible disease interactions between asthma, exercise intolerance and obesity with potential perpetuating or even potentiating effects. These effects may steadily deviate the respiratory system from a healthy state into a disease state with physical inactivity, exercise intolerance and poor asthma control. In analogy to such a systems-based physiological model, a multidimensional treatment approach should again target the parallel improvement of asthma and weight control, exercise training and psychosocial counselling (see also Figure 2).

normal equilibrium states (catch-up growth, early increased weight gain) during vulnerable phases of development (Fig. 1) may provide stimuli for detrimental effects on coupled systems such as lung development and related asthma.

(4) Is it possible that therapeutic interventions are only successful if they target not only individual mechanisms but their interactions and the related disease progression?

In complex networks, the interactions between the network components are very important for the function of the network. Small changes can affect other components or interactions of the network in a highly nonlinear and unpredictable manner. If two disease networks such as asthma and obesity interact, these relationships might be even more complex (99). Based on this hypothesis, we speculate that monitoring of such complex disease processes must be multidimensional (e.g. asthma and weight control, physical activity, quality of life and psychosocial well-being) (Fig. 2). Furthermore, interventions and successful therapy approaches must be multidimensional (inhalation therapy, diet, exercise and counselling) and coordinated, as therapy success is likely to be dependent on these interactions (Fig. 3).

(5) What future research strategies can be proposed to address aspects of complexity in the interaction of asthma and obesity?

Addressing these complex interactions and mechanisms in future research is one of the major challenges of systems biology, systems medicine and personalized health. The key components of systems biology approaches involve the acquisition of large and accurate data sets, model construction, disease simulation, model perturbation, theory formation and prediction, and experimental or clinical validation of theory (112). The acquisition of large and accurate genetic, molecular, cellular, physiological and clinical data sets is often only possible in multicentre research and multidisciplinary consortia. Along with these analyses, epidemiological clustering studies, computer modelling or perturbation studies may facilitate the identification of key components of the system.

Using quantitative, probabilistic approaches, systems biology aims to investigate the spectrum of possible biological effects by, for example, applying high-throughput -omics techniques (e.g. genomics, proteomics, metabolomics). Such data provide information on the complex composition of transcriptional profiles in an unbiased manner. In the field of systems medicine, such approaches are extended to multilevel models (113) including organ physiology or clinical phenotypes, for example, using statistical clustering techniques of larger and well-defined population samples. To achieve this, large patient data sets and clinical data mining strategies are needed, as well as stringent quality control criteria to provide an accurate phenotype of the patients.

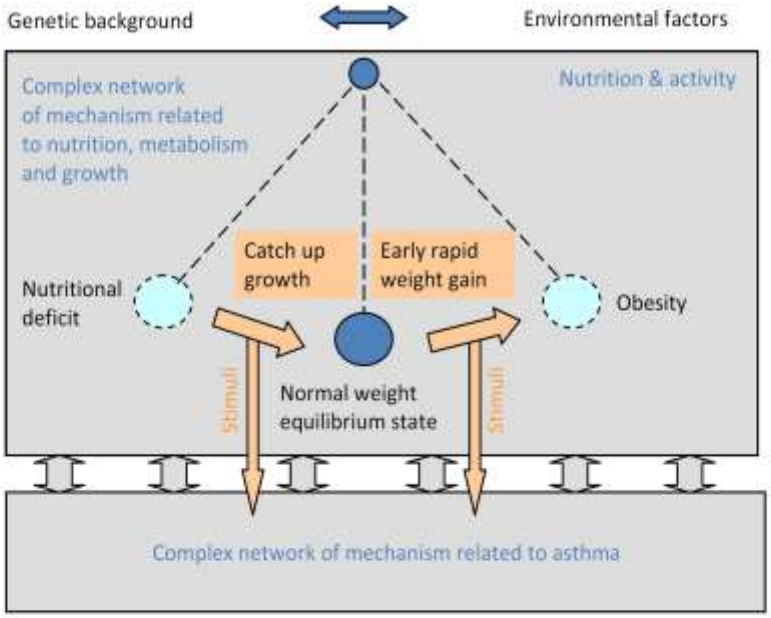

Figure 4 Hypothetical model (of question 3) to illustrate the interaction of a complex network of mechanisms related to metabolism and growth and a complex network of mechanisms related to asthma, assuming weak interactions and coupling between the two networks (grey arrows) based on various mechanisms (immunological, mechanical, hormonal, etc.). Deviation from normal equilibrium states (catch-up growth, early increased weight gain) during vulnerable phases of development may provide strong stimuli also for other coupled complex systems.

Association studies such as these with clinical characteristics are hypothesis generating in nature, but could potentially lead to novel disease models by way of quantitative computer modelling of the involved networks (e.g. gene–gene interactions, cytokine networks). Such computer models could then be used in the simulation and prediction of to simulate disease behaviour, for example, while the system is pertubated by environmental factors. They may also predict how the disease system will evolve over time. Such predictions, however, would then need to be tested and validated by controlled intervention and longitudinal cohort studies. In the field of obesity and asthma, future clinical interventional studies should thus involve detailed -omics data on various levels. As obesity and asthma are two loosely interacting complex disease systems, the situation is even more interesting, as the perturbation of obesity (e.g. dietary measures) can affect the other system (e.g. asthma symptoms).

To better understand how obesity and asthma evolve over time in response to changes in environmental input, longitudinal cohort or time series studies are urgently needed. Ideally, time series analysis studies would investigate fluctuations in various biomarkers and clinical symptoms over time using novel mathematical methods as, for example, summarized in ref. (100, 114). In asthma, such methods facilitate a more accurate description of temporal phenotypes, disease stability, exacerbation risk and treatment response (114). Longitudinal cohort studies optimally use detailed clinical information on obesity and asthma, but should also include -omics information on a functional, cellular, molecular and genetic level. This is particularly true for children, where normal development may be altered by disease.

As both obesity and asthma are strongly dependent on environmental factors, data from the following categories are invaluable: epigenetics (115), human behaviour and social norms, environmental triggers, pollution and global climate change, global food availability and sedentary lifestyle (116). While the computer modelling of complex environmental interaction is challenging, we would like to suggest that future research in environment-dependent complex diseases may profit from models that are currently in use in evolutionary systems biology, in which researchers attempt to understand the evolution of various species (phenotypes) dependent on their interaction with environmental factors (117).

Conclusions

Asthma and obesity are two highly prevalent entities in children in the Western world, sharing similar lifestyle factors, particularly a positive energy balance with physical inactivity and exercise limitations. At this point, the relationship between obesity and asthma is still poorly understood, and no single causal mechanism has been found. It appears much more likely that certain time periods are critical for certain risk factors and their impact on normal growth and development and/or disease progression. What remains unknown is how the multiple mechanisms involved in both diseases interact, whether specific interactions are more important than

others or whether it is the combined effect of multiple interactions that exacerbates these conditions. For this reason, future intervention and longitudinal studies using a systems biology approach are urgently needed. Unfortunately, there is limited evidence in the literature of how to treat the obese asthmatic child. Many experienced clinicians are left with their case-by-case experience and follow an approach of difficult-to-treat or problematic asthma. Here, we have highlighted how little is known in the particular circumstance of asthma in the presence of obesity. Therapeutic evidence of simple successful strategies to not only *treat* but more importantly *prevent* these cases should be the focus of future research.

Author contributions

Urs Frey had overall responsibility for the paper and is the main author, particularly focused on the aspects of complexity and system behaviour. Philipp Latzin was mainly responsible for aspects of reviewing development and growth and epidemiological studies. Jakob Usemann reviewed and contributed to all sections of this article equally. Janet Maccora provided research support and helped with English style. Urs Zumsteg was responsible for the aspects of obesity and endocrinology. Susi Kriemler was responsible for the aspects of exercise and therapy approaches. The literature search and manuscript writing were undertaken through close collaboration between all authors. Due to the complexity of the topic reviewed in this article, the number of authors adds up to a total number of six instead of the five authors recommended in the journals' author guidelines.

Conflicts of interest

The authors declare that they have no conflicts of interest.

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3.2 Methodological challenges of lung function tests

3.2.1 Application of multiple breath wash out

Inert gas washout: background and application in different lung diseases

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Inert gas washout: background and application in different lung diseases

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Keywords: lung clearance index; multiple breath-washout; pediatric, pulmonary function tests, ventilation inhomogeneity.

Abbreviations: AATD: alpha-1-antitrypsin deficiency AHR: hyperresponsiveness BTPS: body temperature, pressure, saturated with water BO: bronchiolitis obliterans CT: computer tomography CDI: convection-dependent inhomogeneity CF: Cystic Fibrosis CFTR: Cystic Fibrosis Transmembrane Conductance Regulator CLD: chronic lung disease COPD: chronic

obstructive pulmonary disease DLCO: diffusion capacity for carbon monoxide CO_2: carbon dioxide FEV_1: forced expiratory volume in one second FVC: forced vital capacity FRC: functional residual capacity He: helium HCT: haematopoietic stem cell transplantation ICS: inhaled corticosteroids ILD: interstitial lung disease LCI: lung clearance index MBW: multiple breath-washout MR: moment ratio MRI: magnet resonance imaging N_2: nitrogen N_2O: nitrogen dioxide O_2: oxygen PCD: primary ciliary dyskinesia RMS: respiratory mass spectrometer Sacin: slope of acinar airways Scond: slope of conducting airways SF_6: sulfur hexafluoride

Abstract

Multiple breath inert gas washout (MBW) is a lung function technique to measure ventilation inhomogeneity in the lungs. The technique was developed more than 60 years ago, but not much used for many decades. Technical improvements, easy protocols, and higher sensitivity compared to standard lung function tests in some disease groups have led to a recent renaissance of MBW.

The lung clearance index (LCI) is a common measure derived from MBW tests, and offers complementary information on lung pathology compared to conventional lung function, such as spirometry. The LCI measures the overall degree of pulmonary ventilation inhomogeneity. Other MBW outcomes, such as slope III derived parameters describe more regional airway ventilation and enable specific information on conductive or acinar ventilation inhomogeneity. How this specific ventilation distribution is exactly related to different disease processes has not entirely been examined yet.

MBW measurements are performed during tidal breathing, making this technique attractive for even young children and infants. These benefits and the additional physiological information on ventilation inhomogeneity early in the course of lung diseases have led to increasing research activities and clinical application of MBW especially in pediatric lung diseases, such as Cystic Fibrosis (CF). In these patients, LCI detects early airway damage and enables the monitoring of disease progression and treatment response. Guidelines for the standardization of the MBW technique were recently published. These guidelines will, hopefully, increase comparability of LCI data obtained in different centers or intervention trials in children and adults.

In this non-systematic review article, we provide an overview of recent developments in MBW. Literature was searched in Pubmed, and in the North American and European clinical trial registries. Search terms were: CF, lung clearance index, lung function test, lung disease, and washout. We specifically focused on literature in children. We first explain the physiological and technical background of this technique with a short explanation of several methodological

aspects. These are important to understand the principle behind the technique and to enable high quality measurements. We then provide examples of MBW application in different lung diseases of children and adults, both with regards to clinical application and research activities. Lastly, we report on ongoing clinical trials using MBW as outcome and give an outlook on possible future developments

1. Introduction

For decades, conventional spirometry has been the standard technique to assess the degree of airway obstruction in most chronic lung diseases, including Cystic Fibrosis (CF), asthma, and chronic lung disease of prematurity (CLD). However, there is mounting evidence that based on its underlying physiological principle, spirometry is insensitive for the assessment of peripheral airway involvement and for the assessment of ventilation distribution. This resulted in an increased interest in gas dilution techniques, in particular multiple breath-washout (MBW), for the assessment of small airway function, i.e. efficient, homogeneous ventilation distribution [1, 2].

MBW was first described more than 60 years ago by Ward S. Fowler [3]. In his pioneering work of 1952, he compared nitrogen (N_2) clearance from single breath washouts between healthy subjects and patients with cardiopulmonary disease, to assess the degree of uneven alveolar gas dilution [3]. However, the technique was little appreciated until gas analyzers and computers were further developed to improve automated analysis of gas and volume signals during measurements [4, 5]. Today, the technique is returning to “prime time”, especially in the pediatric pulmonology community. Recently, an international workshop reviewed current literature on the monitoring of preschool lung disease. Besides detailed recommendations for technical standards and measurement procedures, this report suggested MBW as a promising tool in preschool children with CF, highlighting its importance [6].

2. Physiological background, mechanisms of ventilation inhomogeneity

The main function of the human lung is to homogeneously ventilate the lungs, enabling efficient gas exchange. During fetal lung development, the lungs grow from proximal to distal by a continuous division of the airways, which later form the unique structure of the bronchial tree. The bronchial tree consists on average of 23 bronchial generations, but gas exchange only occurs in approximately the last 9 generations. The bronchial tree resembles a self-similar, so-called fractal structure, enabling efficient gas transport. Normal ventilation distribution occurs by

convection and diffusion. Three main mechanisms of ventilation inhomogeneity are currently known: i) convection-dependent inhomogeneity (CDI) in the conducting airway zone (more proximal located airways); ii) diffusion-limitation related inhomogeneity in the diffusion-dependent airway zone (distal airways, acini); iii) interaction between convection and diffusion in an intermediate zone at the level of the diffusion-convection front, which is thought to arise at the acinar entrance [7]. The acinar compartment, i.e. the alveoli, is separated by a thin tissue layer from a capillary meshwork and forms a large surface for efficient oxygen (O_2) and carbon dioxide (CO_2) gas exchange.

3. Technical background

3.1 MBW testing

Besides analysis devices, MBW tests require only a tight facemask or mouthpiece and quiet tidal breathing for 2-10 minutes per test, making this technique applicable across all age groups, even during infancy. Measurements in infants are done in a supine position during quiet non-rapid eye-movement sleep (or with sedation), using a face mask. In older children and adults, measurements are usually performed in a sitting position with a mouth-piece and nose clip. Differences between sitting and supine position have been described [8] and need to be taken into account when comparing data or in longitudinal studies. To support regular breathing patterns, distraction with videos is recommended in children [9], while visual breathing pattern feedback may be useful in adolescents and adults [10]. Because time for triplicate testing can be demanding in busy outpatient clinics or patients with advanced lung disease, promising, abbreviated protocols were proposed [11, 12].

Each MBW test consists of a wash-in and a wash-out phase. Depending on the gas for the MBW test employed, there are in principle two different ways to perform MBW: 1) When using an inert extrinsic gas (i.e. 4% sulfur hexafluoride (SF_6); 20% helium (He)), the gas mixture is inspired until an equilibrium is reached. Then, the washout phase starts from this point of equilibrium by again breathing room air. 2) For inert intrinsic gas (i.e. N_2), no formal wash-in

phase is required for the first of three tests. For N_2 washout of the airways, 100% oxygen (O_2) is usually used. Regardless of the gas used, the washout is stopped when the test gas reaches $1/40^{th}$ (or 2.5% from the initial starting concentration set to 100%) of the initial gas concentration [7]. This cut-off was recently challenged to improve comparability between different techniques as the role of N_2 is not fully understood yet [13]. Figure 1 shows a typical washout trace of an MBW test using N_2 washout.

3.2 MBW outcomes

There are three main parameters reported from MBW tests: the functional residual capacity (FRC), the lung clearance index (LCI), and moment ratios (MR). The FRC is the volume of air present in the lung after tidal expiration in those open lung regions that are in communication with the mouth. LCI and MR are both measures for global ventilation inhomogeneity. Besides LCI and MR, other parameters assessing specifically peripheral airway ventilation can be calculated, as detailed below.

Given that MBW setups measure inert gas concentrations and the cumulative volume required to washout the resting lung volume (FRC), the latter can be calculated. The FRC is derived from a ratio, i.e. the cumulative expired volume (CEV) of the inert gas over the difference of the end-tidal concentration of the inert gas (Cet) measured at the start (Cet_{start}) and end (Cet_{end}) of the washout. The LCI is a volume ratio, net CEV (including all gas fractions) over FRC: LCI=CEV/FRC. An increased ventilation inhomogeneity would thus result in more tidal breaths (greater net CEV) needed to wash out the inert gas, and in a subsequently increased LCI. To adjust for lung size, net CEV is divided by FRC to obtain LCI.

MR also quantifies ventilation inhomogeneity, but is less commonly used. They have been described in detail elsewhere [4, 14-23]. The advantage of MR over LCI is that they can be weighted to specific parts of the washout curve.

Specific markers for peripheral lung ventilation are the slopes of alveolar phase III (SIII) of the inert gas expirogram. The first SIII value is thought to reflect ventilation inhomogeneity within

diffusion–convection-dependent acinar airways (Sacin), while the subsequent evolution of SIII values from lung turnover 1.5 to 6.0 is thought to reflect ventilation inhomogeneity within convection-dependent conducting airways (Scond) [7, 24]. While these indices were derived from numerical lung models [24], recent comparative data from ventilation imaging techniques are reassuring [25, 26].

3.3 MBW equipment and analysis procedures

One of the earliest and most recognized systems for MBW is based on a respiratory mass spectrometer (RMS), allowing the simultaneous measurement of multiple gases at 33 Hz or higher. Drawbacks of the AMIS 2000 (Innovision, Denmark) relate to the custom design, sophisticated maintenance and costs [27]. Other customized systems have been described in detail elsewhere [7, 28]. Currently, there are at least three commercially available devices which strongly differ i.e. in regards to the inert gas used, the gas analyzer, analysis algorithms, and the age group to which application is recommended (Table 1).

3.4 Gases for MBW testing

Depending on the choice of gas and setup, derived MBW indices significantly differ [29]. For example, He is much lighter than SF_6 and generates systematically higher LCI values [7]. Furthermore, in subjects with emphysematous diseases, the diffusion equilibrium in the enlarged peripheral airways differs across gases. Other aspects regarding the use of SF_6 are its costs and limited availability since it belongs to the most potent greenhouse gases. Applications using lower SF_6 fractions may be more suitable for routine use.

The choice for intrinsic gases (i.e. N_2) for MBW has the advantage that the required O_2 for washout is widely available and affordable. Another strength is that N_2 is resident in all lung units and this gas, therefore, has great sensitivity to detect abnormality compared to extrinsic gases. Further, the wash-in is done using room air and without the need for a tight mouthpiece, making it much easier to apply.

There is clear evidence that breathing patterns may change during MBW. While in adults fixed 1 litre breathing protocols may have some advantages with regards to slope III standardisation and analysis [30], most studies nowadays use free tidal breathing in order to take advantage of natural breathing pattern. This is especially important in children, as fixed breathing protocols have been shown to influence MBW outcome parameters substantially [31]. Application of 100% O_2 in MBW was shown to alter breathing patterns in infants [22], and SF_6 induced transient hypopnea in preterm and healthy infants [32, 33]. However, in school-aged children, MBW indices were not influenced by inhalation of 100% O_2 [34]. In addition, the effect of N_2 back-diffusion from tissue N_2 requires further studies.

3.5 Sample flow and gas analysis

Tidal flow and gases are usually measured within the main path of the respiratory flow (mainstream), or a continuous sample is taken from a capillary (sidestream). Yet sidestream sample flow (suction) may impact upon the analyzer response and add noise to “small” signals i.e. from infants. Flows and integrated volumes have to be further corrected for BTPS (body temperature, pressure, saturated with water) [7]. The gas concentrations can then be measured directly (RMS, infrared, etc.) or indirectly (molar mass, cumulative gas fractions, etc.) [35]. Of note, usually the flow and gas signals are not sampled at the same sensor point. Signals, therefore, need to be aligned in time. Poor BTPS correction or signal misalignment can be a source of error in MBW outcomes [7, 36].

3.6 Impact of dead space on LCI

Dead space roughly consists of two compartments. Technical dead space consists of the volume of MBW hardware (mask, mouthpiece, and tubes) required to transport gases to the sensors. Anatomical and physiological dead spaces refer to the volume of upper and lower airways, respectively, which transport gases but do not participate in gas exchange. Technical dead space is hardware specific and impacts upon LCI [37, 38]. The impact seems larger in younger children

compared to adults, and is apparently independent of lung disease. Thus, a small technical dead space ≤2 ml/kg is recommended [39].

3.7 Software for MBW analysis

For analyses of MBW indices, commercially available online and offline software is available, and several custom-made software applications exist. While offline data analysis was frequently used in the past, it has the disadvantage that analysis is time consuming, and therefore limits its application in clinical settings. Several studies reported an impact on MBW indices due to different software and settings [36, 40, 41]. Current commercial setups usually provide recording and analysis software on-board. Yet these applications undergo constant development and software updates need to be validated in clinical settings for reliability [42-44].

3.8 Normative data for MBW

Depending on the age of subjects and the factors mentioned above (gas, equipment, dead space, software) LCI is usually below 8.5 lung turnovers (LCI units) in healthy subjects. However, normative data for MBW measurements across different age groups are scant [45]. Some data stem from customized setups [46], limiting generalizability. Furthermore, data is not only system, but also gas-specific, and may even be influenced by the analysis approach. The latter has been shown for both, healthy, and CF patients [40, 41]. Two previous studies reported MBW reference values for infants using common available equipment from a large Swiss [45] and African population [47], with measurements conducted at 5 weeks postnatal age. Since LCI is thought to decrease throughout infancy and early childhood, then remains constant and increases in the elderly [46], these reference data cannot be applied to other age groups. There are other relevant factors which impact upon MBW measures including the posture during tests (supine versus seated) [8], the gas choice (SF_6 versus N_2) [37], dead space [38, 48, 49], as well as sedation, which may play a significant role for LCI variability across age.

3.9 Limitations of MBW testing, knowledge gaps

Limitations to MBW application relate to technical and physiological aspects. Much effort has been done to improve standardization of MBW protocols and analysis, but there are still several unanswered questions as mentioned above. Overcoming these knowledge gaps seems difficult, considering the, at times, poor software transparency [43]. Besides software, there are other aspects which may change MBW indices. There is evidence that interventions prior to MBW testing, such as raised volume rapid thoracoabdominal compression [50] or physiotherapy [51-53], subsequently effect MBW outcomes. Other important aspects have been outlined previously [7].

The impact of repeat LCI measurements for the assessment of respiratory disease outcomes is largely unknown. While data suggest a clear association with infection burden, structural airway pathology or later pulmonary exacerbation in CF, it remains unclear what change in LCI should prompt clinicians to intervene. As true for most lung function outcome parameters, the beneficial effect of regularly measurement of LCI in clinics on disease outcome has not been assessed yet. Recent data may help to establish what would constitute a clinically important change in LCI, at least in preschool children [54].

3.10 Single-breath washout

There are techniques other than MBW to assess ventilation inhomogeneity, i.e. single-breath washout (SBW) tests, using a single or double inert tracer gas mixture. Several studies have used this technique, also in younger children. SBW has been used in CF patients to detect early lung disease [55], assess response to airway clearance [56], and to study the involvement of small airways in patients with mild asthma [57], COPD [58-61], PCD [62, 63] and bronchiolitis obliterans (BO) [64-66]. SBW may be attractive for clinical settings, since measurements can be completed more quickly than for MBW, and SBW is applicable during normal tidal breathing or forced maneuvers. While acceptable reproducibility of this test has been reported in adults [60] and children [55], reproducibility is lower compared to MBW. Several unanswered technical

aspects also remain (i.e. impact of breathing pattern) [7, 67, 68], precluding its use for clinical decision making.

4 Application of MBW in lung diseases

4.1 Cystic Fibrosis

CF is an inherited life-limiting disease with a mean prevalence of approximately 0.8/10.000 in Europe and the United States [69]. In Switzerland, CF newborn screening, introduced in 2011, enables early CF diagnosis and follow-up of lung function [44]. CF lung disease is characterized by mucus plugging, chronic infection and inflammation resulting in irreversible lung damage. Treatment advances have resulted in the preservation of normal forced expiratory volume in one second (FEV_1) (>-1.64 z-scores) into young adulthood, but progression of bronchiectasis may be undetected by spirometry [70]. This led to more research to detect early airway abnormalities in CF patients by MBW, and several observational studies and clinical trials support its usefulness, as detailed below.

4.1.1 Observational studies

The majority of longitudinal data from infants and children with CF are currently obtained from two large prospective cohorts, the Australian Respiratory Early Surveillance Team for Cystic Fibrosis (AREST-CF), and the London Cystic Fibrosis Collaboration (LCFC), (reviewed in [71]). There is mounting evidence that compared to healthy controls, LCI is already abnormal shortly after birth in patients with CF [1, 72, 73]. In infancy, LCI is normal in approximately half of the infants. Interestingly, there are infants with normal LCI values in the presence of abnormal forced volume values (as assessed by raised volume rapid thoracoabdominal compression technique) [74]. Several observational studies “tracked” LCI from preschool age to school age [75, 76]. A multi-center study assessed LCI at several time points and was able to identify significant deterioration of LCI in CF over time, which was not detected by spirometry [54]. Another study found that LCI during preschool years was more likely to be abnormal than

spirometry, and an abnormal LCI in preschool children predicted both, abnormal LCI, and abnormal spirometry at school age [75].

Studies in infants [77], children [74, 78], and adults [79] could show that patients with evidence of a bacterial infection were more likely to have abnormal LCI values. Findings from the longitudinal study AREST-CF, suggested that LCI has a more pronounced increase in infants with airway infections compared to those without, and that this increase was long lasting [80]. Several studies used MBW to monitor treatment response to antibiotic therapy in infants and adult patients with CF [81-83]. A systematic review included data from 176 exacerbations and observed an overall decrease, albeit small (~ -3%), in LCI after antibiotic treatment. However, the LCI response to therapy was very heterogeneous in CF patients, and is not fully understood [81].

4.1.2 Clinical trials

There is an ongoing debate about whether or not MBW can be used in multicenter trials. The Cystic Fibrosis Foundation Workshop Report from 2015 concluded that, for example, lack of knowledge on MBW equipment hampers routine application of MBW in clinical trials [6]. On the other hand, the European CF Society Clinical Trial Network Standardization Committee [84] suggested LCI as an outcome measure, especially in young children and those with mild CF disease.

To date, several single- and multicenter trials with CF patients using LCI as outcome have been published. Two multicenter interventional studies investigated the treatment effect of drugs modifying the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) defect in CF patients. One study enrolled patients >6 years, with at least one copy of the rare G551D mutation and normal FEV_1, and assessed the ability of LCI to detect a treatment effect of ivacaftor. Treatment with ivacaftor resulted in significant improvement of LCI compared to placebo [85]. Another study enrolled patients 6-11 years, homozygous for F508del-CFTR, to assess the treatment effect of combined therapy with ivacaftor and lumacaftor on LCI. While no changes in

FEV_1 were observed after 24 weeks of intervention, there was significant improvement in LCI [86].

Two trials examined the usefulness of LCI in infants and preschool children to assess treatment response to hypertonic saline inhalation [87, 88]. One study performed MBW measurements before and after treatment with hypertonic saline (twice daily for 48 weeks) in 25 children <6 years. LCI decreased (improved) significantly more in the hypertonic saline group, compared to controls [87]. Of note, the pattern of LCI change with treatment was age-dependent: when specifically analyzing a subgroup of subjects <1 year of age, LCI was in the normal range at baseline and did not change after treatment [87]. A recent study in 18 older children (mean age 14.0 years) investigated the short term effect of hypertonic saline inhalation [88], and found that LCI did not change after 24 hours treatment [88].

Responsiveness of LCI was assessed in two randomized, double-blind, placebo-controlled trials in older children. While FEV_1 was not systematically affected, LCI improved over a 1-month period after treatment with hypertonic saline [89], and with dornase alfa (Pulmozyme®), an enzymatic agent improving mucus clearance in CF [90].

A cross-sectional study assessed the effect of antibiotic therapy on LCI abnormalities and magnet resonance imaging (MRI) in clinically stable patients, aged 1-20 years. LCI and MRI were sensitive to detect an effect of antibiotic treatment for pulmonary exacerbations [82], indicating that these tools are useful endpoints in intervention trials.

4.1.3 MBW indices and lung imaging

Several recent studies in children and infants compared the association of LCI with structural airway damage or functional correlates using lung imaging techniques, including computer tomography (CT) and MRI. While studies in infants reported poor association between LCI and i.e. bronchiectasis as assessed by CT [91, 92], a closer correlation was found in preschool- and school-aged children [92]. This is not the case for spirometry indices. A strong correlation between LCI and MRI imaging was reported in clinically stable CF patients (age range 2-20

years) [82]. This study could further show that LCI and MRI are sensitive to detect disease severity levels, supporting the application of these tools for diagnostic and therapeutic monitoring. Taken together, these data support the concept of LCI as a sensitive measure of structural airway pathology. However, LCI cannot replace lung imaging yet, as negative predictive values to exclude bronchiectasis appear too low, especially in younger children. An overview of MBW application in CF lung disease is given in table 2.

4.2 Wheezing

Lower respiratory tract infections, or other triggers, such as allergen exposure leading to wheezing episodes in preschool children, are highly prevalent. Some, but not all wheezers experience worsening lung function [93] and involvement of the small airways, as assessed by MBW and lung biopsy [94]. Functional data using MBW are conflicting. One study measured LCI in 110 preterm infants who required respiratory support during the first days of life. Measurements were performed with sedation, at approximately 42 weeks postmenstrual age. The authors found a higher LCI in infants with less (≤3%), compared to infants with more (>3%) expiratory wheezing [95]. These results were supported in a subsequent study reporting a higher LCI in 40 non-wheezing infants, compared to 41 infants with wheezing [38]. These findings contradict a study in preschool children, which reported an increased LCI in wheezers compared to healthy controls [94], but mean LCI differences were small (6.8 versus 6.6.), and within the normal range. Apparently, the effect of bronchodilator inhalation on LCI is somewhat paradoxical. LCI may increase (worsen) after inhaling salbutamol in controls, but not in wheezing subjects [96]. High variability between MBW tests at baseline, and heterogeneous response to bronchodilatators questions the use of MBW to distinguish preschool wheezers from healthy controls [96]. To summarize, current data does not support application of MBW measurements in infants and children to characterize wheezing or to monitor treatment response.

4.3 Asthma

Asthma is the most common chronic respiratory disease in children, typically characterized by eosinophilic airway inflammation, airway hyperresponsiveness (AHR), and reversible airway obstruction. AHR is the ability of airways to narrow excessively due to provoking stimuli. While current guidelines recommend symptom assessment and spirometry for the diagnosis of asthma [97], the value for monitoring asthma is not clear [98]. Spirometry was rather insensitive for peripheral airways, unless severe obstruction was present [99]. LCI in asthmatic subjects was slightly elevated compared to healthy controls, but often within normal range, and therefore not helpful to diagnose asthma at the individual level [100-102]. Few studies assessed the association between MBW measures and AHR. A study in older asthmatic subjects (age range 59-80) found that AHR was predicted by higher Sacin [103], and a study in asthmatic adults (age range 18-66 years) found that AHR was associated with LCI, and that treatment with inhaled corticosteroids (ICS) decreased the LCI [104]. In adults with severe asthma, ICS uptitration resulted in a decrease in Scond with no effect on Sacin [105]. The study further reported that those patients with increased ventilation inhomogeneity at baseline had the greatest improvement following ICS dose uptitration [105]. An effect of bronchodilator response on LCI is still poorly understood. While two studies in children found no effect [100, 102], another study reported that the LCI was elevated in adolescents with asthma, and decreased after treatment with nebulized bronchodilatator [106]. To summarize, while MBW can be useful to explore the exact degree of airway damage and physiological impairment in asthma, until now, the technique is not established for diagnosis or monitoring treatment response in asthma.

4.4 Chronic lung diseases of prematurity

Disruption of normal lung development due to preterm birth results in complex structural changes of airways, lung parenchyma, and vasculature. This may result in smaller airway calibres, and impaired alveolarization, along with a reduced number of alveoli and enlarged airspaces [107].

The functional relevance of disrupted airway development during infancy and thereafter is not fully understood. This can be partly explained since the degree of airway obstruction is underestimated by spirometry. Recent studies in former preterm infants used other outcomes including the LCI [108-113], FRC [110, 113, 114], and parameters specifically assessing peripheral airway damage (Scond, Sacin) [109]. Results are inconsistent, probably reflecting the heterogeneity of the disease and different measurement techniques. Abnormal values for LCI have been reported in preterm infants during infancy [110], and for former preterm infants at school age [112]. Other studies reported no difference in LCI values in former preterm infants compared to healthy subjects during early infancy [113], at 1 year [114], between 9-11 [115], and 7-13 years [109] of age. The latter study further investigated parameters specifically assessing peripheral airway ventilation. In that study, FEV_1 and Scond were often abnormal at school age, while Sacin was comparable between former preterm and healthy subjects. A functionally normal alveolar compartment at school age, together with functionally abnormal central conducting airways suggests a disynaptic growth pattern of the lungs in former prematurely born children [109]. Using MBW measurements, studies assessed the development of lung volume. Prematurity was associated with a reduced FRC during early infancy [110] and at 1 year of age [114].

4.5 Primary ciliary dyskinesia

Primary ciliary dyskinesia (PCD) is a rare congenital disease characterized by defective ciliary function, leading to impaired mucociliary clearance and recurrent chronic upper and lower airway infections [116]. The diagnosis of PCD is challenging due to the heterogeneous nature of the disease and poorly standardized algorithms [116]. Patients with PCD may already have reduced lung function (i.e. FEV_1, and forced vital capacity (FVC)) at preschool age [117]. Similar to CF, spirometry may underestimate the full degree of functional impairment in early PCD lung disease [118, 119]. Recent studies reported marked peripheral airway dysfunction, assessed by MBW, in almost all patients with PCD [62, 63, 120]. Another study compared the

association between LCI and FEV_1 with structural abnormalities, as assessed by CT, in 38 patients. The authors reported a higher concordance between LCI and structural abnormalities compared to FEV_1 (83% versus 53%), suggesting that LCI measurements may be of clinical relevance in PCD patients [119]. Interestingly, studies using SF_6 MBW did not find this association [121]. Taken together, PCD lung disease is characterized by marked peripheral airway dysfunction, and MBW may be a promising tool for early detection of airway damage. Compared to CF, MBW data in PCD are scarce. Whether MBW has utility in the management and/or treatment of PCD still needs to be determined in longitudinal studies.

4.6 Chronic obstructive pulmonary disease

Chronic obstructive pulmonary disease (COPD) is characterized by a variable combination of airway and parenchymal abnormalities. COPD may partly develop irrespective of tobacco smoking, and much earlier in life than previously assumed [122, 123]. The hallmark of COPD is mainly irreversible airflow obstruction resulting from the narrowing of small conducting airways, loss of lung elastic recoil, or both. In the diagnosis of airflow obstruction in COPD, simple spirometry is the main diagnostic tool, although it is recognized that none of the derived parameters are sensitive to peripheral airway narrowing [124, 125].

Within a prospective study of subjects who smoked ≥10-pack years, an increase in ventilation inhomogeneity in both, the conductive and acinar airways was detected, indicated by increased Scond and Sacin values. Moreover, Scond and Sacin were associated with FEV_1/FVC values, but Scond was associated with FEV_1 and Sacin to diffusion capacity for carbon monoxide (DLCO) [126]. Similar results were reported from a cross-sectional study of 57 COPD patients with ≥15-pack years. COPD patients had increased Sacin but not Scond values compared to healthy controls [127], and an association between Sacin and DLCO was also reported [60]. Taken together, these studies suggest that MBW measurements have the potential to diagnose subclinical disease at an earlier state, separate airway from parenchymal abnormalities, and to further characterize clinical features of advanced COPD. While good feasibility and

reproducibility of washout measurements in COPD patients were shown [60], there is still further need to validate this method for non-invasive detection of early abnormalities or peripheral or parenchymal sites [128].

4.7 Other disease groups

In addition to the application of MBW in patients with CF and other common lung diseases, recent studies have applied this technique in less frequent lung disease, of which some studies will be reported.

BO is a small airway disease in which chronic inflammation results in fibrotic remodeling of the peripheral airways. It can occur e.g. after exposure to toxic substances, or following allogeneic haematopoietic stem cell transplantation (HCT). Early detection of BO is important to prevent later lung damage. One study reported abnormal washout indices before a decrease in FEV_1 was observed [129], and measurements may even be used to detect different severity grades of BO, as recently reported [130]. In patients undergoing HCT, a study of 33 clinically stable recipients provided some evidence that measures of MBW were more sensitive than spirometry to detect BO at an early stage [131].

Alpha-1-antitrypsin deficiency (AATD) is a genetically inherited disorder resulting in emphysematous lung changes. Within a heterogeneous group of 193 patients with AATD (age range 4-79 years), LCI was higher in patients compared to controls. Furthermore, LCI was found to be abnormal in patients with normal spirometry measures, indicating that LCI identifies AATD related lung disease earlier than spirometry, comparable to the chronic lung diseases discussed earlier in this article [132].

Bronchiolitis is a common respiratory tract infection during early childhood. A recent study included 29 infants (mean age 3.7 months) with bronchiolitis and reported an increased LCI compared to controls [133]. Another prospective study enrolled infants hospitalized with bronchiolitis due to respiratory syncytial virus (RSV) infection below <1 year of age, and reported no difference in LCI, but FEV_1, between patients and controls at the age of 18 [134].

4.8 MBW in ongoing clinical trials

Currently, there are more than 50 ongoing studies using MBW indices as outcome measures listed in the North American [135] and European [136] clinical trials registries. While most of these trials are performed in patients with CF and asthma, some are performed in less common lung diseases. We present a selection of trials we consider interesting, though it is not a comprehensive overview.

The first trial using LCI as primary outcome in CF patients (aged 6-11 years) homozygous for the F508del mutation after treatment with combined therapy of lumacaftor/ivacaftor (Orkambi®) twice daily over 24 weeks is still ongoing [137]. The study assesses efficacy and safety of the drug, as well as treatment effects on LCI. Two further trials in children are investigating the treatment effect of 8 weeks ivacaftor. One trial is already ongoing, aiming to recruit 50 children 3-5 years with different CFTR mutations to assess changes in LCI[138], and another trial will study the efficacy of ivacaftor in subjects >6 years old, focusing on a specific CFTR mutation [139].

One trial is being conducted in PCD patients within the BESTCILIA (Better Experimental Screening and Treatment for primary CILIAry dyskinesia) study. This is a European multicenter, double-blind, randomized, placebo-controlled trial with the aim to determine the efficacy of azithromycin maintenance therapy for 6 months on respiratory exacerbations in patients with PCD. This interventional trial is currently ongoing and aims to recruit 125 PCD patients, aged 7-50 years old. Besides the quality of life assessment, one of the main outcomes is the LCI [140]. This trial will hopefully help elucidate whether or not maintenance therapy with azithromycin has beneficial effects in patients with PCD.

Several trials assess treatment response to inhaled glucocorticoids, betamimetics or hypertonic saline in adult [141] and pediatric patients with asthma [142] and CF [143]. For example, in patients with CF aged 6-18 years of age, a randomized-controlled trial is assessing the short term effects (within 24 hours post-dose) of hypertonic saline inhalation on LCI [143].

There is an observational, prospective study recruiting children with CF and asthma, but interestingly, also children with BO and sickle cell anemia [144]. This trial will validate the MBW device for different lung diseases in Canada, where it is not currently licensed yet. This study may also provide insights for the application of MBW in pulmonary complications in children with sickle cell disease, which has not yet been investigated.

Little is currently known of the involvement of small airways in patients with interstitial lung disease (ILD). One trial currently underway will perform MBW in adult patients with different manifestations of ILD (i.e. Wegner's Disease, idiopathic pulmonary fibrosis, sarcoidosis), to further understand the complex pathology and heterogeneous presentation of these diseases [145].

5 Conclusion

MBW has regained interest in recent years. Reasons for this renaissance relate to technical and physiological considerations. The technique is sensitive for early detection of lung function impairment often arising in small peripheral airways. MBW provides information that cannot currently be obtained by other lung function tests or chest imaging. MBW is especially attractive for young patients, as measurements require minimal cooperation. There is mounting evidence that the LCI is useful for assessing the extent and progression of lung disease as well as treatment response in patients with CF. LCI is already broadly applied in clinical care for CF patients, yet a number of unresolved questions remain. For example, there are currently poorly defined upper limits of normal for LCI values and its natural variability over time. Further, different software settings and device-setups may impact upon LCI values. Application of MBW tests in other chronic lung diseases may be attractive for research while the impact on clinical management and respiratory disease outcomes require further studies.

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Figure

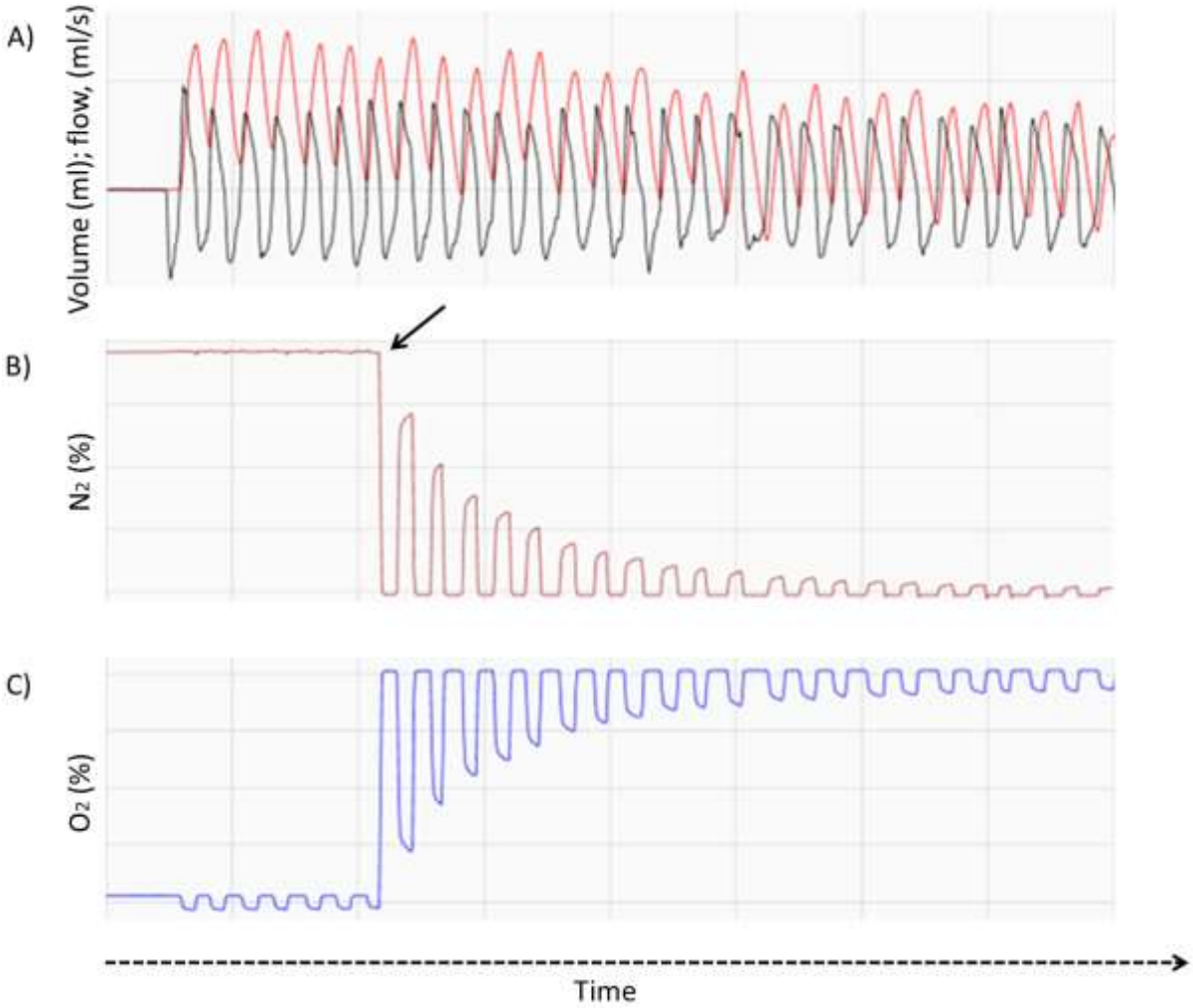

Figure 1 Example of a typical multiple-breath washout (MBW) trace. A) Black line represents volume; red line represents flow signal. B) Nitrogen (N_2) and C) O_2 signal from an N_2 MBW test. Stable breathing pattern can be observed before washout is started (black, continues arrow), and during the time series display of MBW (black, dotted arrow).

Tables

Table 1 Current available multiple-breath washout devices

	Eco Medics infants	**Eco Medics preschool and older**	**ndd EasyOne Pro LAB***	**Innovision Innocor**
Method	SF_6	N_2	N_2	SF_6
Flow / Volume measurement	Ultrasonic flowmeter	Ultrasonic flowmeter	Ultrasonic flowmeter	Pneumotachometer
Tracer gas measurement	Indirect via molar mass mainstream	Indirect via O_2 and CO_2 measurement	Indirect via molar mass, O_2 and CO_2 measurement	Direct via photoacoustic spectroscopy, gas reservoir bag
Gas concentration	4% SF_6	100% O_2	100% O_2	SF6 mixture (0.1% or 0.2% SF6 (27.6% O2, 0.35% N2O))
Validation studies	Lung model Schmidt [146], Gustafsson [147]	Lung model and in vivo Singer [35]	-	In vivo Horsley [10] Lung model and in vivo Gonem [148]
Methodological studies	Anagnostopoulou [40], Latzin [48]	Jensen [37], Summermatter [36]	-	Horsley [149], Downing, [150], Grønbæk [151], Gonem [25], Gonem [148], Nielsen [152], Shawcross [153]
Application in healthy	Fuchs [45], Anagnostopoulou [40], Gray [47] Gray [114]			Downing [150]
Studies in CF	Belessis [77], Simpson [80], Hall [91].	Stanojevic [54], Stahl [82], Amin [88], Ramsey [92]		Davies [85]
CF and controls		Singer [78], Poncin [29]	Poncin [29]	Downing [150]
Other disease groups	Hulskamp [110], Latzin [113]	Yammine [109], Boon [119], Nyilas [118], Madsen [62], Jarenback [127]	Fuchs [132]	Macleod [100]

This table is meant to provide a current overview on available setups and does not necessarily represent the full body of existing literature. Please refer to the respective manufacturer for an update on current studies and recent developments. Manufacture of AMIS 2000 (Innovision) has been discontinued (http://www.innovision.dk/Products/AMIS_2000.aspx, accessed on 1.3.2017). * Before 2012, ndd EasyOne Pro was in use for multiple-breath washout measurement using 4% SF_6 (MM sidestream) (Fuchs [154], Fuchs [155], Ellemunter [156], Fuchs [132]). CF: Cystic Fibrosis: CO_2: carbon dioxide N_2: nitrogen N_2O: nitrogen dioxide O_2: oxygen SF_6: sulfur hexafluoride.

Table 2 Overview of studies in pediatric patients with Cystic Fibrosis using multiple-breath washout measurements					
Study description	**Patient number**	**Age at study**	**Study type**	**Main findings**	**Reference** *
CF versus controls					
Infants	71	3 months	cross-sectional	Higher LCI and FRC in infants with CF compared to 71 controls.	Hoo [74]
Preschool/School age/Adults	40	2.5-5 years	cross-sectional	Higher LCI in patients with CF compared to 37 controls.	Aurora [1]
	48	4.09 (0.7) and 7.83 (1.3) years	prospective	Higher LCI in CF patients at preschool age compared to 45 controls. Preschool LCI predicted abnormal LCI in CF patients at school age.	Aurora [75]
	78	2.5-6 years	prospective	LCI measurements in CF patients and 70 controls at 1, 3, 6 and 12 months detected lung function deterioration over time in CF patients.	Stanojevic [54]
	60	7.8 (1.3) years	cross-sectional	Higher LCI in CF patients versus 60 controls.	Owens [73]
	43	3-18 years	cross-sectional	Higher LCI and MR in CF patients versus 28 controls.	Gustafsson [72]
	47	9.5-16.1 years	cross-sectional	LCI was higher in CF patients compared to 50 controls. Comparison of LCI measured by two different MBW devices revealed poor agreement between different setups.	Poncin [29]
CF with versus without bacterial infection					
	47	0.32-3.24 years	cross-sectional	LCI of 25 healthy subjects was lower compared to CF patients without, and with bacterial infection. In CF patients, inflammatory markers from BAL correlated with LCI.	Belessis [77]
	78	4-16 years	prospective	LCI was higher in CF patients compared to 53 controls. LCI strongly correlated with *Pseudomonas aeruginosa* infection.	Singer [78]
	108	0.1-2.5 years	prospective	LCI measurements at three time points during 2 years revealed a long-lasting increase in LCI after pulmonary infections. *Haemophilus influenzae* infections had in particularly detrimental effects on lung function.	Simpson [80]

	41	8-67 years	cross-sectional	LCI was higher in CF patients compared to 6 controls. A lower colony count of aerobic/anaerobic bacteria was associated with a higher LCI in CF patients.	O`Neill [79]
Assessment of treatment					
Antibiotic treatment for pulmonary exacerbations			systematic review of studies published until March 2014	Overall, LCI decreased after antibiotic treatment but individual response was heterogeneous in CF patients.	Sonneveld [81]
	26	1.8-19.9 years	prospective	LCI was sensitive to detect response to antibiotic therapy for pulmonary exacerbations.	Stahl [82]
Hypertonic saline (7%)	25	1-5 years	observational	Hypertonic saline twice daily for 48 weeks improved LCI.	Subbarao [87]
	18	7-56 years	observational	Hypertonic saline 5 times over 24 hours did not improve LCI.	Amin [88]
	19	10.5 (3.1) years	observational	Hypertonic saline twice daily over 4 weeks improved LCI.	Amin [89]
CFTR regulator Ivacaftor	21	8-43 years	clinical trial	Ivacaftor 150 mg twice daily for 28 days improved LCI.	Davies [85]
CFTR regulators Lumacaftor/ivacaftor combinded	58	6-11 years	clinical trial	Lumacaftor 200 mg/ ivacaftor 250 mg twice daily for 24 weeks improved LCI, sweat chloride and nutritional status.	Milla [86]
Dornase alpha (Pulmozyme®)	17	6-18 years	clinical trial	For weeks 2.5 ml daily Pulmozyme® inhalation improved LCI.	Amin [90]
Lung imaging and MBW					
Magnet resonance imaging	97	0.2-21.1 years	prospective, cross-sectional	LCI correlated with abnormalities in MRI in infants, toddlers and children with CF.	Stahl [82]
Computer tomography	60	7.8 (1.3) years	cross-sectional	Abnormal findings on lung CT correlated strongly with increased LCI in CF patients.	Owens [73]
	49	8.7-112.1 weeks	cross-sectional	Air trapping and bronchiectasis assessed by CT were associated with MR, but not LCI.	Hall [91]

	42, 38, and 39	0-2, 3-6, and 7-16 years	cross-sectional	Agreement between LCI and bronchiectasis in preschool and school age children, but not infants. Air trapping and LCI was only associated in infants.	Ramsey [92]

This table is meant to provide a current overview on studies in pediatric patients with CF and does not necessarily represent the full body of existing literature. *At the time of publication. BAL: bronchoalveolar lavage CF: Cystic Fibrosis. CFTR: Cystic Fibrosis Transmembrane Conductance Regulator LCI: lung clearance index MR: moment ratio. Age at study is given in range or means (standard deviation).

3.2.2 Quality assessment of multiple breath washout

Multiple breath washout analysis in infants: quality assessment and recommendations for improvement

Anagnostopoulou P, Egger B, Lura M, **Usemann J**, Schmidt A, Gorlanova O, Korten I, Roos M, Frey U, Latzin P. Multiple breath washout analysis in infants: quality assessment and recommendations for improvement.

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Fast Track Communication

Multiple breath washout analysis in infants: quality assessment and recommendations for improvement

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Abstract

Infant multiple breath washout (MBW) testing serves as a primary outcome in clinical studies. However, it is still unknown whether current software algorithms allow between-centre comparisons. In this study of healthy infants, we quantified MBW measurement errors and tried to improve data quality by simply changing software settings. We analyzed best quality MBW measurements performed with an ultrasonic flowmeter in 24 infants from two centres in Switzerland with the current software settings. To challenge the robustness of these settings, we also used alternative analysis approaches. Using the current analysis software, the coefficient of variation (CV) for functional residual capacity (FRC) differed significantly between centres (mean ± SD (%): 9.8 ± 5.6 and 5.8 ± 2.9, respectively, $p = 0.039$). In addition, FRC values calculated during the washout differed between −25 and +30% from those of the washin of the same tracing. Results were mainly influenced by analysis settings and temperature recordings. Changing few algorithms resulted in significantly more robust analysis. Non-systematic inter-centre differences can be reduced by using correctly recorded environmental data and simple changes in the software algorithms. We provide implications that greatly improve infant MBW outcomes' quality and can be applied when multicentre trials are conducted.

[4] P A and B E contributed equally to this work.

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Keywords: infant lung function, multiple breath washout, ultrasonic flowmeter

S Online supplementary data available from stacks.iop.org/PM/37/L1/mmedia

(Some figures may appear in colour only in the online journal)

1. Introduction

Infant lung function (ILF) testing is increasingly used nowadays (Davis *et al* 2010, Rosenfeld *et al* 2013, Peterson-Carmichael *et al* 2014) even as an outcome measure in interventional trials (Ramsey *et al* 2012, Stocks *et al* 2012, Subbarao *et al* 2013), thanks to the evolution of the techniques and to commercially available devices.

One commonly used ILF technique is the multiple breath washout (MBW) test that evaluates ventilation distribution in small airways (Robinson *et al* 2013, Vogt *et al* 2014, Subbarao *et al* 2015). It has been already established as a primary endpoint in single-centre clinical studies for infants with cystic fibrosis (CF) (Subbarao *et al* 2013) (Hall *et al* 2011, Kieninger *et al* 2011, Belessis *et al* 2012, Stahl *et al* 2014) and it is now included in multicentre clinical trials (Stick *et al* 2013) (https://clinicaltrials.gov/ identifier: NCT02270476 and NCT01619657). This raises the necessity to obtain comparable outcomes between centres.

To our knowledge, the only multicentre study measuring infant MBW with the ultrasonic flowmeter (Exhalyzer D, Eco Medics AG, Duernten, Switzerland) using sulphur hexafluoride (SF_6) as tracer gas and the WBreath analysis software (ndd Medizintechnik AG, Zürich, Switzerland) was published by Hulskamp *et al* (2009). This is the only commercially available equipment for this kind of measurements, and, thus, the most commonly used (Latzin *et al* 2007, Fuchs *et al* 2011, Hall *et al* 2011, Belessis *et al* 2012, Stahl *et al* 2014, Gray *et al* 2015a). The authors reported large inter-centre differences in the functional residual capacity (FRC) of healthy infants between centres. This observation was unexpected, as the same equipment, software and protocol were used in all centres. The findings were attributed to undetectable between-centre hardware/software differences. If unknown and, more importantly, undetectable factors influence MBW outcomes, it is questionable whether multicentre trials can be performed using this software.

The first aim of this study was to quantify the magnitude of MBW measurement errors using the above-mentioned hardware and software. To address this, we used MBW measurements of healthy infants from two centres recorded from identical devices and following the same protocol. The second aim was to strengthen the quality of MBW outcomes by changing software settings.

2. Materials and methods

2.1. Study design and subjects

This is a retrospective software validation study using data from MBW measurements of healthy term-born infants from the BILD cohort study measured at two centres in Switzerland (Basel and Bern) (Fuchs *et al* 2012) between 1 January 2012 and 31 December 2013. The Ethics Committees of Basel and Bern approved the study and parents gave written consent.

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2.2. MBW measurements

MBW measurements were performed in infants according to current ERS/ATS standards (Robinson *et al* 2013), as previously reported (Latzin *et al* 2007). In brief, unsedated infants were measured while breathing through an infant facemask (Homedica AG, Baar, Switzerland), during natural sleep, in a supine position with the head midline through an ultrasonic flowmeter (Exhalyzer D, Eco Medics AG, Duernten, Switzerland) using 4% SF_6, as previously described (Wauer *et al* 2003, Latzin *et al* 2007). Both centres used identical devices, the same patient interface and followed exactly the same protocol for the calibration and the performance of the measurements. The only part of the equipment that differed slightly between the centres was the deadspace reducer, which is mentioned below in details.

We used three best quality measurements per child during one non-REM sleeping period that is, without awakening between trials. Two investigators (BE and PA) performed the quality control and all analyses, independent of the centre. All analysis steps were double-checked by the other investigator to avoid observer-dependent bias.

2.3. Quantitative parameters for quality control of the analysis

The main MBW outcomes, FRC at mid-sensor point and lung clearance index (LCI) (Robinson *et al* 2013) calculated from the washout, are reported in this study. To evaluate the quality of the analysis, we used the difference in FRC and LCI between washout and washin (Diff-$FRC_{(wo\text{-}wi)}$ and Diff-$LCI_{(wo\text{-}wi)}$, respectively) for each trial, and the coefficient of variation of washout results (CV of FRC and CV of LCI) for the three trials per subject. As all measurements were performed within one sleeping period, the lower those values were, the more accurate the analysis.

2.4. Analysis

2.4.1. Standard analysis. The analysis of the measurements was performed using the software provided by the manufacturer (WBreath Version 3.28.0.0, ndd Medizintechnik AG), if nothing else is reported. More details about the signal processing and other analysis parameters used are reported in the supplementary data. The initial standard analysis was based upon the following settings, as recommended by the manufacturer: use of the original temperature in files, deactivation of the expiratory molar mass (MM) fit option (figure S1(a)) (stacks.iop.org/PM/37/L1/mmedia), and use of 65–95% end expiration (EE) start and stop percentile (figure S1(b)) (stacks.iop.org/PM/37/L1/mmedia) (Latzin *et al* 2007). The deadspace subtraction was performed in the same way at both centres, as previously reported (Latzin *et al* 2007).

2.4.2. Alternative analysis settings

2.4.2.1. Analysis by changing the 'FRC-analysis' parameters. To evaluate the impact of the commonly used analysis settings on MBW outcomes, we repeated the analysis using each time one of the following changes, while the rest of the analysis was performed as before: without temperature simulation; without MM step response correction, with MMFit, EE start and stop percentile set at 60–90%, 55–85%, and 50–80%, respectively (for details see supplementary data and figure S1) (stacks.iop.org/PM/37/L1/mmedia).

2.4.2.2. 'Room-' and 'case-temperature' refinement. 'Room-' and 'case-temperature' values are measured in different compartments of the device (see supplementary data and

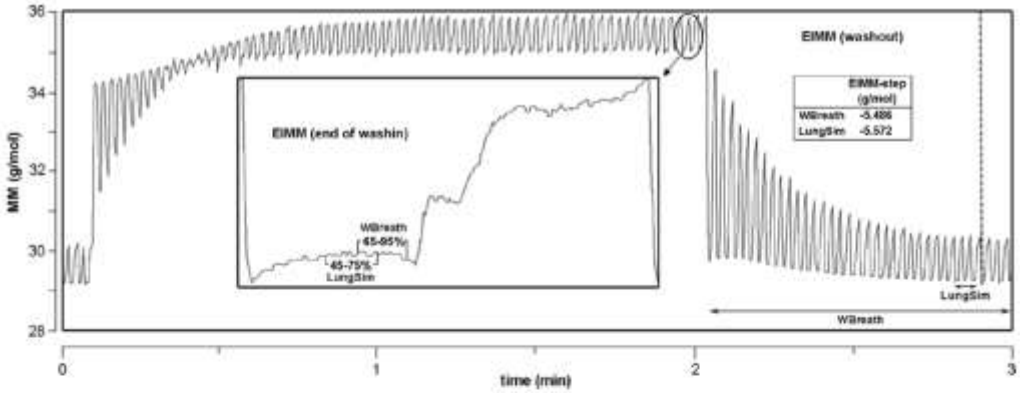

Figure 1. Differences in end-inspiratory molar mass (EIMM) calculations between WBreath and LungSim. Original molar mass (MM) tracing of a SF_6-MBW measurement from a healthy infant. The dashed line indicates the washout breath where the 2.5% end tracer gas concentration is reached. The enlarged part shows the MM signal during a single breath at the end of washin. The main differences between the software are the following: (i) for the EIMM calculation in both washin and washout LungSim uses the 45–75% part of inspiration which is more stable than the 65–95% used by WBreath. (ii) To determine the mean EIMM of the washout, and thus the EIMM-step, LungSim uses the last three inspirations before the end of washout while WBreath uses all washout inspirations. In case of inaccurate step-response correction, this leads to completely different EIMM-step values.

3. Results

Our study included three measurements per subject from 12 infants (six females, median age 5.2 weeks) from Basel University Children's hospital, and 12 infants (six females, median age 4.9 weeks) from Bern University Children's hospital, thus 72 paired washin and washout traces (see table S1 (stacks.iop.org/PM/37/L1/mmedia) for detailed demographic characteristics).

3.1. Intra-subject, intra-centre and inter-centre variability using the standard analysis

We detected an unexpected high intra-subject and intra-centre variability based on the following results: (i). high non-systematic Diff-$FRC_{(wo-wi)}$ in single measurements ranging from −25 to +30% in both centres, as shown in figure 2(a), despite the good quality of the recordings. This difference varied substantially within almost all subjects (SD range from 2.8 to 16.1%, figure 2(a)). Similarly, the Diff-$LCI_{(wo-wi)}$ was often higher than 20% (figure 2(b)). (ii). The CV of FRC per child exceeded 10% for several subjects in both centres (figure 2(c)).

Further, we detected inter-centre differences based on the following results: (i). FRC values were significantly higher in Basel, compared to Bern, whether expressed as absolute values (table S1 (stacks.iop.org/PM/37/L1/mmedia), *t*-test, $p = 0.0097$), or adjusted for body weight (FRC/body weight in table S1 (stacks.iop.org/PM/37/L1/mmedia), *t*-test, $p < 0.0001$) and length (FRC/length in table S1 (stacks.iop.org/PM/37/L1/mmedia), *t*-test, $p = 0.0019$). (ii). The CV for FRC in Basel was almost twice as high as in Bern (mean ± SD (%): 9.8 ± 5.6 and 5.8 ± 2.9, respectively, figure 2(c)).

This non-physiological variation within subjects, between subjects of each centre and between centres raises questions about the robustness of the analysis method, based on our own defined quality control, and suggests that several factors of the analysis algorithms

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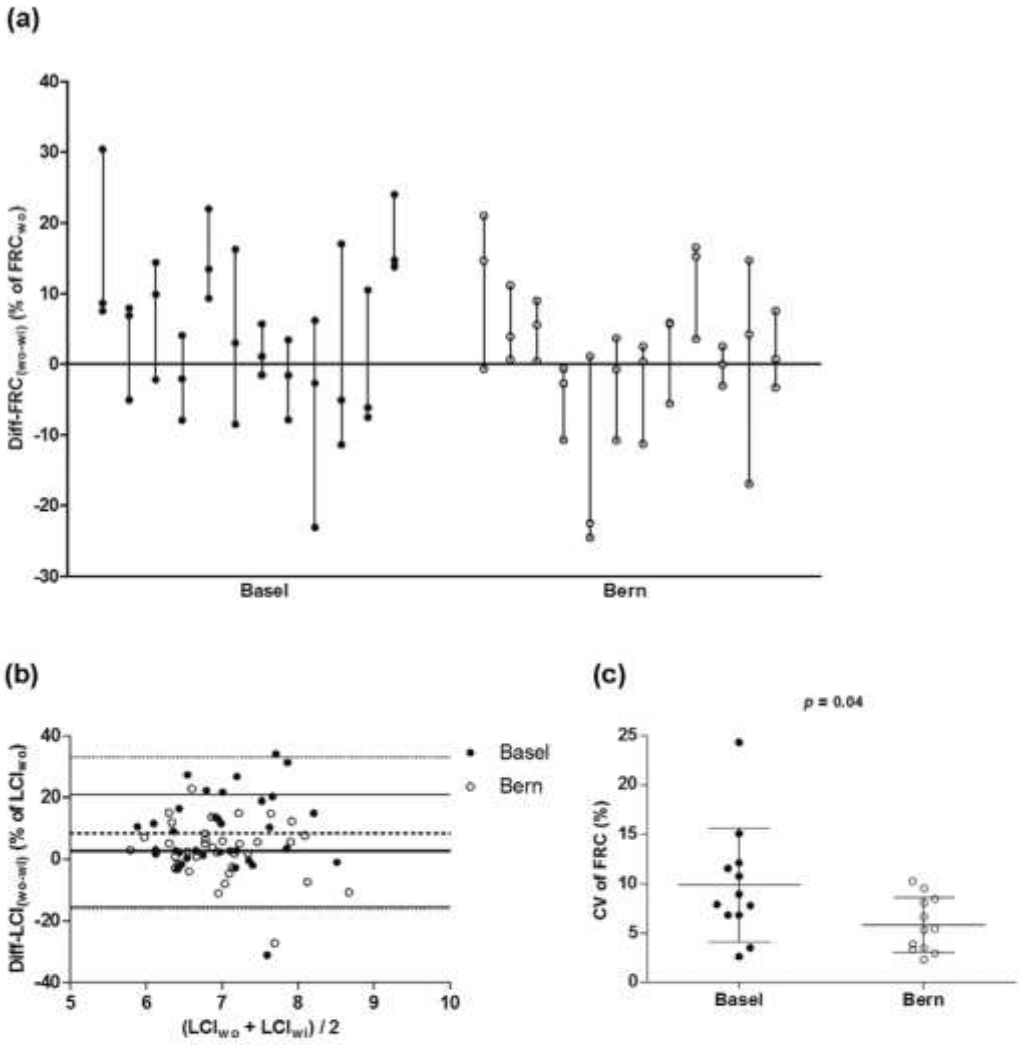

Figure 2. Intra-subject, intra-centre and inter-centre differences in MBW outcomes using the standard analysis. (a) Diff-$FRC_{(wo-wi)}$(% of FRC_{wo}) from Basel (closed circles, $n = 36$) and Bern (open circles, $n = 36$) measurements. Subjects are ordered by measurement date on x axis. (b) Modified Bland–Altman for Diff-$LCI_{(wo-wi)}$(% of LCI_{wo}) from Basel (closed circles, $n = 35$, one with LCI = 0 in washout excluded) and Bern (open circles, $n = 36$) measurements. Dotted lines (Basel) and solid lines (Bern) indicate mean $\pm$ 1.96 SD limits. (c) CV of FRC (%) in Basel and Bern ($N = 12$ infants per centre). Horizontal lines indicate mean and SD values; p value refers to t-test for the inter-centre comparison.

influence the results in a non-systematic way. These factors were further challenged in the following parts.

3.2. Alternative analysis settings

3.2.1. Analysis by changing the 'FRC-analysis' parameters. Each individual change in 'FRC-analysis' parameters resulted in significant changes in FRC and LCI leading to unrealistically

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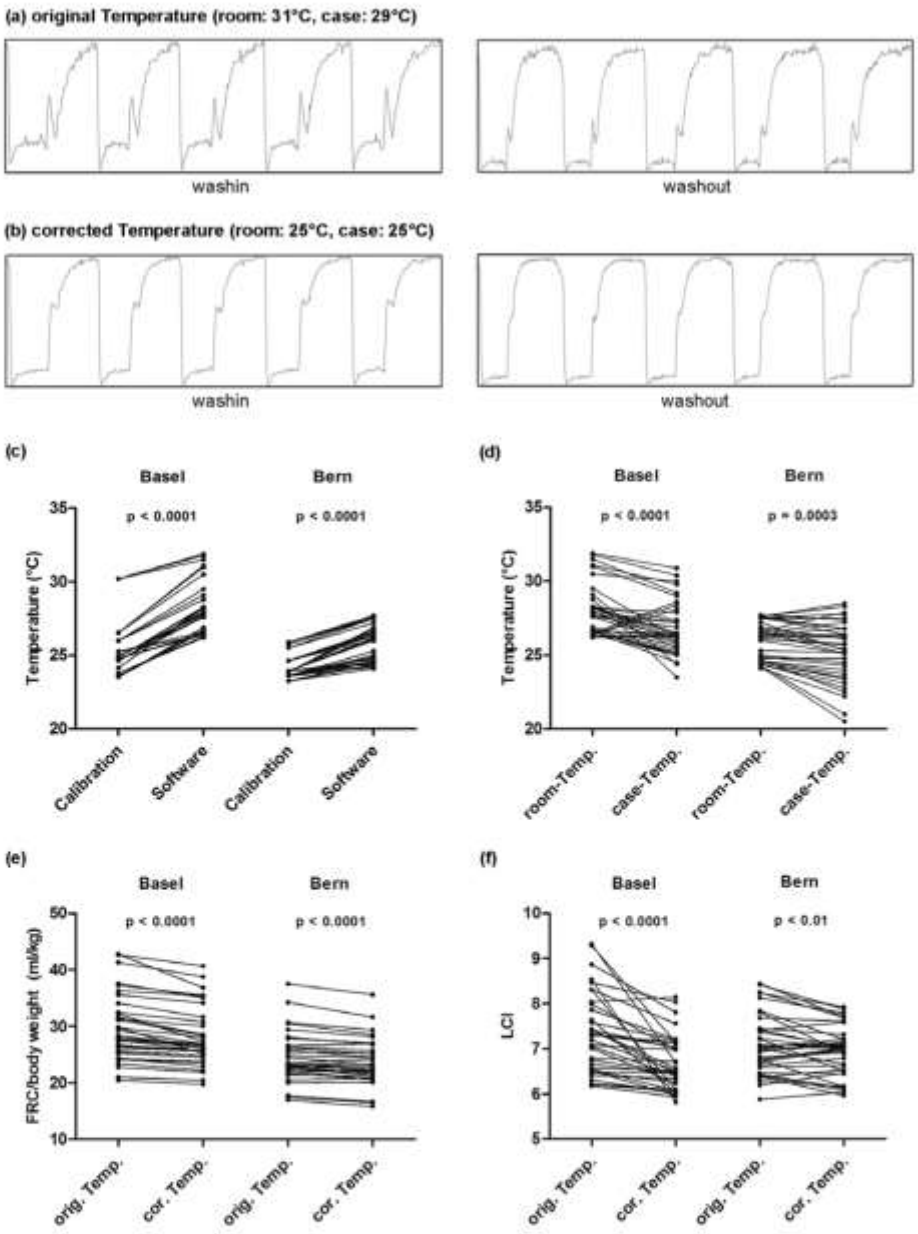

Figure 3. (a-b) Molar mass (MM) signal improves after temperature refinement. MM signal in the last five breaths of the washin and the washout phase respectively in original 'room-' and 'case-temperature' (a) and after temperature refinement (b). (c) 'Room-temperature' (°C) differed between calibration files and software recordings in Basel and Bern (t-test, $n = 36$ measurements per centre). (d) 'Room-temperature' was often found higher than 'case-temperature' as recorded in the software. 'Room-temperature' and 'case-temperature' (°C) in software recordings in Basel and Bern (t-test, $n = 36$ measurements per centre). (e-f) Effects of corrected 'room-' and 'case-temperature' on FRC and LCI values. Comparison of (e) FRC/body weight in ml kg^{-1} and (f) LCI values analysed with original temperature (orig. Temp.) and corrected temperature (cor. Temp.) in Basel (t-test, $n = 35$, one with LCI = 0 excluded) and Bern (t-test, $n = 36$) measurements.

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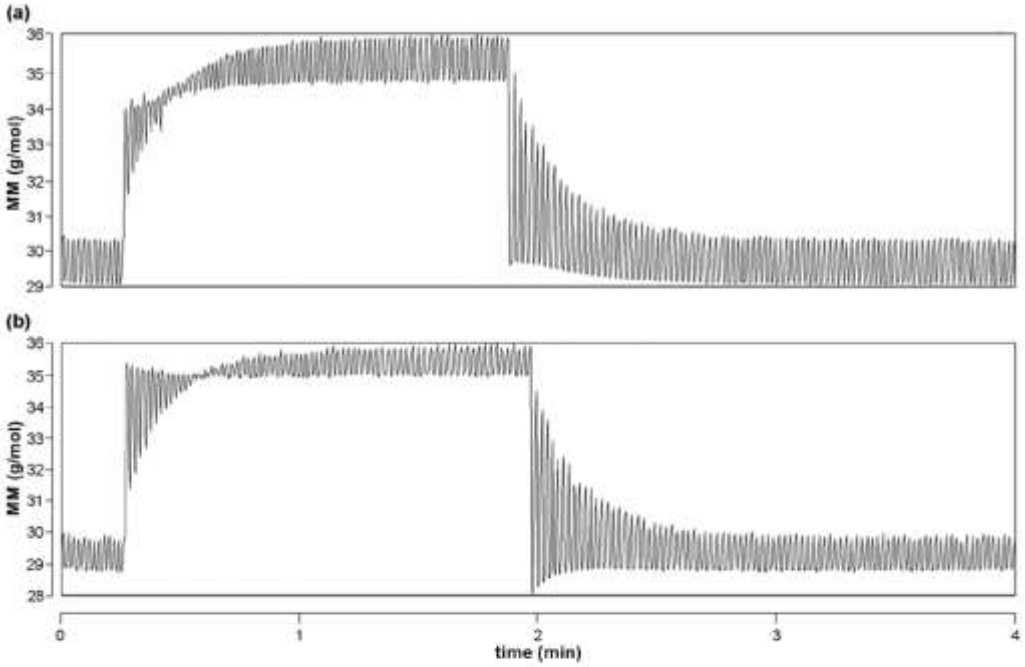

Figure 4. Effect of inappropriate step response correction on molar mass (MM) signal, seen mainly in the beginning of the washout. Original MM tracing curves of MBW measurements from healthy infants with (a) inadequate step response correction and (b) step response overcorrection.

high or low values, as shown in detail in the supplementary data and in table S2 (stacks.iop.org/PM/37/L1/mmedia).

3.2.2. 'Room-' and 'case-temperature' refinement. After the appropriate corrections we still observed several MBW recordings with atypical MM signals (figure 3(a)), and suspected incorrect temperature settings as the underlying reason. Mean 'room-temperature' recorded in the software was 27.0 °C, significantly higher compared to the temperature obtained during calibration for each measurement (mean 24.8 °C, figure 3(c), $n = 36$ measurements per centre, t-test, $p < 0.0001$). This difference was more profound in Basel than in Bern (t-test, $p < 0.0001$). In addition, 'room-temperature' in the software settings was usually equal or higher than the 'case-temperature' (figure 3(d)), which can be attributed to the non-realistic heating of the equipment.

To correct this, we substituted 'room-' and 'case-temperature' in the software with the rounded mean value from the calibration files (25 °C), as was also done by others (Vukcevic *et al* 2015). This temperature refinement normalized the MM curve (figure 3(b)). It resulted further in a rather systematic decrease in FRC/body weight (figure 3(e)), and a more heterogeneous change in LCI values (figure 3(f), t-test, $p < 0.0001$ explained in detail in the supplementary data).

3.2.3. Analysis with alternative MM step response corrections. We observed in several measurements that the MM signal after the step response correction was either inadequately corrected (figure 4(a)), or overcorrected (figure 4(b)). These measurements were not proceeded for further analysis and for this reason were not included in this study. In order to explore the impact of an inaccurate step response correction on the MBW outcomes, we analyzed

the measurements from our study using different MMDifuTau and SPLCham values in the temperature model (see figure S2(a)) (stacks.iop.org/PM/37/L1/mmedia). This changed significantly FRC and LCI values (more detail in the supplementary data and table S3) (stacks.iop.org/PM/37/L1/mmedia).

3.2.4. Analysis using different software versions

3.2.4.1. Analysis with a different software version of the same software package. The analysis with the more recent WBreath software version (3.39.4) resulted in significant changes in FRC and LCI values compared with the older version (3.28.0.0), but the variability remained high (more detail in the supplementary data, table S4 and figure S4) (stacks.iop.org/PM/37/L1/mmedia).

3.2.4.2. Analysis with a new software package. The new analysis software resulted in an impressive decrease in Diff-$FRC_{(wo-wi)}$ in single measurements (figures 5(a), (d), table S5 (stacks.iop.org/PM/37/L1/mmedia), *t*-test, $p < 0.0001$), as well as in the variability of these values within the subjects (SD range from 0.7 to 11.8%, figure 5(a)). The same trend can be assumed for Diff-$LCI_{(wo-wi)}$ (figures 5(b), (e), table S5) (stacks.iop.org/PM/37/L1/mmedia), however we cannot draw any conclusions due to the big heterogeneity in these data. In addition, the intra-subject variability in FRC (CV of FRC) was much lower, with highest value of 10.6% (figure 5(c), table S5) (stacks.iop.org/PM/37/L1/mmedia), compared to 24% with WBreath software (figure 2(c), *t*-test, $p = 0.03$). Also, the inter-centre difference for CV of FRC disappeared (figure 5(c), table S5 (stacks.iop.org/PM/37/L1/mmedia), *t*-test, $p = 0.6$) (see also the supplementary data and figures S5, S6) (stacks.iop.org/PM/37/L1/mmedia).

4. Discussion

4.1. Summary

This study illustrates the difficulties of SF_6-MBW measurements in infants with the only commercially available equipment. More specifically, it reveals several weaknesses of the current software used for the analysis of the measurements. Moreover, it shows the impact of different environmental and software-related parameters on MBW outcomes and provides recommendations that improve the quality of the analysis.

4.2. Interpretation

We found unrealistic high intra-subject and inter-subject variability using original settings, which differed further between centers. The differences in FRC and LCI values between washin and washout of −25% to +30% during quiet sleep without waking up cannot be explained physiologically. In addition, we found differences in FRC values between the two centres that are in accordance with the findings from Hulskamp *et al* (2009). These observations cast doubt on the quality of the current analysis method and raise questions about the strength of the obtained results. With results as variable as those, it is questionable whether clinical studies using MBW outcomes can be performed, especially in multicentre settings.

Previous single-centre studies have reported a much narrower range in intrasubject variability of FRC values (Fuchs *et al* 2011, Gray *et al* 2015a, 2015b,) from infants measured at a similar age with the same device. We attribute this difference to the new analysis approach we used, using the EIMM-step recently shown to be more sensitive in detecting the completion of the measurements, thus providing more accurate results (Anagnostopoulou *et al* 2015).

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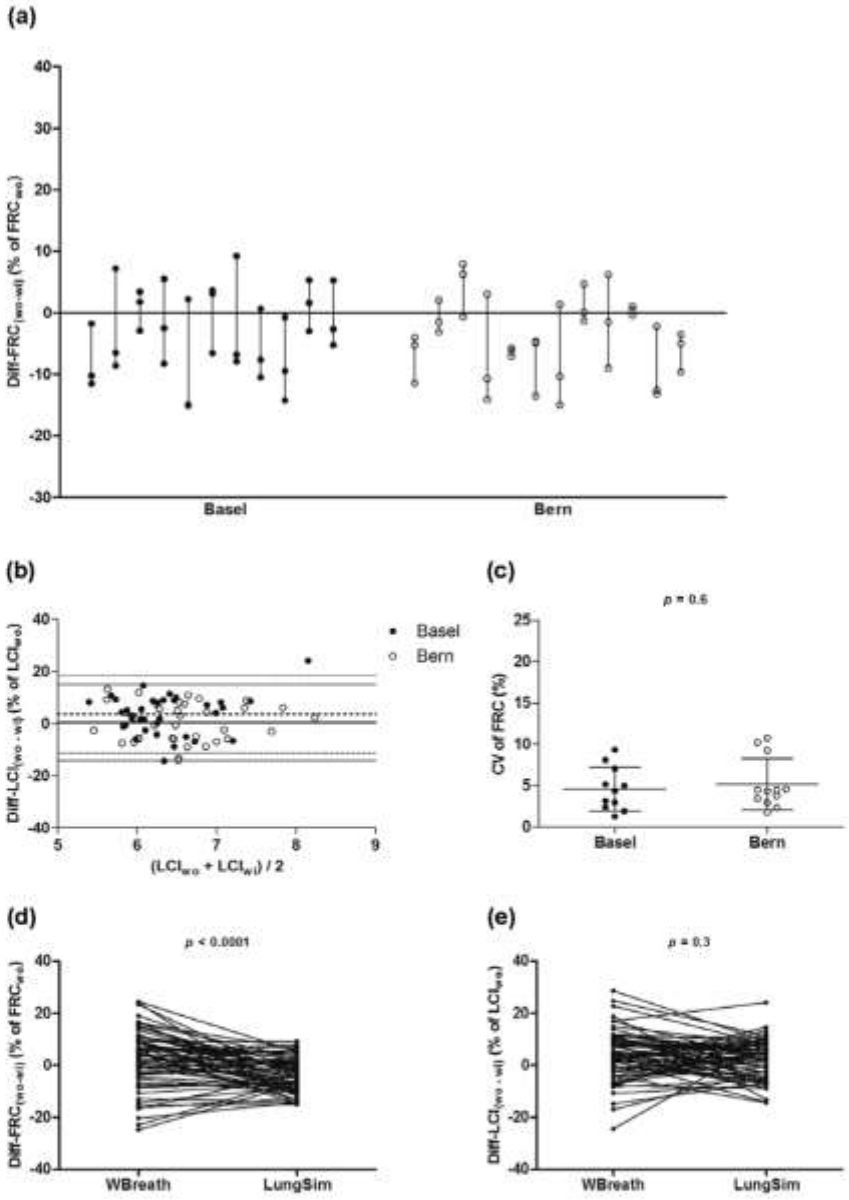

Figure 5. Intra-subject, intra-centre and inter-centre differences in MBW outcomes using the custom-made analysis software, LungSim. (a) Diff-$FRC_{(wo-wi)}$(% of FRC_{wo}) from Basel (closed circles, $n = 35$, one with $LCI = 0$ excluded) and Bern (open circles, $n = 36$) measurements. Subjects are ordered by measurement date on the x-axis. (b) Modified Bland–Altman plot for Diff-$LCI_{(wo-wi)}$(% of LCI_{wo}) from Basel (closed circles, $n = 35$, one with $LCI = 0$ excluded) and Bern (open circles, $n = 36$) measurements. Dotted lines (Basel) and solid lines (Bern) indicate mean ± 1.96 SD limits. (c) CV of FRC (%) in Basel (t-test, $N = 11$, one with $LCI = 0$ excluded) and Bern ($N = 12$) infants. Horizontal lines indicate mean and SD values. (d–e) Comparison between WBreath (version 3.28.0.0) and LungSim of (d) Diff-$FRC_{(wo-wi)}$(% of FRC_{wo}) and (e) Diff-$LCI_{(wo-wi)}$(% of LCI_{wo}) (t-test, $n = 71$ measurements, one with $LCI = 0$ excluded).

4.3. Technical issues

A more systematic analysis approach revealed several factors that substantially influence results. Although certain 'FRC-analysis' settings are recommended by the manufacturer, our study shows for the first time the direct impact of these settings on MBW outcomes and underlines the necessity to stick to these recommendations.

Looking more into the details of these settings, we found that minimal changes in physical constants of the currently used temperature model (Latzin *et al* 2007), such as MMDifuTau and SPLCham, influence MBW outcomes significantly. Thus, the corrected MM signal should be critically evaluated before proceeding to the analysis, and possible atypical signals should be questioned and/or reported. Any change in the settings should be taken seriously into consideration. If needed, the temperature model should be then modified appropriately, under the manufacturer's guidance. Researchers should take special care when analyzing measurements from other centres, and a detailed description of the settings should always accompany the recordings. This is even more important as the temperature model in the particular device is validated only for early infancy (body weight up to 12 kg) (Latzin *et al* 2007, Thamrin *et al* 2015), and it is formally not recommended for older subjects, as the set-up and the settings are slightly different. Further studies are required to implement a temperature model appropriate for older age groups.

According to our findings, invalid 'room-' and 'case-temperature' recordings in the software cause substantial non-systematic changes on the results. We show that 'room-temperature' is affected by temperature changes within the device, which is inevitably heated during the measurement (Latzin *et al* 2007). Temperature sensors are also prone to external temperature conditions, e.g. if the device is located close to a heating system, or in a room with high temperature changes. Therefore, users should be cautious about the temperature recorded in the software. We recommend documenting the 'room-temperature' value during the calibration of the device by all means, and to use this value for both 'room-' and 'case-temperature' (Vukcevic *et al* 2015) instead of the unrealistic temperature recordings in the software for the measurement analysis.

4.4. Clinical relevance

Our study reveals a discrepancy in FRC and LCI values obtained with two different versions of WBreath software. We attribute these differences mainly to the different ways of calculating EIMM and EEMM signals. However, as the exact algorithms used by the software are unknown, we cannot estimate other factors that could play a role. Thus, we conclude that it is not possible to compare results analyzed with different software versions, as reported before in abstract form (Ebdon *et al* 2013). This raises specific concerns about the handling of data from different centres that use different software versions (Hulskamp *et al* 2009, Fuchs *et al* 2011, Hall *et al* 2011, Kieninger *et al* 2011, Belessis *et al* 2012, Stahl *et al* 2014, Gray *et al* 2015b). With the manufacturers' support, we should identify changed parameters in different software versions that could influence the results. In addition, it would be extremely useful if each centre could create its own reference data-set by performing MBW tests in healthy infants as previously recommended (Hulskamp *et al* 2009, Lum *et al* 2010, Stocks *et al* 2010, Peterson-Carmichael *et al* 2014, Gray *et al* 2015a). A combination of normal values from different centres would diminish the risk of systematic bias (Stanojevic *et al* 2008). At present, this is especially important as with the use of the EIMM-step (Anagnostopoulou *et al* 2015), previously published reference values may no longer be entirely valid (Fuchs *et al* 2011).

It is important to mention that these observations account only for the above-mentioned device and analysis software. The reported recommendations do not apply to other devices used for MBW measurements, such as the mass spectrometers (Hoo *et al* 2012, Gustafsson *et al* 2003, Subbarao *et al* 2013) and the Innocor photoacoustic analyzer (Horsley *et al* 2008, Gonem *et al* 2014), as these are independent of the MM signal. However, environmental settings and their influence on lung function measurements is a well-known issue (Frey *et al* 2000) that should always be taken into account.

4.5. Future advances in infant MBW

A new custom-made software, even after the signal corrections were applied in WBreath software, resulted in more robust FRC results, lower intra-subject variability and a lower difference in FRC values between washin and washout, compared to both WBreath software versions. In this proof-of-principle approach, we did not consider the new software package as a new standard analysis software. By applying this new software, our aim was only to show that a few improvements in the software algorithms can substantially increase the robustness of the results. Thus, it seems easily possible for the manufacturer to improve the sensitivity of temperature recordings in the software, to provide more accurate correction steps as well as to implement the analysis algorithms in order to ensure the validity of the obtained MBW outcomes. Nevertheless, the use of new algorithms points towards alternative analysis approaches for future applications.

In order to include infant MBW measurements in multicentre studies, we have to ensure the reliability and the comparability of the results. Experts in the field should work in close cooperation with the manufacturers to diminish technical and methodological difficulties (Thamrin *et al* 2015). Moreover, as the most recent MBW guidelines (Robinson *et al* 2013) (Beydon *et al* 2007) are not applicable for infant measurements, due to test specificities in the early age (Stocks and Lum 2012), it is necessary to implement a universal standard operating procedure for infant MBW testing to address all the above issues (Peterson-Carmichael *et al* 2014, Subbarao *et al* 2015).

4.6. Strengths and limitations of the study

The present study has several strengths. It is a software validation study based on real high quality measurements selected from a large cohort of subjects at the same age. Including only healthy infants, any controversial results cannot be attributed to lung pathology, but to environmental factors and/or factors related to the software or the hardware of the device. As mentioned above, the device and the protocol used were identical in the two centres. Moreover, well-trained and experienced staff performed the measurements, and most of them were supervised by the same person in both centres. Although we acknowledge the low number of subjects in this study as a limitation, this is due to strict selection criteria based on the good quality of the tracings and the prerequisite that all three tests were performed in one sleeping period, in order to ensure to a great extent the stability of environmental conditions.

5. Conclusion

In conclusion, non-systematic inter-centre differences in MBW outcomes in infants can be greatly reduced by including correctly recorded environmental conditions in the software

analysis algorithms as well as by minimal alterations in these algorithms. We provide several simple recommendations for the user in this direction, such as to stick to the recommended 'FRC-analysis' settings, to examine the recorded temperature in the software and correct it, if needed, and to evaluate the validity of the temperature model based on the degree of normalization of the MM curve. We also present specific software characteristics that can be implemented by the manufacturer. The increasing need to include ILF tests in multicentre longitudinal clinical studies for patients with CF (Matecki *et al* 2015) leads to the necessity of obtaining better quality measurements, and both clinical scientists and manufacturing companies should focus on that. Only by improving the reliability of ILF, will we manage to use ILF tests to identify and monitor lung disease from infancy onwards.

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Conflict of interest

The authors declare no conflict of interest.

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Multiple breath washout analysis in infants: quality assessment and recommendations for improvement

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SUPPLEMENTARY DATA

METHODS

1. Standard analysis specifications

As recommended by the manufacturer and the most recent guidelines (Frey *et al.* 2000), the raw signal was corrected as follows: body temperature pressure saturation (BTPS), and flow baseline corrections were applied to the volume signal, and MM temperature simulation and MM step response correction to the MM signal (Latzin *et al.* 2007). After that, the end-inspiratory MM-step (EIMM-step) was used to determine the 100% step of SF_6 (Anagnostopoulou *et al.* 2015).

2. Details to alternative analysis settings

2.1 Analysis by changing the 'FRC-analysis' parameters

We challenged results by repeating the analysis changing each time only one of the parameters, mentioned in details below.

2.1.1 Analysis without MM temperature simulation (figure S1(a)). Temperature simulation aims to correct the MM signal for the influence of temperature and humidity using the temperature model (Latzin *et al.* 2007) (figure S2(a)).

2.1.2 Analysis without MM step response correction. The uncorrected signal leads to different end-inspiratory MM (EIMM) values. We performed the analysis using those values (figure S1(a)).

2.1.3 Analysis with expiratory MMfit. The software uses the MM expirogram instead of the MM curve for FRC computations (figure S1(a)).

2.1.4 Analysis by changing the EE start and stop percentile. These numbers define the part of the inspiration/expiration in proportion used to calculate the EIMM and end-expiratory MM (EEMM) values respectively. We set the EE start and stop percentile at 60-90%, 55-85%, and 50-80%, respectively (figure S1(b)).

2.2 Temperature recordings in the software

Temperature is one of the main factors that influence MM calculation using the ultrasonic flowmeter (Schibler and Henning 2001) and is measured in different compartments of the device (figure S2(a)): the 'body-temperature' is set as being stable at 37 °C, the 'case-temperature' is measured in the side-chamber of the ultrasonic flowmeter, and the 'room-temperature' should represent the temperature of the inspired gas, but in fact is measured inside the device. This means that the device while running leads unavoidably to a non-realistic increase of 'room-temperature'.

2.3 MM step response parameters

MM step response aims to correct the signal for the inhomogeneous gas mixing inside the flowmeter caused by the side-chambers during the measurement (Schmalisch *et al.* 2015). The correction is based on two physical constants included in the temperature model, as shown in figure S2(a), that depend on the set-up of the device and on the tracer gas used (Latzin *et al.* 2007): the MMDifuTau is the time the air needs to diffuse into the side-chamber and the SPLCham accounts for the length of the side-chamber.

3. Description of WBreath software version 3.39.4

Amongst other changes, version 3.39.4 uses the median of data points between 65-95% of inspiration or expiration for EIMM and EEMM calculation respectively, instead of the mean

value. The rest of the analysis was performed as described above, using the refined 'room-' and 'case-temperature' values.

4. Description of the LungSim software (version 2.1.0)

4.1. FRC calculation in the LungSim 2.1.0 software

In order to calculate FRC, the expired tracer gas volume is determined. In WBreath, this quantity is evaluated with help of *prototype* expirograms based on first few appropriately normalized washout breaths. With the EEMM value of any breath, this prototype is then scaled and integrated to provide its contribution to the expired tracer gas.

In LungSim, the expired tracer gas volume is calculated as follows:

- The volume flow $I_V(t)$ and MM signal $MM(t)$ are used to calculate the *mean molar mass* defined by $MM_{mean} = \frac{1}{V_{exp}} \int_{t_s}^{t_e} MM(t) \cdot I_V(t)dt$ for each breath (Figure S3). t_s and t_e signify the start and end time of the expiration and $V_{exp} = \int_{t_s}^{t_e} I_V(t)dt$ is the expired volume.
- During normal breathing, this value represents the mean MM signal due to the expiration of CO2, N2 and O2. Typically, the MM_{mean} values of the last three breaths of a washout (normal breathing again) are averaged to provide MM_{mean}^{avrg}.
- $V_{tracer} = \int_{t_s}^{t_e} MM(t) \cdot I_V(t)dt - V_{exp} \cdot MM_{mean}^{avrg}$ approximates the expired tracer gas volume, as the contributions from the normal air gas species in $MM(t)$ are subtracted.

4.2. Other advantages of the LungSim software

LungSim uses automatically the EIMM-step to define the completion of the measurement. Further, it provides for the user the opportunity to choose either the washin or the washout part of a measurement for analysis, without prior manual cutting.

RESULTS

1. Analysis by changing the 'FRC-analysis' parameters

1. Analysis without MM temperature simulation. By deactivating the temperature simulation correction we found significantly higher FRC/body weight values compared with the standard analysis (table S2, paired t-test, $p < 0.0001$). LCI was lowered to 4.1 ± 5.6 (mean ± SD, table S2, paired t-test, $p < 0.0001$). In 45 out of 72 measurements (62.5%) the LCI was zero, because the software considered them as incomplete (end tracer gas concentration higher than 2.5%).

2. Analysis without MM step response correction. The analysis without using the MM step response correction changed the EIMM-step significantly, as expected (paired t-test, $p < 0.0001$). This changed further FRC/body weight values from 26.9 ± 5.8 to 23.0 ± 3.6 ml/kg (mean ± SD, table S2, paired t-test, $p < 0.0001$) and LCI from 7.1 ± 1.2 to 5.7 ± 0.5 (mean ± SD, table S2, paired t-test, $p < 0.0001$). Apart from that, the software considered several measurements as invalid, due to a high variability in EIMM values in successive breaths.

3. Analysis with expiratory MMfit. When the expiratory MMfit was activated, FRC/body weight (table S2) and LCI values (table S2) differed significantly from the ones derived from the standard analysis in both centres (paired t-test, $p < 0.0001$). An extreme example shows a decrease of 8.4 ml/kg in FRC/body weight (from 31.6 to 23.2), followed by an increase of 3.3 LCI units (from 9.3 to 12.6).

4. Analysis by changing the EE start and stop percentile. The reduction in EE start and stop percentile led to progressively lower values for FRC and higher values for LCI, which differed significantly between them and systematically in comparison with the standard analysis (table S2, ANOVA-test, $p < 0.0001$).

2. 'Room-' and 'case-temperature' refinement

The temperature refinement changed significantly the mean EIMM-step (from -5.618 ± 0.1 to -5.601 ± 0.09 (mean ± SD, g/mol), *t*-test, $p < 0.0001$). Consequently, it changed the breath

number where the 2.5% LCI is determined. This explains why the decrease in FRC/body weight seems rather systematic and smaller (figure 3(e)), while at the same time the change in LCI values was very heterogeneous and larger (figure 3(f), *t*-test, $p < 0.0001$).

3. Analysis with alternative MM step response corrections

The analysis using alternative MM step response corrections resulted in significantly different EIMM-step values, and subsequently different end tracer gas concentrations. Further, FRC and LCI values were significantly changed. Interestingly, the more distant MMDifuTau and SPLCham values were from the original ones, the more washouts were detected as incomplete (end tracer gas concentration > 2.5%) (table S3).

4. Analysis with a different software version of the same software package

The more recent WBreath software version (3.39.4) decreased FRC and FRC/body weight values in a rather systematic way (figure S4(a), table S4, *t*-test, $p < 0.0001$)) and increased LCI (figure S4(b), table S4, *t*-test, $p < 0.0001$)), compared with the old version (WBreath 3.28.0.0). However, the variability of the results remained high, as shown by differences in FRC and LCI between washin and washout ranging from -14% to +26% (figures S4(c), S4(d) and table S4).

5. Analysis with a new software package (LungSim)

In terms of the absolute values, we found a rather systematic decrease in FRC (figure S5(a), table S5, *t*-test, $p < 0.0001$)) when using the new analysis software compared with the WBreath software (version 3.28.0.0). LCI changes were more heterogeneous (figure S5(b), table S5, *t*-test, $p = 0.0016$), attributable probably to the EIMM changes (mean ± SD (g/mol): -5.648 ± 0.1, *t*-test, $p < 0.0001$, compared with WBreath version 3.28.0.0 with corrected temperature). The comparison with FRC and LCI values obtained with the 3.39.4 WBreath version gave similar results (figure S6).

Table S1: Demographic characteristics and MBW outcomes of study subjects in children's hospitals from Basel and Bern.

	Basel	Bern	Between-centre comparison (unpaired t-test)
Number (male/female)	12 (6/6)	12 (6/6)	
Age, wks	5.1 (0.8)[a]	4.9 (0.4)[a]	$p = 0.51$
Gestational age, wks	44.6 (1.2)[a]	45.1 (0.9)[a]	$p = 0.3$
Weight, kg	4.3 (0.6)[a]	4.6 (0.6)[a]	$p = 0.19$
Length, cm	54 (2.6)[a]	55 (2.5)[a]	$p = 0.47$
FRC, ml	126 (24)[b]	111 (22)[b]	$p = 0.0097$
FRC/body weight in ml/kg	29.6 (5.8)[b]	24.2 (4.4)[b]	$p < 0.0001$
FRC/length in ml/cm	2.3 (0.4)[b]	2.0 (0.4)[b]	$p = 0.0019$
LCI	7.1 (1.5)[b]	7.1 (0.7)[b]	$p = 0.88$

[a] at the time of the measurement, data are given in mean (SD)

[b] analysed with WBreath version 3.28.0.0 using the original temperature and the standard analysis method, data are given in mean (SD)

Table S2: Impact of changing 'FRC-analysis' parameters on FRC/body weight and LCI in 24 healthy infants (n = 72 measurements). Values are reported as mean (SD).

	Analysis methods[a]						
	Standard	no TempSim	no step-response	with MMfit	MM calculation at (%) of inspiration/expiration		
EE start and stop percentile[b]	Standard (65-95%)				60-90%	55-85%	50-80%
FRC/body weight in ml	26.9(5.8)	31.3(7.1)[c]	23.0(3.6)[c]	24.5(4.9)[c]	26.4(5.6)[d]	25.7(5.4)[d]	25.0(5.2)[d]
LCI	7.1 (1.2)	4.1 (5.6)[c, e]	5.7 (0.5)[c]	7.8 (1.4)[c]	7.2 (1.2)[d]	7.4 (1.2)[d]	7.4 (1.3)[d]

[a] results obtained with the standard analysis, and when using the following changes in analysis parameters: inactive temperature simulation (no TempSim), inactive step response (no step-response), active MMfit (with MMfit), and percentage of inspiration/expiration used for MM calculations set at 60-90%, 55-85% and 50-80% respectively

[b] End Expiratory (EE) start and stop percentile

[c] in comparison with the standard analysis, paired t-test, $p < 0.0001$

[d] one-way ANOVA, $p < 0.0001$

[e] n = 27 measurements (42 measurements considered as incomplete with LCI = 0)

Table S3: Impact of different SPLCham and MMDifuTau values on MBW outcomes from Basel and Bern (n = 36 measurements per centre, if nothing else is reported), data are given in mean (SD). EIMM-step: end inspiratory molar mass step, End conc.: Tracer gas concentration at the end of washout.

SPLCham [1/100 mm]		1620[a]	1770	1920	1920[b]	1920	2070
MMDifuTau [ms]		4500[a]	6000	6000	7500[b]	9000	9000
Basel	EIMM-step [g/mol]	-5.687 (0.031)	-5.738 (0.031)	-5.754 (0.032)	-5.794 (0.034)	-5.833 (0.038)	-5.853 (0.040)
Bern	EIMM-step [g/mol]	-5.418 (0.048)	-5.465 (0.044)	-5.479 (0.043)	-5.515 (0.040)	-5.552 (0.038)	-5.569 (0.038)
Basel	End conc.[%]	0.20 (0.4)	0.76 (0.65)	1.10 (0.68)	1.72 (0.73)	2.34 (0.76)	2.75 (0.79)
Bern	End conc.[%]	0(0)	0.01 (0.06)	0.03 (0.12)	0.14 (0.27)	0.48 (0.45)	0.84 (0.52)
Basel	FRC/body weight [ml/kg]	27.9 (5.1)	28.4 (5.2)[d]	29.4 (5.6)[d]	29.3 (5.7)[d]	29.1 (5.6)[d]	30.2 (5.7)[d]
Bern	FRC/body weight [ml/kg]	24.3 (3.7)[d]	23.7 (3.8)[e]	24.2 (4.0)[d]	23.6 (4.0)	23.2 (4.0)[d]	24.1 (4.2)[d]
		n	n	n	n	n	n
Basel	LCI	36 6.7 (0.6)	36 7.6 (1.0)[d]	35[c] 7.9 ((1.0)[d]	30[c] 9.0 (1.5)[d]	27[c] 10.5 (2.2)[d]	19[c] 11.7 (2.7)[d]
Bern	LCI	36 6.0 (0.4)[d]	36 6.4 (0.5)[d]	36 6.5 (0.5)[d]	36 6.9 (0.6)[d]	36 7.3 (0.8)[d]	36 7.5 (0.9)[d]

[a] configuration values for Basel
[b] configuration values for Bern
[c] number of measurements reaching the 2.5% end tracer gas concentration at the washout
[d] $p < 0.0001$, paired t-test, compared with the analysis using the original configuration values for each centre
[e] $p = 0.3$, paired t-test, compared with the analysis using the original configuration values for Bern

Table S4: MBW outcomes in Basel and Bern (n = 36 measurements per centre) analysed with different WBreath software versions (WBreath 3.28.0.0 and WBreath 3.39.4). Data are reported as mean (SD). We used the refined 'room-' and 'case-temperature' for both analyses.

	WBreath 3.28.0.0		WBreath 3.39.4	
	Basel	Bern	Basel	Bern
FRC (ml)	119 (21)	108 (20)	109 (17)[c]	101 (16)[c]
FRC/body weight (ml/kg)	28 (5)	24 (4)	26 (4)[c]	22 (3)[c]
Diff-$FRC_{(wo-wi)}$ (ml/kg)	1.2 (3)	0.6 (2.7)	1.4 (2.3)[a]	0.6 (1.8)[a]
CV of FRC (%)	9 (6)	6 (3)	7 (6)[b]	4 (3)[b]
LCI	6.7 (0.6)	6.9 (0.5)	7.3 (0.7)[c]	7.5 (0.6)[c]
Diff-$LCI_{(wo-wi)}$	0.3 (0.6)	0.2 (0.6)	0.3 (0.6)[a]	0.2 (0.5)[a]
CV of LCI (%)	7 (4)	4 (3)	6 (3)[a]	4 (3)[a]

[a] comparison with WBreath 3.28.0.0, t-test, non-significant

[b] comparison with WBreath 3.28.0.0, t-test, $p < 0.05$

[c] comparison with WBreath 3.28.0.0, t-test, $p < 0.0001$

Table S5: MBW outcomes in Basel (n = 35 measurements, N = 11 infants, one measurement did not reach the 2.5% of SF_6 in the washout) and Bern (n = 36 measurements, N = 12 infants) analysed with a custom-made software package (LungSim 2.1.0). FRC (ml), FRC/body weight (ml/kg), FRC/length (ml/cm), CV% of FRC, Diff-FRC $_{(wo - wi)}$ (% of wo), LCI, CV% of LCI and Diff-LCI $_{(wo - wi)}$ (% of wo) are reported as mean (SD).

	LungSim 2.1.0			
	Basel	Comparison with WBreath 3.28.0 (t-test)	Bern	Comparison with WBreath 3.28.0 (t-test)
FRC (ml)	106 (10)	$p < 0.0001$	99 (14)	$p < 0.0001$
FRC/body weight (ml/kg)	25 (4)	$p < 0.0001$	22 (3)	$p < 0.0001$
FRC/length (ml/cm)	2.0 (0.2)	$p < 0.0001$	1.8 (0.2)	$p < 0.0001$
CV% FRC	5 (3)	$p = 0.042$	5 (3)	$p = 0.044$
Difference $FRC_{(wo - wi)}$ (% of wo)	-3.5 (6.6)	$p = 0.0002$	-4.0 (6.2)	$p = 0.004$
LCI	6.5 (0.7)	$p = 0.2$	6.6 (0.7)	$p = 0.0014$
CV% LCI	6 (4)	$p = 0.6$	6 (4)	$p = 0.2$
Difference $LCI_{(wo - wi)}$ (% of wo)	3.6 (7.6)	$p = 0.7$	0.4 (7.5)	$p = 0.4$

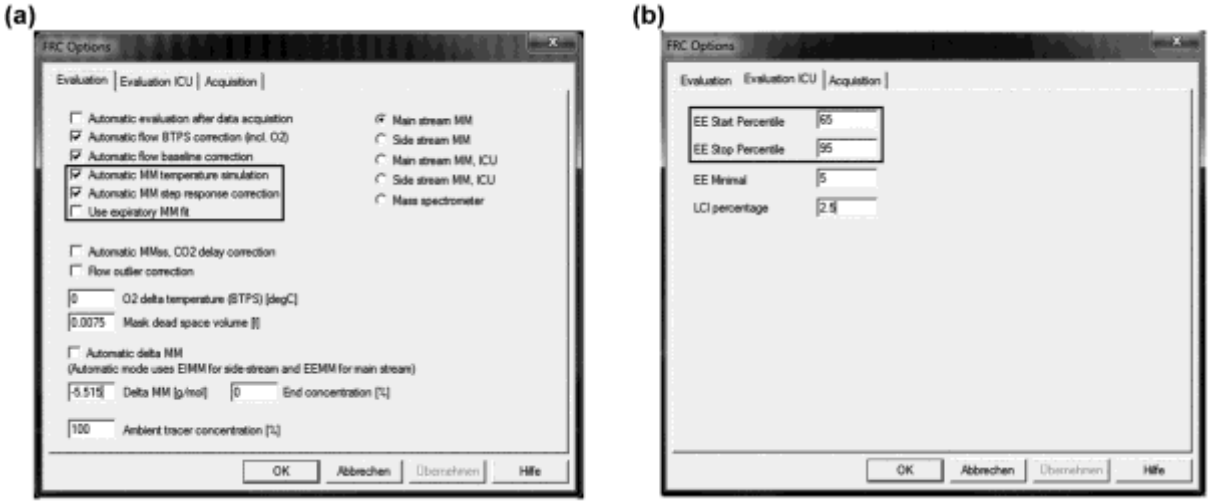

Figure S1: Schematic illustration of the 'FRC analysis' parameters as shown in the software during the standard analysis (WBreath, ndd Medizintechnik AG, Zuerich, Switzerland). The parameters included in boxes were changed manually for the alternative ways of analysis, as described in methods, parts 2.1.1-2.1.4.

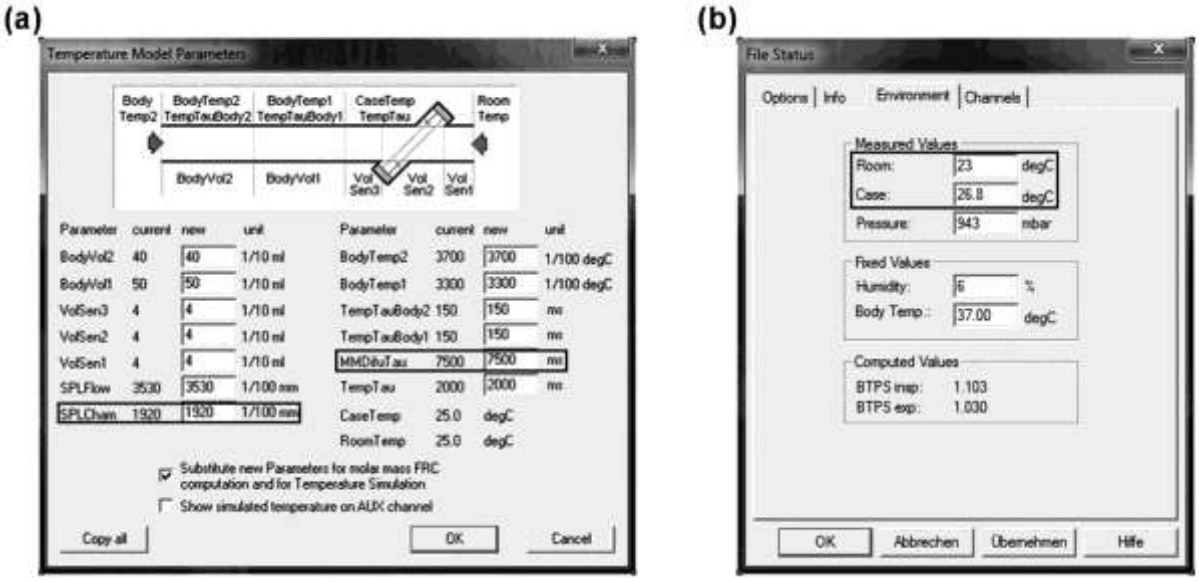

Figure S2: Schematic illustration of (a) the 'room-' and 'case-temperature' settings and (b) the temperature model as shown in the software (WBreath, ndd Medizintechnik AG, Zuerich, Switzerland). The values in white background are user-changeable. The values included in a box were manually changed as described in the part 2.3 in methods for panel (a) and in the part 2.2 in methods for panel (b), respectively.

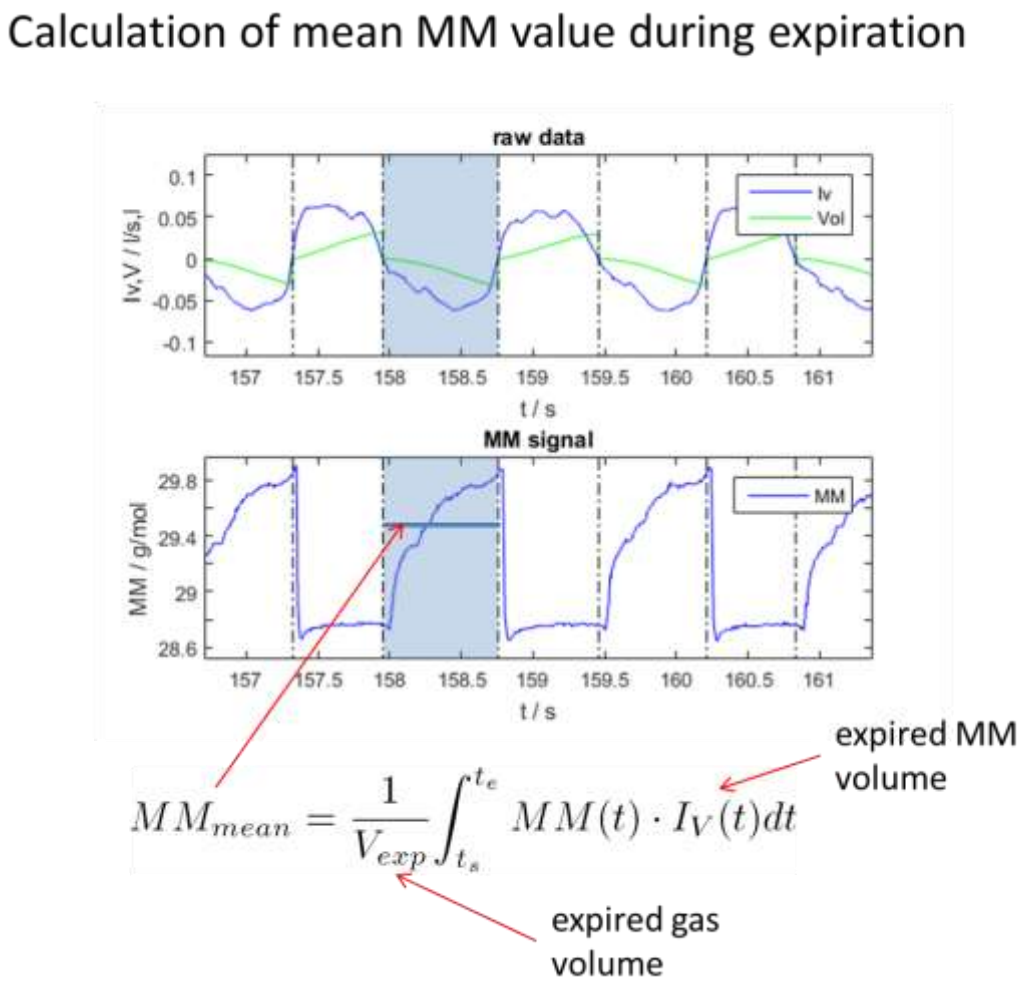

Figure S3: Original SF_6-MBW tracing from a healthy infant analysed with the LungSim 2.1.0 software. The algorithm for the calculation of the mean molar mass during expiration is shown. This quantity is used to evaluate the expired tracer gas volume.

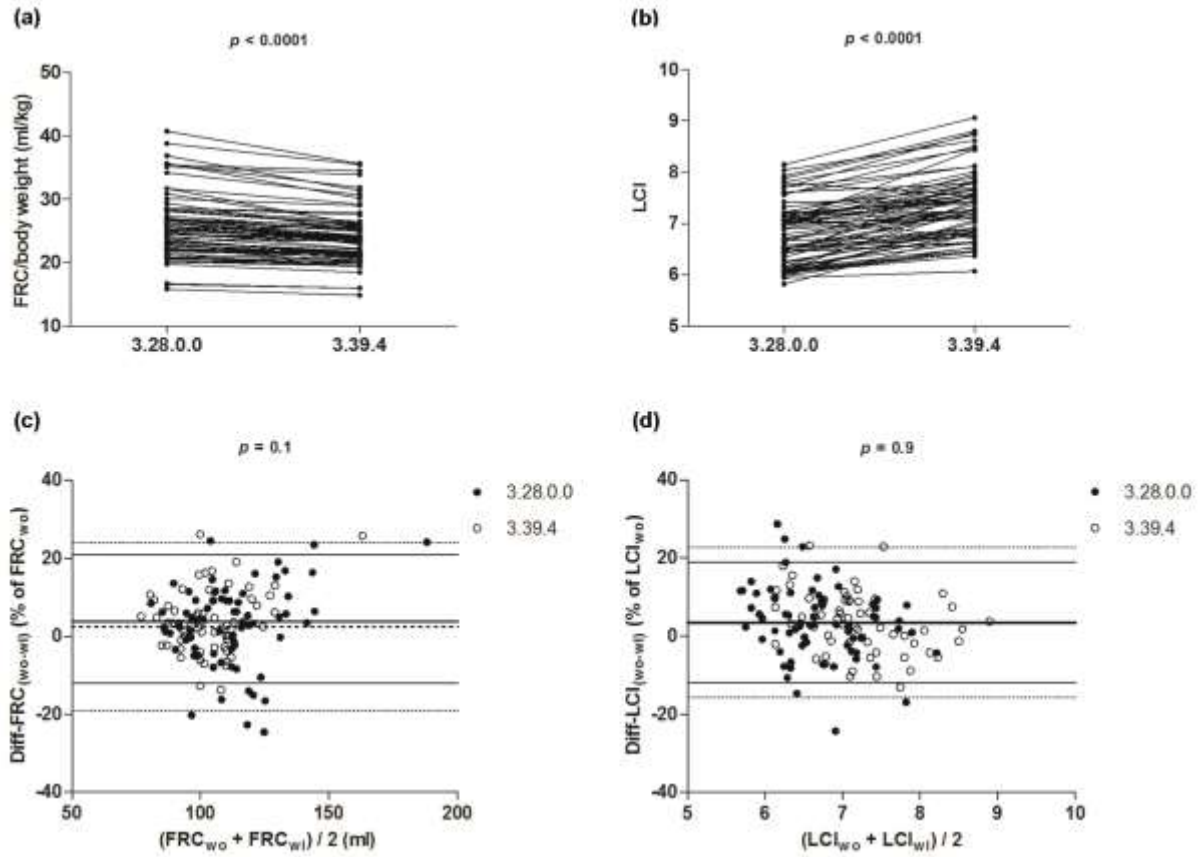

Figure S4: MBW outcomes using the newer version of the WBreath analysis software (3.39.4). (a-b) Comparison of (a) FRC/body weight in ml/kg and (b) LCI analysed with WBreath version 3.28.0.0 and 3.39.4 (*t*-test, n = 72 measurements). (c-d) Modified Bland Altman plot for (c) Diff-$FRC_{(wo-wi)}$ (% of FRC_{wo}) and (d) Diff-$LCI_{(wo-wi)}$(% of LCI_{wo}) analysed with WBreath version 3.28.0.0 (closed circles) and 3.39.4 (open circles) (*t*-test, n = 62 measurements, 10 excluded as incomplete in washin when analysed with version 3.39.4). Dotted lines (version 3.28.0.0) and solid lines (version 3.39.4) indicate mean ± 1.96 SD limits.

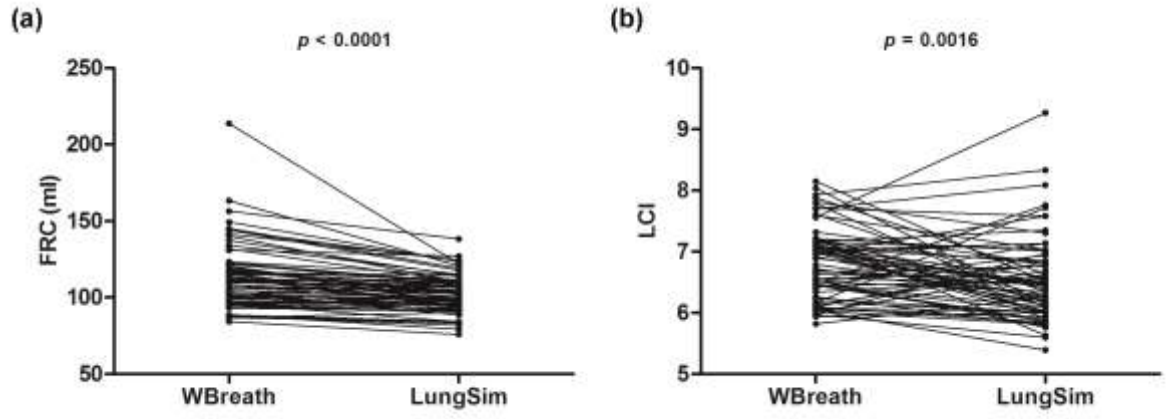

Figure S5: Comparison of MBW outcomes analysed with two different softwares (WBreath version 3.28.0.0 and LungSim 2.1.0) in healthy infants from Basel and Bern. (a) FRC (ml) and

(b) LCI values in 71 measurements (one measurement excluded because it did not reach the 2.5% of SF_6 concentration when analysed with LungSim) analysed with WBreath and LungSim, *p* values for paired *t*-test for the comparison between the two softwares.

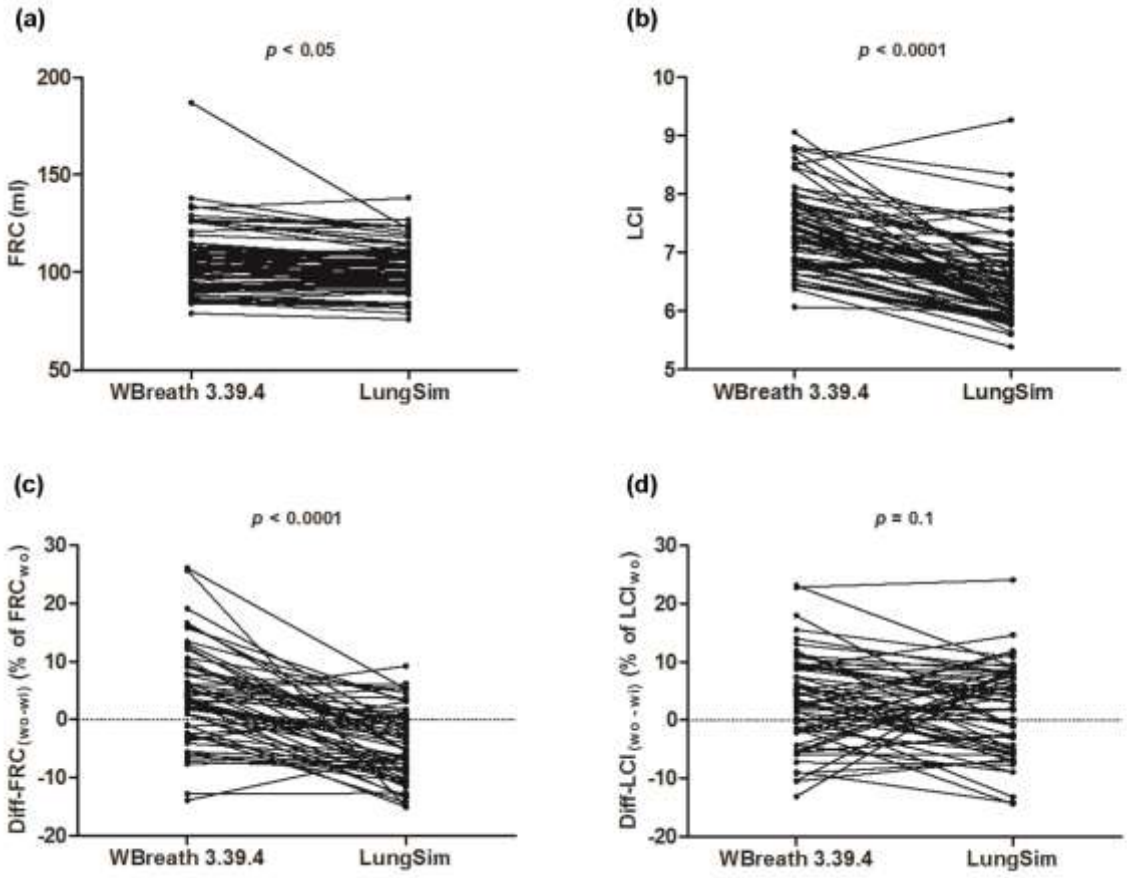

Figure S6: Comparison of MBW outcomes analysed with two different softwares (WBreath version 3.39.4 and LungSim) in healthy infants from Basel and Bern. (a) FRC (ml), (b) LCI values in 71 measurements and (c) Diff-$FRC_{(wo-wi)}$ (% of FRC_{wo}) and (d) Diff-$LCI_{(wo-wi)}$ (% of LCI_{wo}) in 58 measurements (1 washout and 13 washin measurements excluded as incomplete when analysed with the one or the other software) analysed with WBreath 3.39.4 and LungSim, *p* values for *t*-test for the comparison between the two softwares.

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3.2.3 Interrupter technique in newborn infants

Interrupter technique in infancy: higher airway resistance and lower short-term variability in preterm versus term infants

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Interrupter technique in infancy: higher airway resistance and lower short-term variability in preterm versus term infants

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Running head: Interrupter technique in term and preterm infants

ABSTRACT

Background: In preschool children, measurement of airway resistance using interrupter technique (Rint) is feasible to assess the degree of bronchial obstruction. Although some studies measured Rint in infancy, values of Rint and its variability in preterm infants are unknown. In this study, Rint and its variability was measured at infancy and compared between healthy term and preterm infants.

Methods: High quality Rint measurements in term (n=50) and preterm (n=48) infants were obtained at postmenstrual age of 42-50 weeks in two study centers in Switzerland. Intra-measurement variability of Rint in one measurement and inter-measurement variability between two subsequent measurements was assessed by coefficient of variation (CV).

Results: Mean Rint in term infants was 4.2 ± (SD; 1.9) $kPa \cdot s \cdot L^{-1}$ and in preterm infants was 5.6 ± (2.8) $kPa \cdot s \cdot L^{-1}$. Mean CV in term infants was 29.6 ± (14.9) % and in preterm infants was 20.2 ± (8.4) %. Rint was significantly lower (95% CI -2.31 to -0.38; P=0.007) and CV significantly higher (95% CI 4.53 to 14.3; P<0.001) in term compared to preterm infants. There were no differences in mean Rint and mean CV between the first and the second measurement obtained in a subgroup of term (n=24, 48%) and preterm (n=22, 45%) infants.

Conclusions: Our results suggest that differences in airway mechanics between term and preterm infants can be assessed with the interrupter technique during early infancy. Before clinical application of Rint measurements in this age group, reasons underlying the variability of measurements should be further investigated.

INTRODUCTION

Prospective studies have demonstrated that reduced lung function after birth is associated with increased respiratory morbidity during infancy [1], throughout childhood, and even into adulthood [2]. Prematurely born infants are at increased risk for later respiratory morbidity [3] and reduced lung function compared to term infants that can be detected shortly after birth [4,5]. Therefore, early identification of infants at risk is crucial to implement preventive strategies to reduce long-term morbidity.

Airway obstruction in infants can be measured using the interrupter technique (Rint). Neergard and Wirz described this method almost a century ago [6], but Rint was not routinely used in pediatric studies before measurements were standardized [7,8] and ATS/ERS recommendations for pre-school children were published [9]. Rint was measured in few studies during infancy [10-13], but studies comparing Rint between term and preterm infants are lacking despite the fact that measurements can be done during tidal breathing.

Studies systematically assessing Rint in infants and children reported poor repeatability [12,14] and high variability of measurements [10,11,15]. This indicates that Rint measurements require further quality assessment, which is important since Rint was previously used to assess bronchodilator response in asthmatic [16,17] and prematurely born children [18].

Based on studies reporting increased airway resistance in former preterm born children in early childhood [4], and the high variability of Rint measurements reported from pre-school children [15], we hypothesized that: i) preterm infants have increased airway resistance compared to term infants in infancy and ii) measurements have high variability in both study groups. The aim of this study was to compare Rint measurements between term and preterm infants. In addition, we aimed to assess variability of Rint in both study groups.

METHODS

Study design and subjects

This prospective, nested, case-control study included term (≥37 weeks) and preterm (<37 weeks) infants from the Basel-Bern infant lung development (BILD) birth cohort [19]. Rint was measured at two centers in Switzerland (Basel and Bern) at 42–50 weeks postmenstrual age. Inclusion criteria were ≥5 interruptions during one measurement [9], Rint ≥1 $kPa \cdot s \cdot L^{-1}$ and occlusion time <200 ms. In Basel, all infants from 2011–2015 were included. In Bern from 2003-2007, unequally more term than preterm infants were measured. Therefore, to obtain equal group sizes in Bern, all preterm infants were included and matched to term infants according to study year and number of interruptions. The Ethics Committees of Basel and Bern approved the study and parents provided written consent.

Rint measurements

Rint was measured according to ATS/ERS guidelines for preschool children [9] and recommendations for infants [8,20] during behaviorally defined, unsedated non-REM sleep in supine position using a rigid face mask (Teleflex medical AG, Ruesch, Silicone, size 2, Belp Switzerland) lined with putty to ensure a leak-free seal and to reduce dead space. Interruptions were manually triggered (Exhalyzer D, Eco Medics AG, Duernten Switzerland) every 3-6 breaths. Rint was calculated using the linear back-extrapolation method, applied to the trace of pressure at airway opening between 30 and 70 ms post-interruption [10].

Data recording and processing

Rint was recorded and processed [10,21] using WBreath (Version 3.28.0.0, ndd Medizintechnik AG, Zurich, Switzerland). Before data analysis, values were corrected to body temperature pressure saturation (BTPS) and zero-offset of flow baseline was performed, as described previously [22]. Breath detection was manually adapted to manufacturer's recommendations to increase analysis sensitivity. The first measurement was designated Rint1, containing all interruptions of the first measurement. After a 2-minute break, a second measurement was completed in a subgroup

designated Rint2, containing all interruptions of the second measurement. Rint1 was divided into groups of 5 successive interruptions named in alphabetical order. Therefore, $Rint\__a$ represents the first 5 interruptions of Rint1 and $Rint\__b$ represents the following 5 interruptions of Rint1 and so forth. The last subgroup ($Rint\__i$) contained interruption numbers 40–45. Rint2 was not divided into subgroups (Supplementary E-Figure 1).

Rint variability

Intra-measurement and inter-measurement variability (coefficient of variation (CV) (%) = standard deviation (SD)/mean×100), were used to evaluate variability of Rint within one measurement and between measurements.

Outcome parameters

Outcomes are visualized in Supplementary E-Figure 1.

Rint and CV

Main outcomes were mean Rint1 and mean CV1. The number of interruptions of Rint1 varied between term and preterm infants. Therefore, Rint and CV from the first group of interruptions of Rint1 ($Rint\__a$) were analyzed to control for different interruption numbers. Further, the 9^{th} group ($Rint\__i$) was analyzed to assess potential differences between groups at different time points.

Trends of Rint during one measurement

Trends of Rint over time during one measurement were assessed using visual inspection and detrended analysis.

Comparison of Rint and CV between successive measurements

To assess differences between two subsequent measurements, Rint1 and Rint2 were compared in those infants that completed two measurements.

Statistics

After inspection of data for normal distribution, paired *t*-tests were used to compare Rint and CV between measurements in one infant and unpaired *t*-tests to compare Rint and CV between term

and preterm infants. For analysis of trends during repeated measurements, Kernel density estimations [23] were applied to compare original Rint to linear-detrended-Rint [24] by ANOVA test. To control for center differences and confounders, linear regression analysis was done to study the association between prematurity separately with Rint and CV, adjusted for study center, sex, and weight at study date. The association between clinical parameters (e.g. oxygen therapy, mechanical ventilation) with Rint and CV in preterm infants was analyzed using linear regression. $P<0.05$ defined statistical significance. Data was analyzed using Stata® (Stata Statistical Software: release 11. STATA Cooperation; College Station, TX) and Matlab R2015ab (The MathWorks, Inc., Natick, MA, USA).

RESULTS

In Basel, 16 (16%) of 100 term infants, and 14 (14.7%) of 95 preterm infants fulfilled the inclusion criteria. In Bern, 49 (26.3%) of 186 term infants, and 34 (15.1%) of 225 preterm infants fulfilled inclusion criteria. To obtain equal group sizes in Bern, 34 of the 49 term infants were matched to 34 preterm infants. For final analysis, 50 term, and 48 preterm, infants from both centers were included (Figure 1). Mean postmenstrual age at study was 44 weeks (range 42-47) for term, and 45 weeks (range 40-50) for preterm, infants (Table 1). Mean number of occlusion during Rint measurements was 38 in term (range 5-252), and 44 in preterm, infants (range 6-230). The distribution of sex and gestational age was similar at both centers, but differences in weight and length were noted (Supplementary E-Table 1). There was no difference in success rate of measurements between centers for term (P=0.635) and preterm infants (P=0.865). Anthropometrics, Rint, and CV between included and excluded term infants from Bern did not differ (Supplementary E-Figure 2, Supplementary E-Table 2).

Comparison of Rint and CV in term and preterm infants

In general, Rint was lower and CV higher in term compared to preterm infants. Mean Rint1 in term infants was 4.2 ± (SD; 1.9) $kPa \cdot s \cdot L^{-1}$ and in preterm infants 5.6 ± (2.8) $kPa \cdot s \cdot L^{-1}$, resulting in a mean difference of -1.35 (95% CI -2.31 to -0.38) $kPa \cdot s \cdot L^{-1}$. The corresponding mean CV in term infants was 29.6 ± (SD; 14.9) % and in preterm infants 20.2 ± (8.4) %, resulting in a mean difference of 9.41 (95% CI 4.53 to 14.3) % (Table 2 and Figure 2). For both Rint and CV, associations with prematurity were similar after adjustment. In preterm infants, Rint was 1.2 (95% CI 0.3 to 2.2) $kPa \cdot s \cdot L^{-1}$ higher and CV was -9.8 (95% CI -14.8 to -4.7) % lower compared to term infants (Supplementary E-Table 3). Very similar results were obtained when stratifying the analysis by center and at different time points during one measurement (Supplementary E-Table 3, Table 2, Supplementary E-Figure 3, Supplementary E-Results). In preterm infants, oxygen therapy was associated with an increased Rint. Adjusted coefficient was

0.31 (95% CI 0.03 to 0.56) kPa·s·L^{-1} per ten day increase in oxygen therapy (Supplementary E-Table 4).

Trends of Rint during one measurement

Trends of Rint were assessed in 13 term, and 14 preterm, infants completing 45 interruptions. Comparison of original Rint versus linear-detrended Rint revealed no differences in term (*P*=0.939) and preterm infants (*P*=0.932) (Supplementary E-Figure 4).

Comparison of Rint and CV between successive measurements

There was no difference in Rint and CV between two successive measurements in term (n=24) and preterm (n=22) infants. Difference in mean Rint1 compared to mean Rint2 in term infants was 0.05 (95% CI -1.25 to 1.35) kPa·s·L^{-1} and in preterm infants -0.13 (95% CI -1.67 to 1.4) kPa·s·L^{-1}. Difference in mean CV1 compared to mean CV2 in term infants was -1.57 (95% CI -9.92 to 6.78) % and in preterm infants 0.003 (95% CI -5.75 to 5.76) %. A high intra-subject variability of Rint and CV between two successive measurements was observed. Intra-subject variability of Rint and CV in term infants ranged from -86.5% to 90.6% and from -101.5% to 77.5%, respectively. Intra-subject variability of Rint and CV in preterm infants ranged from -47.1 to 56.3% and from -87.2% to 105.7%, respectively (Figure 3).

DISCUSSION

This study of healthy term and preterm infants found that Rint measured during natural sleep was increased in preterm compared to term infants, even over subsequent measurements. A high CV was observed in both groups with preterm infants having a lower CV compared to term infants.

Few studies measured Rint in healthy newborns without sedation [10-12]. Using the same occlusion time, Rint values reported by Fuchs et al. (3.64 $kPa \cdot s \cdot L^{-1}$) [11] were comparable to the current study (4.2 $kPa \cdot s \cdot L^{-1}$). This was not unexpected since the previously studied subjects [11] were a subset of this study. Adams et al. found a higher Rint measured between days 1-4 after birth [12]. Diverging results might be due to differences in study age, data acquisition and analysis software, occlusion time, and quality control. In another study, Rint measured in preterm infants during sedation at one year [18] was lower than in our study. This difference might be explained by the older age of study participants, since Rint decreases with increasing age [25]. Airway resistance in infants can be assessed with other methods, e.g. the single occlusion technique (SOT) [9], but methodological differences limit comparison. Van Putte-Katier et al. measured airway resistance with SOT in term infants [26] reporting a higher Rint compared to this study, possibly explained by methodological differences [26].

Rint values did not change significantly over subsequent measurements. Furthermore, raw Rint, versus linear-detrended Rint, did not differ, indicating no significant impact of trends during measurements. In children, Rint was stable over time [15,27] and results of this study suggest that longitudinal Rint measurements in infants might be feasible.

A relatively high CV in term, and preterm infants, during and between measurements was observed. Previous studies in preschool children reported a CV between 8% and 23% [14,28,29], and it was shown that age is inversely associated with CV of Rint [14,28]. The high CV could therefore be due to young age during measurements, but also due to methodological reasons. For example, during measurements in school-aged children the investigator supports the cheeks and throat to decrease upper airway compliance [30], while measurements in newborns are performed without

manual support. The use of a facemask did not result in more variability than a mouthpiece, but this was shown only for pre-school children [31], not for infants. Maturation processes of motor activity during infancy might further explain differences in CV: a study in healthy infants reported that variability of spontaneous movements decreased with increasing age [32]. We speculate that an age-dependent variability of spontaneous motor activity similarly applies for breathing parameters, which may further explain a higher CV in infants compared to children [14,15].

Reduced airway function at school age in former preterm infants is well-documented [33-35]. Increased specific respiratory resistance [33] and decreased expiratory flows were reported in former preterm infants assessed at 11 years [33] and 6 years of age [35]. However, it was recently reported that Rint measured at 6 years was not associated with prematurity [36]. The latter contradicts previous studies [33,35], and the authors speculated that the small number of preterm infants in their study might have resulted in insufficient power to detect differences in Rint [36]. Despite methodological differences in assessing airway obstruction in this study compared to studies in former preterm children [33,35], the higher Rint of our preterm infants indicate that airway obstruction reported in former preterm children at school-age can be observed at early at infancy.

Variability of measurements could be considered noise, but could also be regarded as a biological signal *per se*. We observed a lower CV of Rint in preterm compared to term born infants, and a similarly, lower short-term variation in preterm infants was reported [37]. Thus, less variability during tidal breathing in preterm infants suggests an association between immaturity and breathing control. It is unclear whether this finding is due to residual lung disease, differences in central regulation, or both. In biological systems in general, variability of physiological parameters are considered a healthy state [38]. Studies comparing other physiological parameters (tidal breathing [37], heart rate [39]) between term and preterm infants reported less variability in preterm infants. The question whether or not lower Rint variability in preterm

infants could have prognostic relevance for subsequent respiratory morbidity can only be addressed in longitudinal studies.

Strengths and limitations

Strengths of this study are that measurements were performed according to current ATS/ERS standards [8] using the same equipment throughout the study. Data quality was further improved by manual inspection and rigorous assessment. Only high quality measurements were included, diminishing the influence of technical issues, which could have influenced e.g. intra-test variability. Measurements were performed during unsedated sleep, reflecting a more natural state of breathing, which is relevant to reliably study breathing mechanics [40].

A limitation is that no quality criteria were applied during measurement recording. With the only available software, however, data analysis can only be performed after measurements. Success rate in this study (14% to 26%) was low, but a similar rate was previously reported in infants (12%) [7]. The low success rate is not unsurprising, since measurements were performed during natural sleep without sedation. Rint in term and preterm infants was higher in Basel compared to Bern. This may be partly explained by differences in postmenstrual age and weight at study between centers. However, these inter-center differences are unlikely to bias our results since associations with prematurity were similar after adjustment and stratification by center. Since this matched case-control study was embedded within a prospective cohort, 15 term infants from Bern were excluded to obtain similar group sizes. However, a selection bias is unlikely, since Rint, CV and anthropometrics of included versus excluded subjects did not differ. Mean number of interruptions in preterm infants were higher compared to term infants. One could speculate that this resulted in a lower CV in preterm infants. We feel that this is unlikely, since CV during the first 5 interruptions was also higher in preterm infants, and a similar trend was observed in a subgroup of infants at interruptions 40-45. We further acknowledge the relatively low sample size which limits our conclusions, particularly in regards to inter-measurement variability.

Conclusion

In conclusion, this study provides first evidence of higher airway resistance and lower variability during interrupter measurements in unsedated preterm compared to term infants, suggesting differences in breathing mechanics during early infancy. Measurements were performed during natural sleep, which is more representative of normal breathing in these infants, but is consistent with findings of increased Rint in sedated pre-school children. Future studies need to assess whether Rint can be used as a prognostic marker for subsequent respiratory morbidity taking into consideration the high variability of measurements *per se* which require further interpretation.

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

Contributions PL and UF conceived the study. Measurements were recorded by P.A., I.K., O.G., D.D., J.U., P.L. and U.F. Statistical analyses were done by D.D. and J.U.; S.S. assisted in patient recruitment. J.U, D.D. S.S. and P.L drafted the manuscript; all authors have seen and approved the final manuscript.

Patient consent Obtained.

Ethics approval Ethical approval for the study was obtained at each site.

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Table 1 Population characteristics

	Term (n=50)	Preterm (n=48)	*P*-value
Anthropometrics			
Male sex	21 (42)	29 (60.4)	0.068
Postmenstrual age at birth, weeks	39.7 ± 1.2 (37–41.9)	30.1 ± 3.0 (24.6–35.4)	<0.001
Postmenstrual age at study date, weeks	44. ± 1.2 (41.8–47.0)	44.6 ± 1.6 (39.6–50.6)	0.319
Length at birth, cm	49.5 ± 2.6 (42–57)	39.2 ± 4.7 (31–50)	<0.001
Length at study date, cm	55 ± 2.3 (50 –59)	53.4 ± 2.8 (47–60)	0.002
Weight at birth, kg	3.4 ± 0.5 (2.1–4.3)	1.4 ± 0.6 (0.6–2.8)	<0.001
Weight at study date, kg	4.4 ± 0.6 (3.1–5.7)	4.3 ± 0.7 (3.0–5.4)	0.123
Clinical parameters			
Mechanical ventilation, days	n/a	1.9 ± 4.2 (0–27)	
Oxygen therapy, days	n/a	32.4 ± 34 (0–133)	
CRIB score	n/a	2.5 ± 2.9 (0–9)*	
Apgar score at 5 min	9.2 ± 0.9 (5–10)	7.6 ± 1.4 (5–9)	<0.001
Chorioamnionitis	1 (2)	20 (41.7)	<0.001
Patent ductus arteriosus	0 (0)	3 (3.1)	0.073
Atrial septal defect	0 (0)	2 (4.2)	0.145

Values are means ± SD (range) or number (percentage) of infants. *Data available on n=45 infants, missing data on n=3. Differences between term and preterm infants were tested with *t*-test and χ^2-test.

CV, coefficient of variation; CRIB, clinical risk index for babies; n/a, not applicable; Rint, airway resistance; SD, standard deviation.

Table 2 Rint and coefficient of variation of measurements

	Term (n=50)	Preterm (n=48)	Mean difference (95% CI)	*P*-value
Rint1	4.2 ± 1.9 (1.8–11.4)	5.6 ± 2.8 (2.0–15)	-1.35 ± 0.49 (-2.3 to -0.38)	0.007
CV1	29.6 ± 14.9 (7.2–70.4)	20.2 ± 8.4 (6.5–39.6)	9.41 ± 2.46 (4.53 to 14.30)	<0.001
$Rint_{\_a}$	3.9 ± 1.7 (1.8–8.8)	5.2 ± 2.7 (1.7–14.9)	-1.35 ± 0.46 (-2.26 to -0.45)	0.004
$CV_{\_a}$	24.6 ± 16.4 (4.2–82.3)	17.8 ± 8.3 (4.8–43.8)	6.83 ± 2.64 (1.59 to 12.07)	0.011
$Rint_{\_i}$	4.6 ± 2.1 (3.0–8.9)‡	6.6 ± 3.7 (2.2–14.3)‡‡	-1.94 ± 1.17 (-4.36 to 0.47)	0.11
$CV_{\_i}$	16.7 ± 7.6 (3.3–29.1)‡	12.2 ± 7.7 (1.7–31.4)‡‡	4.47 ± 2.95 (-1.62 to 10.55)	0.143
Rint2	4.2 ± 2.2 (1.8–11.8)*	5.3 ± 2.7 (1.7–11.1)**	-1.02 ± 0.72 (-2.47 to 0.44)	0.167
CV2	28.3 ± 11.4 (7.3–55.4)*	20.9 ± 9.3 (8.9–42.3)**	7.36 ± 3.08 (1.16 to 13.56)	0.021

Values are means ± SD (range). Rint values are given in $kPa \cdot s \cdot L^{-1}$ and CV in %. Rint1 and CV1 contain all data from the first measurement. $Rint_{\_a}$ and $CV_{\_a}$ contain data from the first 5 interruptions, whereas $Rint_{\_i}$ and $CV_{\_i}$ contain data from the 9^{th} group of interruptions. Rint2 and CV2 contain all data from the second measurement (described in Methods and Supplemental Figure S1). ‡Data available on n=13 infants, missing data on n=37. ‡‡Data available on n=14 infants, missing data on n=34. *Data available on n=24 infants, missing data on n=26. **Data available on n=22 infants, missing data on n=26. Differences between term and preterm infants were tested with *t*-test.

CV, coefficient of variation; Rint, airway resistance; SD, standard deviation.

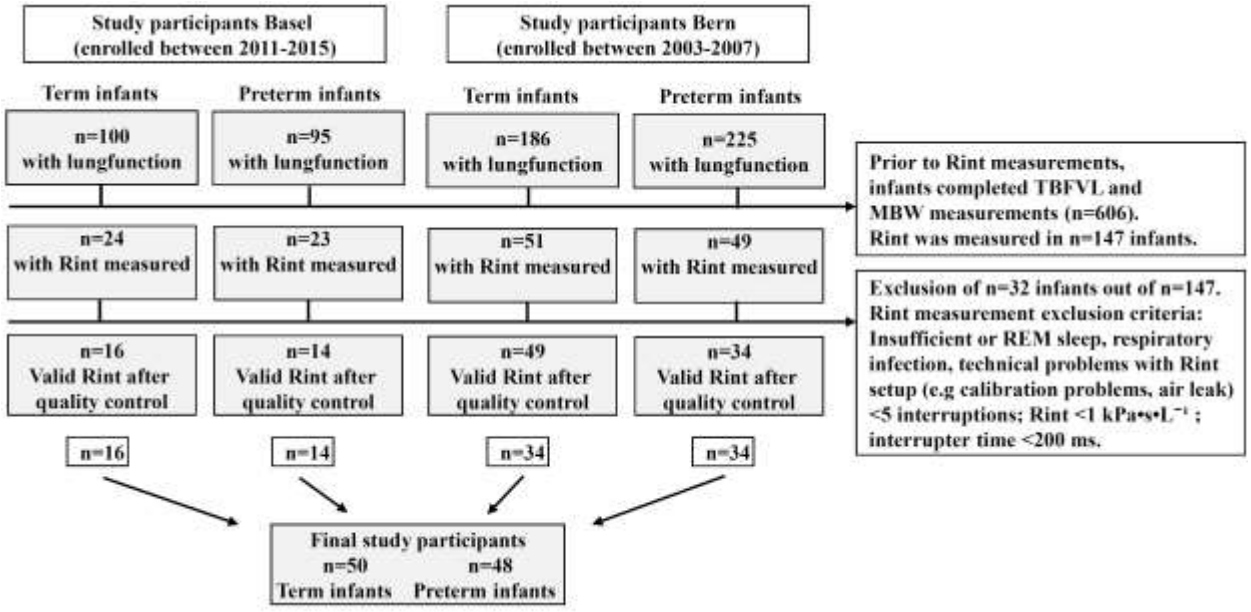

Figure 1 Flow chart of the study population. Numbers represent valid lung function tests and Rint measurements and datasets excluded from analysis. To obtain equal group sizes from term and preterm infants enrolled in Bern, 34 out of 49 term infants were matched to 34 preterm infants according to study year and number of interruptions.

MBW, multiple breath washout; REM, rapid eye movement; TBFVL, tidal breathing flow–volume loop; Rint, airway resistance.

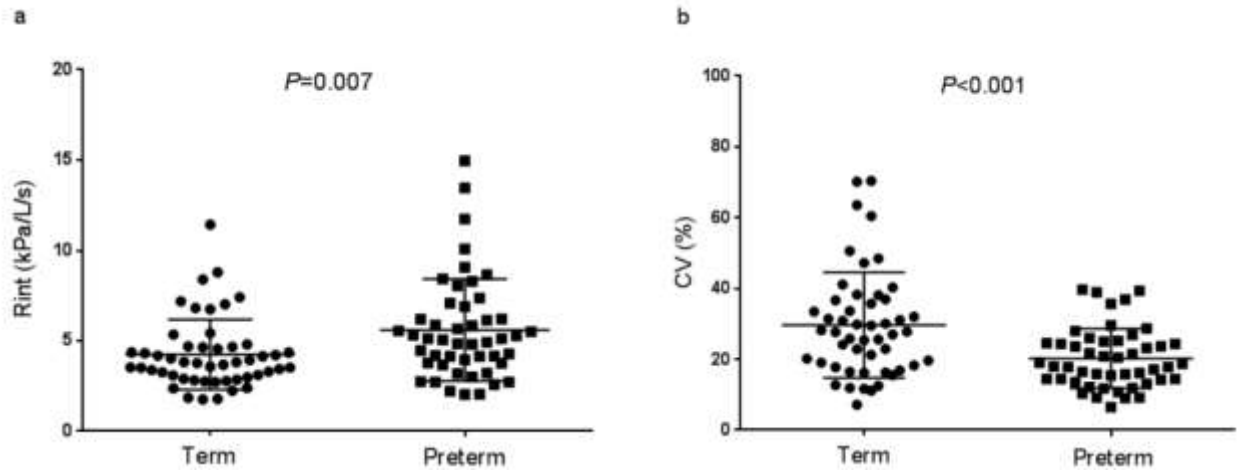

Figure 2 Comparison of Rint and CV between term and preterm infants. (a) Mean Rint1 from all interruptions in term infants was 4.2 ± (SD; 1.9) kPa·s·L^{-1} and in preterm infants was 5.6 ± (2.8) kPa·s·L^{-1}. (b) Mean CV1 from all interruptions in term infants was 29.6 ± (14.9) % and preterm infants was 20.2 ± (8.4) %. Horizontal line represents mean ± SD values; *P*-values refer to *t*-test comparing term versus preterm infants.

CV, coefficient of variation; Rint, airway resistance; SD, standard deviation.

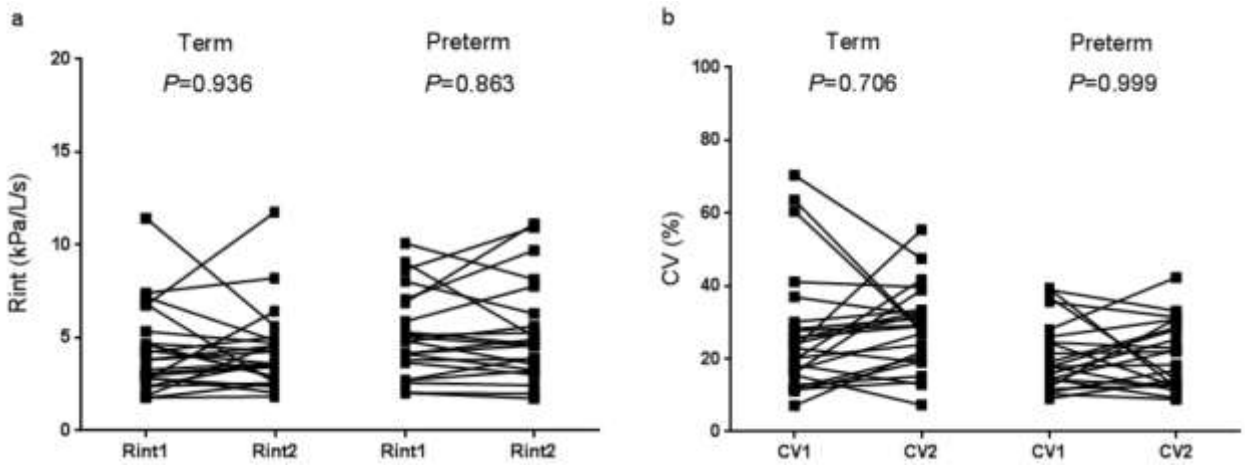

Figure 3 Inter-measurement and intra-subject variability of Rint and CV in term and preterm infants. Left panel (a) Mean Rint of the first measurement (Rint1) in term infants (n=24) was 4.3 ± (SD; 2.3) kPa·s·L^{-1} and of the second measurement (Rint2) was 4.2 ± (2.2) kPa·s·L^{-1}. Right panel (a) Mean Rint of the first measurement in preterm infants (n=22) was 5.1 ± (2.3) kPa·s·L^{-1} and of the second measurement was 5.3 ± (2.7) kPa·s·L^{-1}. Left panel (b) Mean CV of the first measurement (CV1) in term infants was 26.7 ± (16.9) % and of the second measurement (CV2) was 28.3 ± (11.4) %. Right panel (b) Mean CV of the first measurement in preterm infants was 20.9 ± (9.6) % and of the second measurement was 20.9 ± (9.3) %. Variability between two successive measurements for Rint ranged in term infants from -86.5% to 90.6% and in preterm infants from -47.1 to 56.3%. Variability between two successive measurements for CV ranged in term infants from -101.5% to 77.5% and in preterm infants from -87.2% to 105.7%. *P*-values refer to *t*-test comparing the first and the second measurement.

CV, coefficient of variation; Rint, airway resistance; SD, standard deviation.

SUPPLEMENTARY INFORMATION

Interrupter technique in infancy: higher airway resistance and lower short-term variability in preterm versus term infants

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Supporting results

Rint and CV at different time points of one measurement

We first compared the first group of interruptions of one measurement between term and preterm infants: Mean $Rint_{\_a}$ in term infants was 3.9 ± (SD; 1.7) $kPa \cdot s \cdot L^{-1}$ and in preterm infants was 5.2 ± (2.7) $kPa \cdot s \cdot L^{-1}$. Mean $Rint_{\_a}$ was significantly lower in term compared to preterm infants with a mean difference of -1.35 (95% CI -2.26 to -0.45) $kPa \cdot s \cdot L^{-1}$. The corresponding mean $CV_{\_a}$ in term infants was 24.6 ± (16.4) % and in preterm infants was 17.8 ± (8.3) %. Mean $CV_{\_a}$ was significantly higher in term compared to preterm infants with a mean difference of 6.83 (95% CI 1.59 to 12.07) %. Secondly, we compared the 9^{th} group of interruptions of one measurement obtained in a subgroup of 13 term and 14 preterm infants. Mean $Rint_{\_i}$ was lower in term compared to preterm infants: $Rint_{\_i}$ mean difference was -1.94 (95% CI -4.36 to 0.47) $kPa \cdot s \cdot L^{-1}$. Mean $CV_{\_i}$ was higher in term compared to preterm infants: $CV_{\_i}$ difference was 4.47 (95% CI -1.62 to 10.55) % (Table 2).

E-Table 1 Characteristics of study participants in children's hospitals from Basel and Bern

	Basel		Bern	
	Term n=16	Preterm n=14	Term n=34	Preterm n=34
Male sex	5 (31.3)	8 (57.1)	16 (47.1)	21 (61.8)
Postmenstrual age at birth, wks	39.4 (1.2)	31.7 (1.7)	39.9 (1.2)	29.4 (3.2)
Postmenstrual age at study date, wks	44.7 (1.2)	44.6 (1.9)	45 (1.2)	44.6 (1.6)
Length at birth, cm	47.9 (2.8)*	42.4 (4.1)	50.2 (2.2)*	37.9 (4.4)§
Length at study date, cm	54.2 (2.7)	54.6 (3.4)*	55.4 (2.0)	52.8 (2.3)*
Weight at birth, kg	3.2 (0.5)	1.7 (0.5)*	3.4 (0.4)	1.3 (0.6)*
Weight at study date, kg	4.3 (0.6)	4.5 (0.6)	4.5 (0.5)	4.2 (0.7)
Rint1, $kPa \cdot s \cdot L^{-1}$	3.7 (1.7)	4.3 (1.5)*	4.5 (2.0)	6.1 (3.1)*
CV1, %	33.0 (14.4)	22.9 (9.2)	28.1 (15.1)	19.1 (8.0)

Values are numbers (percentage) or means ± (SD).

§ Data available for n=33 infants, missing data on n=1. $Rint_{\_1st\ \_round}$ and $CV_{\_1st\ \_round}$ were calculated from all data from the first round of measurements. Differences between centers for term and preterm infants were tested with *t*-test and χ^2-test; *P-values <0.05.

Rint, airway resistance; CV, coefficient of variation; SD, standard deviation; wks, weeks.

E-Table 2 Comparison of characteristics between all and selected Bernese term infants

	All subjects n=49	Selected subjects n=34	*P*-value
Male sex	26 (53.1)	16 (47.1)	0.289
Postmenstrual age at birth, wks	35.0 (4.4)	35.8 (4.8)	0.477
Postmenstrual age at study date, wks	39.9 (1.1)	39.9 (1.2)	0.995
Length at birth, cm[§]	50.1 (2.2)	50.2 (2.2)	0.774
Length at study date, cm	55.3 (2.1)	55.4 (2.0)	0.840
Weight at birth, kg	3.5 (0.5)	3.4 (0.4)	0.649
Weight at study date, kg	4.5 (0.5)	4.5 (0.5)	0.878
Rint1, $kPa \cdot s \cdot L^{-1}$	4.4 (1.9)	4.5 (2.0)	0.773
CV1, %	27.7 (13.7)	28.1 (15.1)	0.906

Values are numbers (percentage) or means ± (SD).

[§]Missing data on n=1 infant. Rint1 and CV1 were calculated from all data from the first round of measurements. Differences between centers for all subjects and selected subjects were tested with *t*-test and χ^2-test.

Rint, airway resistance; CV, coefficient of variation; SD, standard deviation; wks, weeks.

E-Table 3 Association of prematurity with Rint and CV by study center

	Unadjusted model			Adjusted model[+]		
	Coefficient	95% CI	*P*-value	Coefficient	95% CI	*P*-value
	All infants[*]					
Rint1	1.3	0.4 to 2.2	0.007	1.2	0.3 to 2.2	0.012
CV1	-9.3	-14.1 to -4.4	<0.001	-9.8	-14.8 to -4.7	<0.001
	Infants from Bern					
Rint1	1.6	0.4 to 2.9	0.011	1.5	0.3 to 2.2	0.032
CV1	-8.9	-14.8 to -3.1	0.003	-9.0	14.8 to -4.7	0.006
	Infants from Basel					
Rint1	0.6	-0.7 to 1.8	0.356	0.9	-0.3 to 2.1	0.139
CV1	-10.1	-19.3 to -0.9	0.032	-10.7	-20.4 to -0.9	0.003

Coefficient and 95% CI for the association of prematurity with Rint ($kPa \cdot s \cdot L^{-1}$) and CV (%) derived from linear regression analysis. [+] This model was adjusted for sex and weight at study date. [*]For analysis of all infants, the adjusted model was additionally adjusted for study center. Mean Rint1 and mean CV1 contain all data from the first measurements.

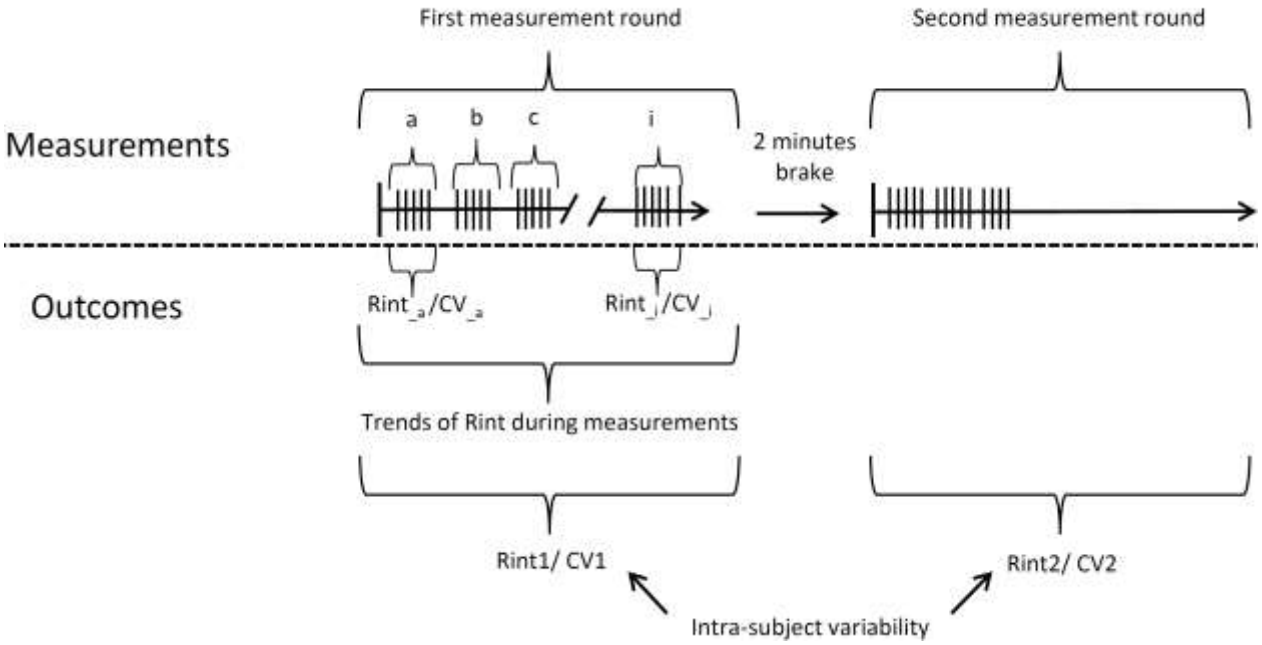
Rint, airway resistance; CV, coefficient of variation.

E-Table 4 Association of clinical parameters with Rint and CV in preterm infants

	Unadjusted model			Adjusted model[+]		
	Coeff,	95% CI	*P*-value	Coeff.	95% CI	*P*-value
			Outcome Rint1			
Mechanical ventilation	0.32	0.11 to 0.54	0.570	0.24	-0.17 to 0.22	0.804
Oxygen therapy therapy, days	0.32	0.19 to 0.54	0.004	0.31	0.03 to 0.56	0.029
CRIB score	0.09	-0.19 to 0.39	0.503	-0.04	-0.37 to 0.28	0.503
			Outcome CV1			
Mechanical ventilation	-4.33	-10.1 to 1.35	0.132	-3.93	-9.81 to 1.95	0.185
Oxygen therapy therapy, days	-0.43	-1.12 to 0.28	0.234	-0.19	-1.05 to 0.66	0.651
CRIB score	-0.21	-1.07 to 1.13	0.503	-0.21	-1.14 to 0.73	0.665

Coefficient and 95% CI for Rint ($kPa \cdot s \cdot L^{-1}$) and CV (%) per ten day increase in mechanical ventilation/oxygen therapy or for one score in increase in CRIB derived from linear regression analysis. [+] This model was adjusted for sex and weight at study date. Mean Rint1 and mean CV1 contain all data from the first measurements.

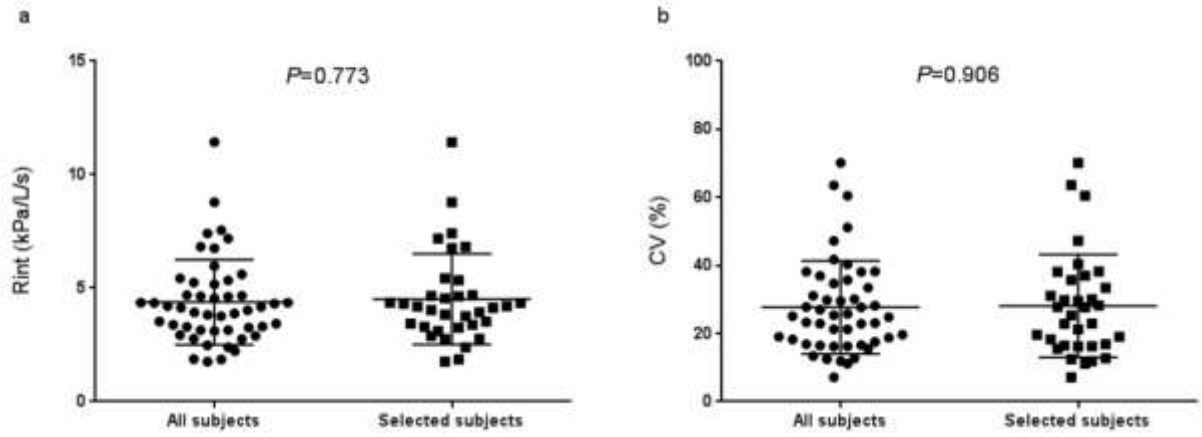
Rint, airway resistance; Coeff, coefficient; CV, coefficient of variation; CRIB: clinical risk index for babies.



E-Figure 1 Classification of Rint measurements and outcomes assessed. First measurement was designated Rint1, containing all interruptions of the first measurement. The second measurement was designated Rint2, contained all interruptions of the second measurement. Rint1 was further divided into groups of 5 interruptions named in alphabetical order ($Rint_{a}$ to $Rint_{i}$). Thus, $Rint_{a}$ to $Rint_{i}$ contained 9×5=45 interruptions.

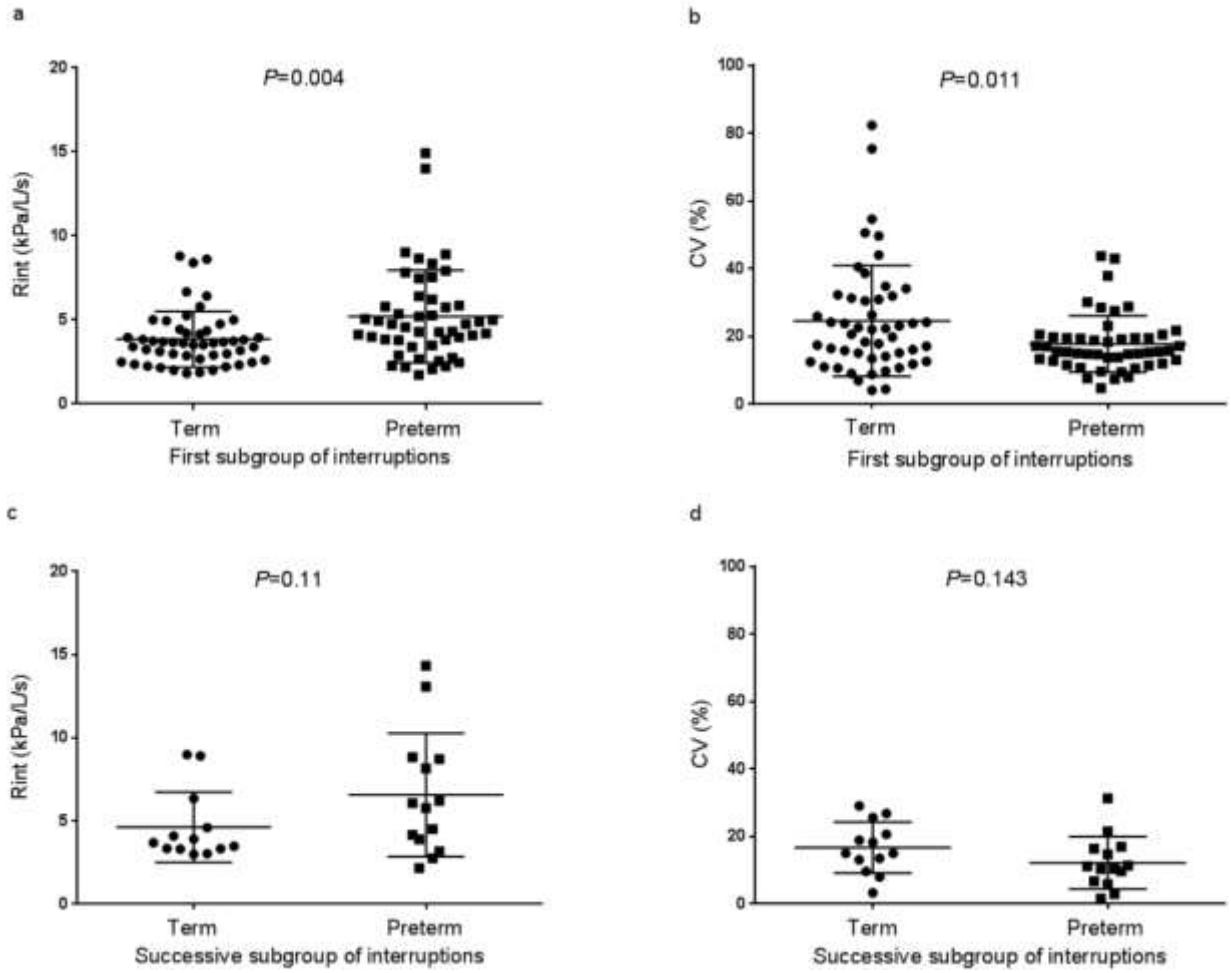
The following outcomes were compared between term and preterm infants: i) Rint and corresponding CV at different time points during measurements (Rint1/CV1, $Rint_{a}/CV_{a}$ $Rint_{i}/CV_{i}$ and Rint2/CV2) ii) trends of Rint during one measurement iii) and intra-subject variability in term and preterm infants between two subsequent measurements.

Rint, airway resistance.



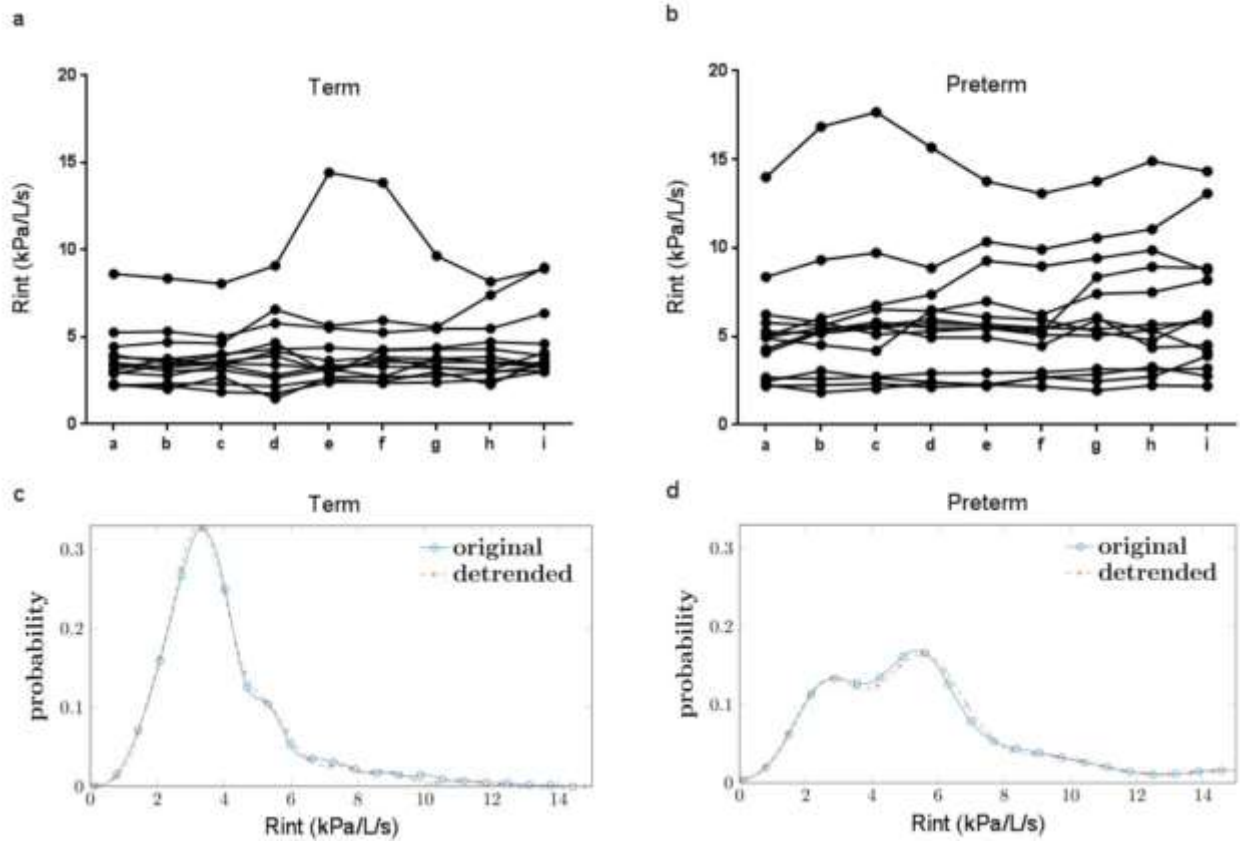
E-Figure 2 Comparison of Rint between all and selected Bernese term infants. (a) Mean Rint of all term infants (n=49) was 4.4 ± (SD 1.9) kPa·s·L^{-1} and of selected term infants (n=34) 4.5 ± (2) kPa·s·L^{-1} (*P*=0.773). (b) Mean CV of all term infants was 27.7 ± (13.7) % and of selected term infants was 28.1 ± (15.1) % (*P*=0.905). Horizontal line represents mean ± SD values. *P*-values refer to *t*-test comparing all infants versus selected term infants.

CV, coefficient of variation; Rint, airway resistance; SD, standard deviation.



E-Figure 3 Robustness of differences in Rint and CV between term and preterm infants. (a) Mean Rint of the first five interruptions ($Rint_{\_a}$) in term infants (n=50) was 3.9 ± (SD; 1.7) kPa·s·L^{-1} and in preterm infants (n=48) was 5.2 ± (2.7) kPa·s·L^{-1}. (b) In these infants, mean CV ($CV_{\_a}$) in term infants was 24.6 ± (16.4) % and in preterm infants was 17.8 ± (8.3) %. (c) Mean Rint of interruptions 40-45 ($Rint_{\_i}$) in term infants (n=13) was 4.6 ± (2.1) kPa·s·L^{-1} and in preterm infants (n=14) was 6.6 ± (3.7) kPa·s·L^{-1}. (d) In these infants, mean CV ($CV_{\_i}$) in term infants was 16.7 ± (7.6) % and in preterm infants was 12.2 ± (7.7) %. Horizontal line represents mean ± SD values; *P*-values refer to *t*-test comparing term versus preterm infants.

CV, coefficient of variation; Rint, airway resistance; SD, standard deviation.



E-Figure 4 Trends of Rint during one measurement in term and preterm infants. (a-b) Trends of Rint were assessed using 9 groups of Rint measurements ($Rint_a$ to $Rint_i$, resulting in 45 interruptions) in 13 term and 14 preterm infants. (c-d) The probabilities between raw Rint values and linear-detrended-Rint values were compared in term and preterm infants using the Kernel smoothing density function. Open circle line represents original values and crossed line represents linear-detrended-Rint values. There was no difference between original Rint and linear-detrended-Rint for (c) term (P=0.939) and (d) preterm infants (P=0.932). P-values refer to ANOVA-test comparing original versus linear-detrended values in term and preterm infants.

CV, coefficient of variation; Rint, airway resistance.

3.3 Methodological challenges to assess airway inflammation

3.3.1 Exhaled nitric oxide and asthma development

Predictive value of exhaled nitric oxide in healthy infants for asthma at school age

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RESEARCH LETTER
IN PRESS | CORRECTED PROOF

Predictive value of exhaled nitric oxide in healthy infants for asthma at school age

To the Editor:

Exhaled nitric oxide fraction (F_{ENO}) is a noninvasive biomarker that is elevated in subjects with asthma and allergic diseases [1]. Nitric oxide (NO) is produced by three NO synthase (NOS) enzymes: neuronal, endothelial and inducible (iNOS), all present in the human lung [2]. The increase of F_{ENO} in asthmatic patients has mainly been attributed to an activation of iNOS, mediated through proinflammatory cytokines in the airways [3].

Early monitoring of airway inflammation assessed by elevated F_{ENO} provides information on asthma evolvement and helps to identify subjects at risk. Few studies have investigated the association between F_{ENO} during early childhood and asthma at school age. In pre-school children with recurrent respiratory symptoms, those with higher F_{ENO} were at an increased risk of asthma at 6 years [4]. In a selected cohort of infants with eczema, increased F_{ENO} prior to any wheezing episodes was associated with an increased risk of asthma at 5 years [5]. Notably, in these studies, F_{ENO} was measured in high-risk children who were already exposed to environmental factors known to modify F_{ENO} levels [6–8].

The value of F_{ENO} after birth to predict later symptoms before relevant exposure to environmental factors has been prospectively investigated in only two studies [9, 10]. LATZIN *et al.* [9] reported that infants born to atopic mothers had increased F_{ENO} prior to respiratory symptoms and that this association was enhanced in mothers who smoked. In infants born to asthmatic mothers, CHAWES *et al.* [10] showed that increased F_{ENO} was associated with recurrent wheezing episodes during the first year of life but not thereafter.

Taken together, while there is cumulative evidence that elevated F_{ENO} in high-risk children after a possible impact by environmental exposures is associated with later asthma [4, 5], it is unknown if F_{ENO} after birth, prior to a possible influence by post-natal environmental exposures and first respiratory symptoms, is associated with asthma. Given that environmental factors are known to induce NOS activity and modify F_{ENO} [6, 7, 11, 12], we hypothesised that F_{ENO} measured after birth, and before relevant exposure to these factors, is not associated with asthma at school age.

The aim of this prospective cohort study was to investigate if F_{ENO} levels after birth in unselected newborns are associated with asthma or atopy at school age. This prospective birth cohort study comprised of unselected, healthy, term-born infants recruited in the region of Bern, Switzerland [13]. At 5 weeks of age, F_{ENO} was measured from multiple breaths during natural sleep, as previously described [9, 14], with a rapid response chemiluminescence analyser (CLD 77; EcoMedics, Duernten, Switzerland) (analysis software: WBreath version 3.28.0.0; ndd, Zurich, Switzerland).

At 6 years of age, asthma was assessed by study physicians with questions adapted from the ISAAC (International Study of Asthma and Allergies in Childhood) questionnaire [15], defined as a history of wheezing within the 12 months prior to follow-up. Study physicians assessed atopy, defined as allergic asthma, allergic rhinitis, atopic dermatitis or positive skin-prick test (SPT). An SPT including seven common allergens was determined to be positive in the case of hives bigger than positive control, histamine, with any of the tested allergens [13]. We assessed risk factors for asthma or atopy of the child: parental asthma was defined as self-reported or doctor-diagnosed asthma; parental atopic disease was defined as allergic asthma, hay fever or eczema by history.

The Governmental Ethics Committee of the Canton of Bern, Switzerland approved the study and informed, written consent was obtained at enrolment.

Logistic regression was used for analysis of an association between F_{ENO} at birth and asthma, atopy and positive SPT at school age. Adjustment for confounders (*e.g.* parental asthma and atopy) and nonconfounding factors (minute ventilation, which is known to modify F_{ENO} [16]) was done. Linear regression was used for the analysis of an association between parental variables and F_{ENO} at birth. Data are presented as odds ratios or difference in F_{ENO} (in parts per billion) with 95% confidence intervals. Data were analysed with STATA 13 (STATA Corporation, College Station, TX, USA). Patient characteristics for those with and without follow-up were compared using Mann–Whitney U-test and Chi-squared test.

We measured F_{ENO} in 278 infants with 44 (16%) being excluded for technical reasons, resulting in 234 subjects. Of those 26 (12%) were lost to follow-up, resulting in 208 final study participants. Demographics,

exposure to risk factors and F_{ENO} levels did not differ between subjects followed up and those lost to follow-up (data not shown). For the entire group, neither maternal atopy nor maternal smoking was associated with postnatal F_{ENO}. Smoking during pregnancy was only associated with decreased F_{ENO} levels in infants of nonatopic mothers (−2.91 ppb, 95% CI −5.76–−0.048 ppb). These findings were similar to previously published data [16], although the fraction of smoking mothers was lower in this study (9% *versus* 13% [16]). At 5 weeks of age, mean F_{ENO} was 13.9 ppb (range 1.8–32.9 ppb). Parents of 43 (20%) children had asthma and those of 126 (61%) were atopic; 19 (10%) mothers smoked during pregnancy. Among 6-year-olds (age range 5–7 years), 31 (15%) had asthma with 13 (6%) allergic asthma cases, and none of the children used corticosteroids. There were 62 (30%) atopics and in 164, an SPT was completed with 26 (19%) being positive.

F_{ENO} at birth was not associated with asthma, atopy or positive SPT at school age. Per 1-ppb increase in F_{ENO}, the simple and adjusted odds ratios for asthma were 0.99 (95% CI 0.92–1.07) and 0.97 (95% CI 0.89–1.06); for atopy, 0.99 (95% CI 0.94–1.05) and 0.99 (95% CI 0.94–1.06); and for positive SPT, 0.95 (95% CI 0.88–1.03) and 0.95 (95% CI 0.87–1.03), respectively (table 1).

Our study is the first to investigate the association between F_{ENO} at birth and asthma at school age in unselected infants. It was previously shown that the association of elevated F_{ENO} after birth with respiratory symptoms is restricted to infancy [10]. In this study, we found supporting evidence for this finding, since F_{ENO} after birth was not associated with diagnosis of school age asthma.

Based on this finding, and on previous studies in selected high-risk populations, we propose two different models of F_{ENO} metabolism in early infancy (figure 1). In model 1, elevated F_{ENO} is an expression of an intrinsic mechanism, determined by pre- and early postnatal risk factors. In this model, F_{ENO} levels during infancy would then not be altered by environmental factors, and F_{ENO} measured at birth could serve as a predictor for later asthma and atopy. In model 2, environmental factors (*e.g.* infections or air pollution) are needed to induce iNOS [6, 7, 11], which then results in elevated F_{ENO}. In this hypothesis, we would expect no association between F_{ENO} at birth (measured before environmental exposures) and school-age outcomes. F_{ENO} may then only serve as a phenotype-specific biomarker in infants after the first activation of the environmentally or genetically induced iNOS. We speculate that the second, rather than the first, model better explains NO metabolism after birth, since studies measuring F_{ENO} after environmental exposures found an association between F_{ENO} and asthma development, while our study, measuring F_{ENO} before environmental exposures, did not find any association.

Measurements were performed using a face mask, which is the only available technique for measuring F_{ENO} in infants at this time. This introduces the possibility that NO from the upper airways contributes to the overall F_{ENO} measured. However, we believe this potential contribution is unlikely due to the fact that the nasal sinuses of infants are not developed.

Asthma prediction with F_{ENO} might further be hampered by the physiological variability of F_{ENO} *per se*, by an intersubject variability of up to 50% [18], and influenced by different measurement techniques. In school-aged children, comparison of F_{ENO} measured from single *versus* multiple breaths resulted in higher F_{ENO} values using the latter technique [19]. Multiple-breath F_{ENO} measurement technique is, at this time, the only one available for infants. The present study is limited by the low number of asthmatics (n=31) and the questionnaire-based assessment for diagnosis, which could lead to possible misclassification. In general, the cohort reflects the epidemiological situation in Switzerland, with a low prevalence of mild-to-moderate

TABLE 1 Association between exhaled nitric oxide fraction (F_{ENO}) in newborns and subsequent diagnoses of asthma, atopy and positive skin-prick test (SPT) in school-aged children

Outcome	Exposure F_{ENO}			
	Simple[#] model		Adjusted[¶] model	
	OR (95% CI)	p-value	OR (95% CI)	p-value
Asthma[+]	0.99 (0.92–1.07)	0.970	0.97 (0.89–1.06)	0.586
Atopy[§]	0.99 (0.94–1.05)	0.976	0.99 (0.94–1.06)	0.971
SPT positive[f]	0.95 (0.88–1.03)	0.285	0.95 (0.87–1.03)	0.256

Odds ratios and 95% confidence intervals per 1-ppb increase in F_{ENO} were determined by logistic regression. [#]: F_{ENO} was adjusted for minute ventilation [16]; [¶]: additionally adjusted for sex, parental asthma, parental atopy and smoking during pregnancy; [+]: defined as a history of wheezing within the 12 months prior to follow-up; [§]: defined as the presence of asthma, allergic rhinitis, atopic eczema or positive prick test; [f]: positive in case of hives bigger than positive control, histamine, with any of the tested allergens.

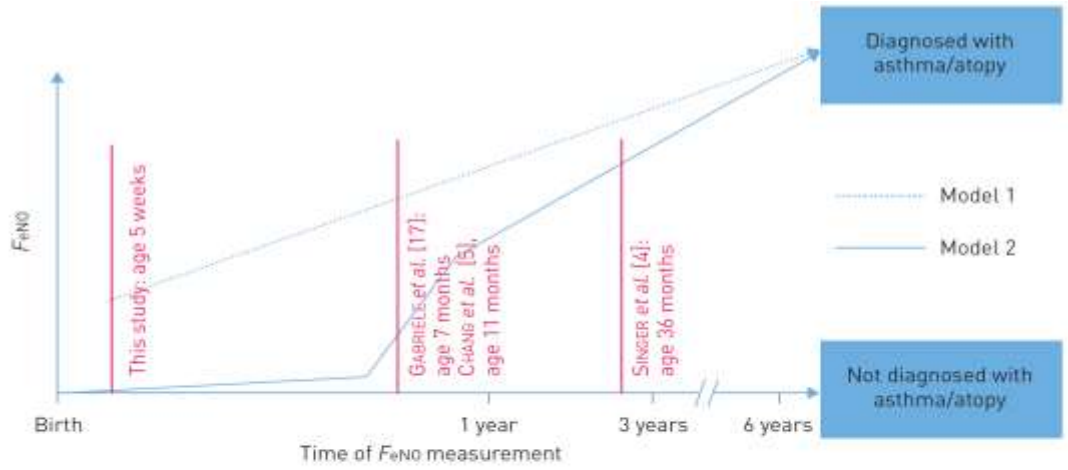

FIGURE 1 We measured exhaled nitric oxide fraction (F_{ENO}) at 5 weeks of age, while previous studies measured F_{ENO} in older children during time ranges. Based on this and previous studies, we propose two hypotheses for F_{ENO} metabolism during infancy. In model 1, elevated F_{ENO} is an expression of an intrinsic mechanism determined by pre- and early postnatal risk factors. In this model, F_{ENO} levels during the first year of life would then not be altered by environmental factors and F_{ENO} measured at birth could serve as a predictor for asthma/atopy at school age. In model 2, environmental factors (*e.g.* infections or air pollution) are needed to induce nitric oxide synthases, which then results in elevated F_{ENO}. In this model, we would expect no association between F_{ENO} at birth (measured before environmental exposures) and asthma/atopy at school age. F_{ENO} may then only serve as a phenotype-specific biomarker in infants after the first activation of environmentally induced nitric oxide synthesis.

asthmatics. Coincidentally, however, in our study sample, only mild intermittent asthmatics (without corticosteroid use in the last 12 months) were included, but this study was conducted in a prospective, unselected cohort, representing the general population. In contrast to previous studies in high-risk populations, we measured F_{ENO} at a single point in time after birth, excluding possible age- or time-dependent effects on F_{ENO}.

The interpretation of F_{ENO}, its predictive value and its modifiers are age dependent [4, 5, 7, 9–11]. Postnatal F_{ENO} metabolism seems to be modified by various environmental factors. On a cellular level, maternal tobacco smoke modified NOS activity in the fetal vascular bed in newborns [12]. Consistent with this observation, postnatal F_{ENO} is modified by prenatal tobacco smoke exposure in offspring [16, 17], interestingly enough, in an interaction with maternal atopy [16]. In contrast to the pre- and early postnatal situation, infancy and preschool age seems to be critical for further gene–environment interactions through exposures other than smoking and maternal atopy and their impact upon NO metabolism [4, 5].

In summary, we show that postnatal F_{ENO} measured in unselected healthy newborns is not associated with asthma diagnosis at school age. We speculate that NO metabolism may play a role in the pathophysiology of childhood asthma and atopy only after exposure to environmental factors at preschool age. To confirm that environmental exposures indeed modify NOS expression during infancy, frequent longitudinal assessment of F_{ENO} levels and NOS expression would be necessary. Our findings should encourage further research on factors impacting upon NO metabolism during infancy that can serve as targets for new preventive strategies on childhood asthma development.

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F_{ENO} in newborns before exposure to environmental factors is not associated with school-age asthma development http://ow.ly/ShQQ300xiN2

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3.3.2 Exhaled nitric oxide and Cystic Fibrosis

Lower exhaled nitric oxide in infants with Cystic Fibrosis compared to healthy controls

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Accepted J Cystic Fibr

Lower exhaled nitric oxide in infants with Cystic Fibrosis compared to healthy controls

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Abstract

Exhaled nitric oxide (FE_{NO}) is a well-known marker for airway inflammation. However, despite chronic airway inflammation, in patients with Cystic Fibrosis (CF) FE_{NO} is decreased. To understand if reduced FE_{NO} is primary related to Cystic Fibrosis Transmembrane Regulator (CFTR) dysfunction or an epiphenomenon of chronic inflammation, we measured FE_{NO} in infants with CF prior to clinical symptoms. FE_{NO} was lower in CF infants compared to healthy controls ($p=0.0006$) and the effect was more pronounced in CF infants without residual CFTR function ($p<0.0001$). This suggests that FE_{NO} is reduced in CF *a priori*, possibly associated with underlying CFTR dysfunction.

Introduction

The fractional concentration of exhaled nitric oxide (FE_{NO}) is a well-known biomarker for airway inflammation and elevated in a number of inflammatory disorders of the lung (1). However, despite chronic severe airway inflammation, in patients with Cystic Fibrosis (CF) FE_{NO} is decreased (2-4). The following underlying causes have been discussed: (i) reduced NO synthase isoenzyme (NOS) expression (ii) lack of NOS substrates, (iii) NO decomposition by bacterial reductases or (iv) impaired NO diffusion through viscous mucus (1, 5, 6). Reduced levels of NO or NOS have been related to a number of adverse effects, such as increased airway narrowing, reduced ciliary motility and susceptibility to infections (2, 7).

Absent or residual function of the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) protein in CF patients results in insufficient NOS induction, which however appears reversible (8). Reduced FE_{NO} normalizes in patients with CF after treatment with Ivacaftor, one of the first approved CFTR-targeting drugs for certain gating mutations (9). This suggests that decreased FE_{NO} in CF is not an epiphenomenon of chronic inflammation or infection, but might be reduced in CF airways *a priori*, associated with the defect in CFTR.

In order to understand whether reduced FE_{NO} in CF airways is primary related to CFTR mutation independent of secondary inflammation, FE_{NO} measurements in early life before the onset of first infections need to be explored. We thus measured FE_{NO} in infants with CF and healthy controls at five to twelve weeks of age.

Methods

We enrolled infants with CF diagnosed by newborn screening and contemporary healthy infants aged five to twelve weeks, matched 1:2 based on season of birth and sex, from two ongoing birth cohort studies, the Swiss Cystic Fibrosis Infant Lung Development (SCILD) cohort (10)) and the Basel Bern Infant Lung development cohort (11) born between 2011 and 2015.

Exclusion criteria was a history of respiratory symptoms suggesting upper- or lower respiratory tract infection prior to the study. Clinical examination was normal in all infants. Infants with

asymptomatic bacterial colonization were not excluded (n=5). Lung function measurements were performed five and twelve weeks of age in regular, quiet sleep as previously described (12). In brief, FE_{NO} measurements were obtained online using a tight fitting mask, with a rapid response chemiluminescence analyser (CLD 77; Eco Medics AG, Duernten, Switzerland; analysis software: WBreath 3.28, ndd, Zurich, Switzerland). To avoid contamination by ambient nitric oxide (NO), we used NO-free air for inspiration. FE_{NO} was measured during multiple breaths in quiet sleep, during the third quartile of expiration. Mean FE_{NO} was then calculated over 100 breaths. As FE_{NO} is flow dependent, results are presented for both FE_{NO} and V'NO (FE_{NO} multiplied with corresponding expiratory flow), which were the primary outcomes. Additionally, flow and respiratory rate are displayed as secondary outcomes. Details have been previously described (12-15). We compared NO levels between healthy controls and infants with CF. Subsequently, patients were stratified into CFTR groups with (i) no residual function (two copies of class I and/or class II mutations), (ii) residual function (infants with at least one copy of a class III-VI mutation, and (iii) unclassified mutations. As FE_{NO} data were not perfectly normally distributed in visual inspection, we illustrate average estimates in both mean and median. We thus performed both Wilcoxon-Man-Whitney test and linear regression after log transformation of variables and adjusted for possible confounders (age, sex, maternal atopy and smoking during pregnancy). The study was performed in Bern and approved by the Ethics committee Bern, Switzerland. Informed consent was obtained from the parents.

Results

We analysed 102 measurements in 34 infants with CF and 68 healthy controls. For anthropometric data and results of measurements, see Table 1.

Table 1: study population and FE_{NO} measurements

	healthy infants (n = 68)	infants with CF (n = 34)
Gender, female	33 (48.5)	12 (35.29)
Age at study date, wk	5 (1.2)	8 (2.5)
Gestational age, wk	39.5 (1.4)	38.7(1.7)
Length at birth, cm	50 (2.3)	50 (2.8)
Weight at birth, g	3400 (567)	3310 (569)
Weight at study date, g	4430 (485)	4970 (1413)
CF mutation*:		
No residual CFTR function (class I and/or II)		19 (56)
Residual CFTR function (class I/II and III-VI)		7 (21)
Unknown CFTR function		8 (23)
FE_{NO} ppb	17.0 (5.0)	13.7 (5.3)
FE_{NO} ppb median (IQR)	16.4 (13.8 – 18.7)	12.2 (10.2 – 16.3)
V'NO nl/min	46.3 (11.5)	38.5 (15.7)
Flow exp. ml/s	47.8 (11.0)	48.2 (11.1)
Respiratory Rate (1/min)	43.1 (8.5)	43.0 (8.5)

results are displayed in number (%) and mean (+/- SD) if not stated otherwise
*CF infants were grouped in 1: two known copies of class I and/or II mutations 2: one class I or II mutation plus one other mutation 3. >=1 mutation not classified or unknown mutation. All children in group #3 however had two copies of disease causing mutations.
wk= weeks

Significantly lower FE_{NO} and V'NO values were found in infants with CF compared to healthy controls (median difference for FE_{NO} 4.25 ppb, p=0.0006 and for V'NO 12.15 nl/min, p=0.002). Flow and respiratory rate were comparable between groups, thus lower FE_{NO} values were not due to differences in breathing patterns, confirmed by significant differences for V'NO. The stratified analysis based on CFTR function revealed that in infants without residual CFTR

function, FE_{NO} levels were even lower compared to healthy controls (median difference 4.4 ppb, p<0.0001; V'NO 13.25 nl/min, p=0.0001), see Figure 1.

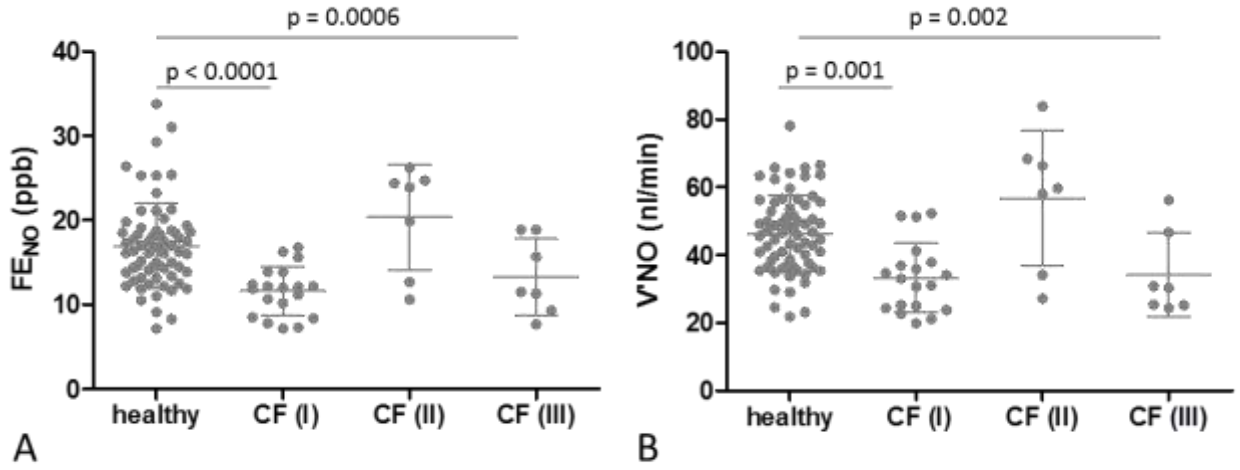

Figure 1: (A) FE_{NO} and (B) V'NO measurements in healthy infants (n = 68) and infant with CF with (I) no CFTR residual function (n=19), (II) with CFTR residual function (n=7) and (III) unknown CFTR function (n=8). Each dot symbolizes one infant. Lines indicate mean +/-SD.

FE_{NO} levels between infants with and without residual CFTR function also differed significantly (p=0.005), however numbers for analysis were low. All findings were confirmed when analysed in linear regression and after adjustment for confounders, assuring robustness of our results (see Table 2A and 2B).

Table 2A : Comparison of lung function measurements between healthy infants and CF infants

	unadjusted model			adjusted model		
	Coef	95% CI	p-value	Coef	95% CI	p-value
FeNo (log)	-0.24	[-0.37,-0.11]	<0.0001	-0.32	[-0.50,-0.15]	<0.0001
V'No (log)	-0.23	[-0.35,-0.10]	0.001	-0.27	[-0.44,-0.11]	0.002
Flow (log)	0.01	[-0.09,0.11]	0.85	0.05	[-0.09,0.18]	0.5
respiratory rate (log)	0	[-0.08,0.08]	0.97	0.03	[-0.07,0.14]	0.53

linear regression model comparing measurements between healthy infants (n = 58) and infants with CF (n= 34), after log transformation of variables to obtain normal distributuion. baseline are healthy infants, adjusted model : adjusted for gender, age at measurement, maternal atopy and smoking in pregnancy, Coef = beta Coefficient; 95% CI = 95% Confidence interval

Table 2B : Comparison of lung function measurements between healthy infants and CF infants with class I/II mutation

	unadjusted model			adjusted model		
	Coef	95% CI	p-value	Coef	95% CI	p-value
FeNo (log)	-0.37	[-0.52,-0.22]	<0.0001	-0.35	[-0.54,-0.16]	<0.0001
V'No (log)	-0.34	[-0.48,-0.20]	<0.0001	-0.2	[-0.37,-0.03]	0.03
Flow (log)	0.02	[-0.10,0.15]	0.73	0.15	[-0.02,0.31]	0.08
respiratory rate (log)	0.02	[-0.07,0.12]	0.63	0.09	[-0.04,0.22]	0.12

linear regression model comparing measurements between healthy infants (n = 58) and infants with CF and class I/II mutations (n= 19) , after log transformation of variables to obtain normal distributuion. baseline are healthy infants, adjusted model : adjusted for gender, age at measurement, maternal atopy and smoking in pregnancy, Coef = beta Coefficient; 95% CI = 95% Confidence interval

Five infants received antibiotic therapy prior or during measurements, indication was asymptomatic bacterial colonization (throat swab), but there was no association between therapy and FE_{NO} levels (results not shown).

Discussion

We show that exhaled FE_{NO} is reduced in young infants with CF and that this effect is more pronounced in infants without residual CFTR function. Importantly, FE_{NO} measurements were performed prior to first respiratory symptoms, suggesting absence of significant lower airway disease in the majority of children. Data were also robust after adjusting for other known

confounders such as atopy. Our results are in line with a previous preliminary study (five infants with CF) (16) and support our hypothesis that FE_{NO} is reduced in CF *a priori*. Low NOS expression seems related to CFTR mutation and residual function. Increase in FE_{NO} values following treatment with Ivacaftor in patients with a class IV mutation (9, 17) further supports the hypothesis that FE_{NO} is a proxy of CFTR function. It may be an important player in progression of CF lung disease, as reduced FE_{NO} is associated with reduced neutrophil sequestration, bacteriostatic properties, and mucociliary transport (2).

Thus, FE_{NO} levels in early life might be of prognostic value for disease development. Furthermore, treatment response in FE_{NO} after CFTR modifier therapy (9, 18) might be a promising biomarker as the measurement is fast and easy.

Although this clearly has to be confirmed in larger prospective studies, our results of lower FE_{NO} in CF airways early after birth, likely associated with underlying CFTR dysfunction, might open up a new chapter in the field of early FE_{NO} measurements in patients with CF.

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Disclosures

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3.4 Translational research: assessing complexity of airway disease

3.4.1 Multivariable approach: immune function and genetics

***CHI3L1* polymorphisms, cord blood YKL-40 levels and later asthma development**

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CHI3L1 polymorphisms, cord blood YKL-40 levels and later asthma development

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Abstract

Background: Single nucleotide polymorphisms (SNPs) in chitinase 3-like 1 (*CHI3L1*), the gene encoding YKL-40, and increased serum YKL-40 levels are associated with severe forms of asthma. It has never been addressed whether SNPs in *CHI3L1* and cord blood YKL-40 levels could already serve as potential biomarkers for milder forms of asthma. We assessed in an unselected population whether SNPs in *CHI3L1* and cord blood YKL-40 levels at birth are associated with respiratory symptoms, lung function changes, asthma, and atopy.

Methods: In a prospective birth cohort of healthy term-born neonates ($n = 260$), we studied *CHI3L1* polymorphisms, and measured cord blood YKL-40 levels by ELISA in ($n = 170$) infants. Lung function was performed at 5 weeks and 6 years. Respiratory health during the first year of life was assessed weekly by telephone interviews. Diagnosis of asthma and allergic sensitisation was assessed at 6 years ($n = 142$).

Results: The SNP rs10399805 was significantly associated with asthma at 6 years. The odds ratio for asthma was 4.5 (95 % CI 1.59–12.94) per T-allele. This finding was unchanged when adjusting for cord blood YKL-40 levels. There was no significant association for cord blood YKL-40 levels and asthma. SNPs in *CHI3L1* and cord blood YKL-40 were not associated with lung function measurements at 5 weeks and 6 years, respiratory symptoms in the first year, and allergic sensitisation at 6 years.

Conclusion: Genetic variation in *CHI3L1* might be related to the development of milder forms of asthma. Larger studies are warranted to establish the role of YKL-40 in that pathway.

Keywords: Asthma, *CHI3L1* protein, Children, Cohort study, Cord blood, Genetic association study, Genetic variation, Infants, YKL-40 protein

Background

The incidence of childhood asthma is increasing [1], and early identification of infants at risk could help in the prevention and treatment of this disease. Several studies have proposed that the recently discovered biomarker YKL-40 could be useful in the diagnosis of asthma [2]. The chitinase-like protein, YKL-40, is secreted by macrophages, neutrophils and epithelial cells: particularly in people with severe asthma [3, 4]. In serum, YKL-40 was elevated in children and adults with severe asthma [5–7], and inversely correlated with lung function measures [6, 7]. It was recently suggested that YKL-40 could be involved directly in airway remodeling [4, 5, 8]. Genetic studies revealed that variation in the gene encoding YKL-40, chitinase 3-like 1 (*CHI3L1*), contributes to the pathogenesis of asthma [6]. Genetic variation in *CHI3L1* was associated with pathological lung function values in adults [6], and correlated with poor asthma control and inflammatory markers in severe asthmatic children [5].

Direct involvement of YKL-40 in airway remodeling [4, 5, 8] lead us to hypothesise that early measurement of YKL-40 levels might help identify infants at risk for asthma. Until now, only one longitudinal study in high-risk asthmatic children assessed single nucleotide polymorphisms (SNPs) in *CHI3L1* and cord blood YKL-40 levels at birth. The authors identified genotype-specific effects on circulating YKL-40 levels, but no association

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of SNPs in *CHI3L1* and cord blood YKL-40 with asthma at school age was found [6]. It remains unknown if genetic variations in *CHI3L1* or cord blood YKL-40 levels assessed at birth in unselected infants are associated with milder forms of childhood asthma.

We studied in a birth cohort of unselected infants the association of SNPs in *CHI3L1* and cord blood YKL-40 levels with asthma development. We further assessed the correlation of SNPs in *CHI3L1* and cord blood YKL-40 levels with respiratory symptoms in the first year of life, lung function measures, and allergic sensitisation.

Methods

Methods are detailed in Additional file 1.

Study design and subjects

This prospective birth cohort study comprised a group of unselected, healthy neonates recruited antenatally in the region of Bern, Switzerland. The Ethics Committee of the region of Bern approved the study, and written consent from all parents was acquired at enrolment.

Genotyping and marker selection

Genome-wide SNP genotyping was conducted in collaboration with asthmagene.de (University of Regensburg, Germany) using Illumina HumanOmniExpress Bead Chips (Illumina Inc., San Diego, USA) according to the manufacturer's instructions. Details on genotyping and quality control methods are given in the online supplement. SNPs from a region 13 kb upstream and 1.2 kb downstream of CHI3L1 were chosen, as previously described [9]. The SNPs selection was done on HapMap CEU data (www.hapmap.org) PhaseII + III Rel28 10th of August 2015, on NCBI B36 assembly, dbSNP b126. Haploview [10] was used to calculate linkage disequilibrium (LD) and to select tagging SNPs with a minor allele frequency (MAF) >5 % and r^2 > 0.8. In total 7 SNPs were represented on the Chip and included in the analysis (Fig. 1).

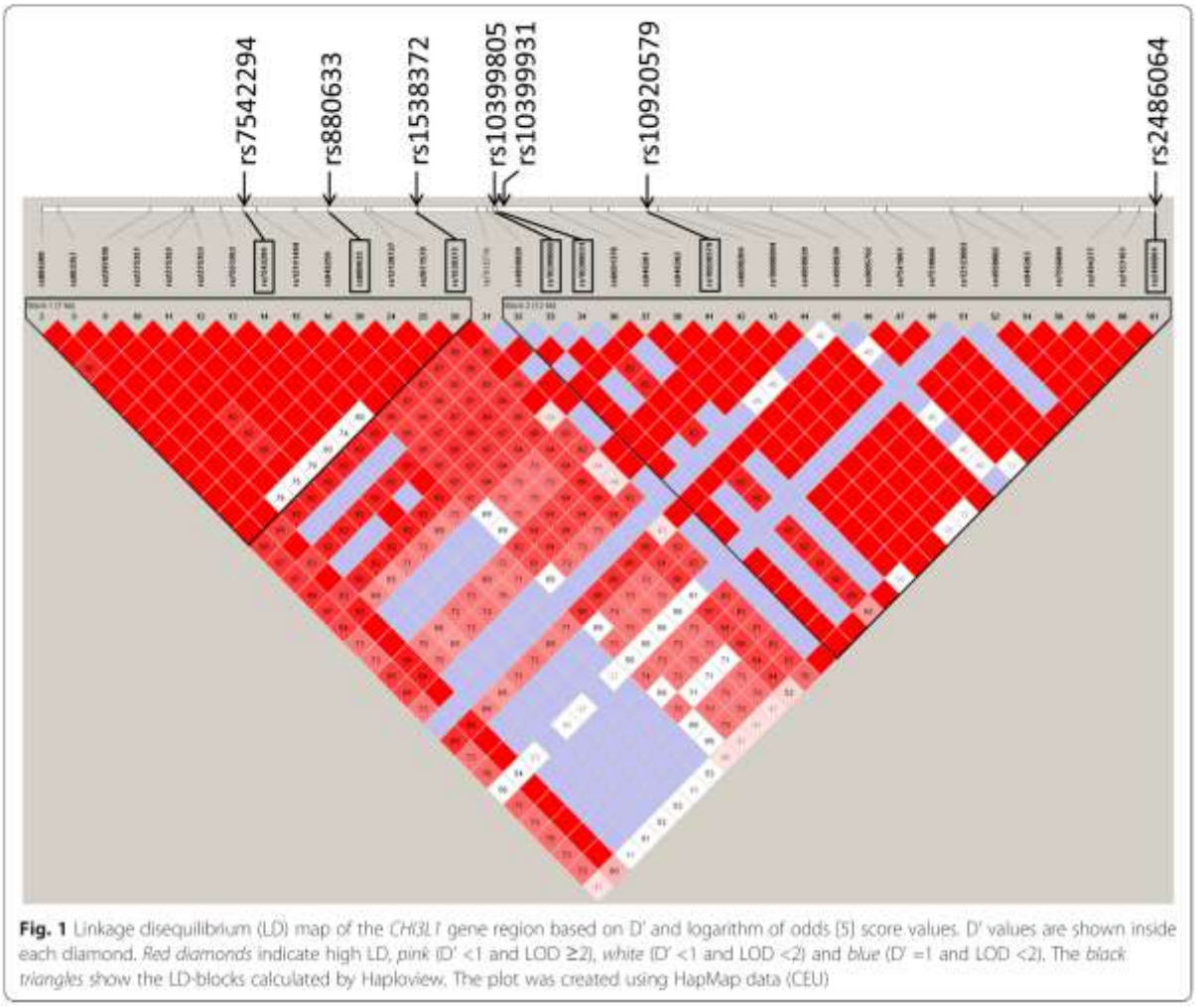

Fig. 1 Linkage disequilibrium (LD) map of the *CHI3L1* gene region based on D' and logarithm of odds (LOD) score values. D' values are shown inside each diamond. *Red diamonds* indicate high LD, *pink* (D' <1 and LOD ≥2), *white* (D' <1 and LOD <2) and *blue* (D' =1 and LOD <2). The *black triangles* show the LD-blocks calculated by Haploview. The plot was created using HapMap data (CEU)

YKL-40 measurement

Cord blood YKL-40 was measured in duplicates by an enzyme-linked immunosorbent assay (ELISA) (R&D Systems, USA). In order to capture the elevated cord blood YKL-40 levels with the limited range of the ELISA, samples had to be pre-diluted 1:20 before measurement. For consistency, all samples were pre-diluted at the same ratios. Data is presented as ng/ml; minimum detection limit of the assay is 3.55 pg/ml.

Outcomes during the first year

Respiratory symptoms were assessed weekly by telephone interviews [11, 12]. Lung function was performed according to ERS/ATS standards [13]. Tidal volume (V_T), mean tidal expiratory flow, time to peak tidal expiratory flow (T_{PTEF})/expiratory time (T_E) ratio and minute ventilation (V'_E) were measured (Exhalyzer D; Eco Medics AG, Duernten, Switzerland).

Outcomes at 6 years

Respiratory health was assessed using questions from the International Study of Asthma and Allergies in Childhood [14]. Asthma was diagnosed if one on the following was present in the previous year: (1) physician diagnosis of asthma or (2) episodic wheeze. Atopy was defined by allergic rhinitis, allergic asthma, or atopic dermatitis. A skin-prick test was done for the following allergens: (Dog dander, cat dander, *Dermatophagoides pteronyssinus*, mixed tree pollens, mixed grass pollens, *Alternaria tenuis*, positive control (histamine), negative control (NaCl), Allergomed, Switzerland) positive in case of hives bigger than histamine in any of the tested allergens. Forced expiratory volume in 1 s (FEV_1), forced vital capacity (FVC) and forced expiratory flow at 25–75 % of FVC ($FEF_{25-75\%}$) was measured according to ATS standards [15]. Data are expressed as z-scores using normative data from the Global Lung Function Initiative [16].

Risk factors

Exposure to pre- and postnatal risk factors [11, 14, 17] on outcomes are given in Additional file 1: Table S1. We validated maternal smoking by cotinine levels in the first urine of the newborn (gas–liquid chromatography, IST, Lausanne, Switzerland). Maternal asthma (self-reported or doctor-diagnosed), maternal atopic disease (history of allergic rhinitis, allergic asthma or atopic dermatitis), and parental education were assessed.

Statistical analysis

Anthropometric and clinical outcomes were compared with *t*-test and Mann–Whitney *U*-test. We conducted Poisson, logistic and linear regression analysis. For regression models, associations were calculated: (a) unadjusted and, (b) adjusted for known and potential confounders. YKL-40 was categorised in quintiles and associations of YKL-40 levels were calculated using the Cochran-Armitage trend test, shown as P_{trend}. A trend of association was defined for P_{trend} <0.2. We used an additive genetic model and corrected for multiple comparisons according to Benjamini-Hochberg [18]. A *P*-value <0.05 was considered significant. Data was analysed with STATA®, R [19], PLINK [20]. Power was calculated with Quanto [21].

Results

From 1999 to 2007 the study enrolled n = 260 infants with genotyping performed in n = 225. After genetic quality control and exclusion of individuals without YKL-40 measurements, n = 170 remained for analysis. Of these, n = 28 children were lost to follow-up resulting in n = 142 school-aged children (Additional file 1: Figure S1). Population characteristics and clinical outcomes are given in Table 1 (equally distributed by sexes), and possible risk factors in Additional file 1: Table S1. Characteristics of the SNPs are shown in Additional file 1: Table S2. None of the SNPs were associated with cord blood YKL-40 levels (Additional file 1: Table S3).

Outcomes during the first year

SNPs and cord blood YKL-40 levels were neither associated with 'any respiratory symptoms' nor with 'severe respiratory symptoms' during the first year of life in the univariable analysis. When adjusting for potential confounders on respiratory symptoms [12] this association remained non-significant (Additional file 1: Table S4). Associations between SNPs and cord blood YKL-40 with lung function at 5 weeks are given in Additional file 1: Table S5. There was no association in either the univariable or adjusted models for any of the examined parameters. Sensitivity analysis with adjustment for e.g. maternal asthma and delivery type revealed similar results (data not shown).

Outcomes at 6 years

At follow-up, there were 76 (53 %) males, 15 (11 %) asthmatics, 18 (17 %) had a positive prick test and 53 (39 %) were atopic. The SNP rs10399805 [T] was significantly associated with asthma at 6 years (Benjamini-Hochberg adjusted P = 0.031). The odds ratio (OR) for asthma was 4.5 (95 % CI 1.59–12.94) per T-allele in the univariable assocation. This study was sufficiently powered (80 %) at the 5 % level of significance for an OR >4. When adjusting for maternal atopy, parental smoking and parental education, this association was non-significant (Table 2). A protective effect of the A-allele of rs10399931 for asthma and atopy in the adjusted model [OR asthma 0.21 (95 % CI 0.04–0.98), OR atopy

Table 1 Population characteristics and distribution of clinical outcomes

	Mean ± S.D.	Median (IQR)	Range	N (%)
Anthropometrics at birth[a]				
Gestational age weeks	39.7 ± 1.1	40 (39.1–40.7)	37.0–42.0	
Weight kg	3.4 ± 0.4	3.4 (3.1–3.7)	2.2–4.9	
Length cm	49.5 ± 1.9	50.0 (48–51)	45–55	
Anthropometrics at follow-up[b]				
Age years	6.0 ± 0.3	6 (5.9–6.2)	5.1– 6.9	
Weight kg	22.7 ± 3.8	22.2 (20.0–24.5)	16–35.8	
Length cm	117.5 ± 5.6	117.5 (114–121)	(104–107)	
YKL-40 in cord blood[a]				
YKL-40 ng/ml	42.4 ± 28.0	43.5 (23.3–63.3)	0–97.2	
Clinical outcomes during the first year[a]				
Wks with daytime resp. sympt.	4.6 ± 4.6	3 (1–7)	0–24	
Wks with nighttime resp. sympt.	3.8 ± 3.8	3 (1–6)	0–21	
Wks with severe daytime resp. sympt.	0.5 ± 0.9	0 (0–1)	0–5	
Wks with severe nighttime resp. sympt.	0.6 ± 1.1	0 (0–1)	0–8	
Lung function at 5 weeks[c]				
Tidal volume ml	32.5 ± 5.5	32.5 (28.1–36.4)	21–51	
Mean tidal expiratory flow	43.3 ± 10.4	41.6 (35.7–49.3)	21–79	
T_{PTEF}/T_E	36.2 ± 10.8	34.7 (28.7–41.8)	16–73	
Minute Ventilation ml · min^{-1}	1427 ± 270	1405 (1239–1586)	870–2333	
Clinical data at 6 years				
Asthma[b]				15 (12 %)
Atopy[c]				53 (39 %)
Positive prick test[d]				18 (17 %)
Lung function at 6 years				
FVC z-score[e]	−0.41 ± 0.98	−0.04 (−1.18–0.32)	−2.25–2.02	
FEV_1 z-score[f]	−0.09 ± 0.96	−0.21 (−0.83–0.73)	−0.25–2.02	
$FEF_{25–75\%}$ z-score[g]	−0.04 ± 0.94	−0.04 (−0.68–0.55)	−2.95–2.27	

FEV_1 Forced expiratory volume in 1 s, *FVC* Forced vital capacity, *$FEF_{25–75\%}$* Forced expiratory flow at 25–75 % of FVC, *IQR* Interquartile range, *N* Number, *S.D.* Standard deviation, *Wks* Weeks, *resp. sympt* Respiratory symptoms. Data are given as mean (S.D.) median (IQR) or number (percentage) of infants. [a]Data on n = 142 infants. [b]Missing data on *n* = 7; data available for n = 135. [c]Missing data on *n* = 4; data available for *n* = 138. [d]Missing data on *n* = 38; data available for *n* = 104. [e]Missing data on *n* = 51; data available for *n* = 91. [f]Missing data on *n* = 66; data available for *n* = 76. [g]Missing data on *n* = 62; data available for *n* = 80

0.43 (95 % CI 0.21–0.86)] was observed. These results were non-significant when adjusting for multiple comparisons (Table 2). Since asthma is known to be associated with YKL-40 levels (5, 6), we adjusted the associations of SNPs with asthma for cord blood YKL-40 levels, which revealed similar results (Table 3). This indicates that YKL-40 does not seem to be involved in the pathway responsible for the observed association between SNPs and asthma (Fig. 2). Positive prick test results were not associated with any of the SNPs in the uni- and multivariable analysis (Additional file 1: Table S6).

We observed a trend of association of YKL-40 levels with the OR for asthma and positive prick test results (P_{trend} = 0.169) (Table 2, Fig. 3 and Additional file 1: Table S6). Associations of SNPs and cord blood YKL-40 levels with lung function measures are given in Additional file 1: Table S7. There was no association in the univariable or adjusted models for any of the examined parameters.

Discussion

Summary

In this cohort study, we examined the effect of genetic variation of *CHI3L1* and cord blood YKL-40 levels of unselected infants. We demonstrate that genetic variation of *CHI3L1* is associated with asthma in early childhood. In particular, the T-allele of rs10399805 significantly

Table 2 Associations between cord blood YKL-40 levels and SNPs with asthma and atopy at school age

	Univariable association			Multivariable[a] association		
	OR	95 % CI	*P*-value	OR	95 % CI	*P*-value
Exposure	Outcome asthma[b]					
SNP[*]						
rs10920579	0.25	0.06–1.15	0.305	0.23	0.05–1.2	0.365
rs880633	1.33	0.63–2.89	0.777	1.05	0.46–2.38	0.901
rs10399931	0.21	0.05–0.95	0.212	0.21	0.04–0.98	0.287
rs10399805	4.50	1.59–12.94	0.031	3.42	1.12–10.56	0.224
rs1538372	0.74	0.31–1.72	0.777	0.68	0.28–1.63	0.901
rs7542294	2.80	1.29–7.01	0.158	2.37	0.87–6.46	0.365
rs2486064	0.89	0.42–1.91	0.777	0.94	0.42–2.19	0.901
YKL-40 (ng/ml)						
YKL-40 non-detects	1	reference	P_{trend}	1	reference	P_{trend}
YKL-40 (7–37.9)	0.86	0.13–5.54		0.82	0.12–5.67	
YKL-40 (38–49.9)	1.21	0.22–6.43		1.45	0.24–8.61	
YKL-40 (50–65.9)	1.72	0.35–8.38		2.27	0.41–12.2	
YKL-40 (66–98)	2.24	0.49–10.24	0.218[**]	2.52	0.49–13.01	0.169[**]
Exposure	Outcome atopy[c]					
SNP[*]						
rs10920579	0.56	0.26–1.14	0.691	0.50	0.24–1.05	0.714
rs880633	0.92	0.55–1.51	0.96	0.94	0.57–1.56	0.913
rs10399931	0.45	0.24–0.91	0.169	0.43	0.21–0.86	0.251
rs10399805	1.43	0.64–3.19	0.96	1.63	0.71–3.75	0.913
rs1538372	0.83	0.48–1.39	0.96	0.81	0.47–1.39	0.913
rs7542294	1.25	0.62–2.55	0.96	1.37	0.66–2.81	0.913
rs2486064	0.98	0.61–1.59	0.96	0.89	0.54–1.47	0.913
YKL-40 (ng/ml)						
YKL-40 non-detects	1	reference	P_{trend}	1	reference	P_{trend}
YKL-40 (7–37.9)	1.15	0.42–3.13		1.09	0.43–3.21	
YKL-40 (38–49.9)	1	0.37–2.67		0.98	0.37–2.71	
YKL-40 (50–65.9)	1.44	0.54–3.83		1.35	0.55–3.91	
YKL-40 (66–98)	1.76	0.67–4.61	0.188[**]	1.69	0.66–4.58	0.232[**]

CI Confidence interval, *OR* Odds ratio. *SNP* Single nucleotide polymorphism. Cord blood YKL-40 levels are compared with YKL-40 non-detects. [a]Adjusted for the following additional risk factors: sex, parental smoking during childhood, maternal atopy, parental education. [b]Asthma was diagnosed if one on the following was present in the previous year: (1) physician diagnosis of asthma or (2) episodic wheeze. Missing data on $n = 7$; data available for $n = 135$. [c]Defined if one of the following was present: asthma, allergic rhinitis, atopic eczema or positive prick test. Missing data on $n = 4$; data available for $n = 138$. [*]*P*-values for SNPs are shown after correction for multiple testing according to Benjamini-Hochberg. [**]P_{trend}-values were calculated with the Cochran-Armitage trend test

increased the risk for asthma, even after correction for multiple testing. Cord blood YKL-40 levels were not associated with any of the investigated outcomes.

Limitations and strenghts

The main limitation is the small sample size, which restricts power to identify weak associations. However, in contrast to previous cross-sectional studies [5, 9, 22, 23], this study was conducted in a cohort with a highly elaborate design, which naturally limits sample size. Unlike a previous prospective study [6], we diagnosed asthma based on one definition and assessed further outcomes on respiratory health (lung function at 5 weeks and 6 years, respiratory symptoms during the first year). Nevertheless, most of the correlations in this study were negative and only some findings indicated a trend of association. Trends of associations might be rendered to significant findings with more asthma cases. In an unselected population, this could be achieved by increasing the sample size or by studying a

Table 3 Associations between SNPs and asthma at school age adjusted for cord blood YKL-40 levels

	Univariable association			Univariable association adjusted for YKL-40 levels		
	OR	95 % CI	*P*-value	OR	95 % CI	*P*-value
Exposure	Outcome asthma[a]					
SNP[*]						
rs10920579	0.25	0.06–1.15	0.305	0.23	0.05–1.08	0.254
rs880633	1.33	0.63–2.89	0.777	1.27	0.59–2.75	0.654
rs10399931	0.21	0.05–0.95	0.212	0.2	0.04–0.89	0.189
rs10399805	4.50	1.59–12.94	0.031	4.76	1.66–13.62	0.026
rs1538372	0.74	0.31–1.72	0.777	0.71	0.31–1.68	0.654
rs7542294	2.80	1.29–7.01	0.158	2.66	1.06–6.72	0.189
rs2486064	0.89	0.42–1.91	0.777	0.84	0.39–1.81	0.654

CI Confidence interval, *OR* Odds ratio. *SNP* Single nucleotide polymorphism. [a]Asthma was diagnosed if one on the following was present in the previous year: (1) physician diagnosis of asthma or (2) episodic wheeze. Missing data on $n = 7$; data available for $n = 135$. [*]*P*-values for SNPs are shown after correction for multiple testing according to Benjamini-Hochberg

high-risk population. Both options have their drawbacks since larger birth cohort studies with a similar elaborate design are lacking, while studies in high-risk populations are predominantly multi-center studies, introducing a bias on asthma diagnosis due to different study sites.

Associations between genetic variation in *CHI3L1* and asthma are controversial in the discussion, and the

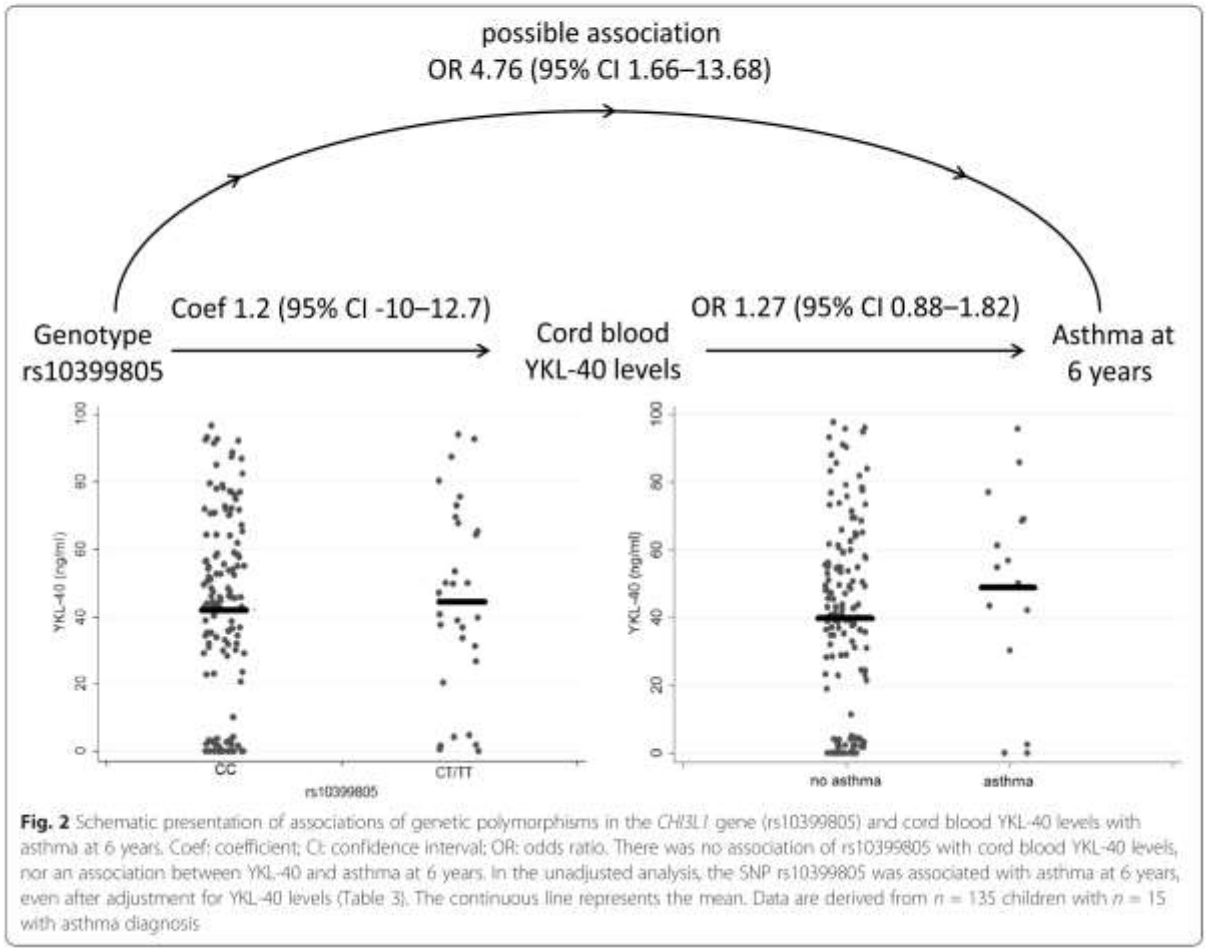

Fig. 2 Schematic presentation of associations of genetic polymorphisms in the *CHI3L1* gene (rs10399805) and cord blood YKL-40 levels with asthma at 6 years. Coef: coefficient; CI: confidence interval; OR: odds ratio. There was no association of rs10399805 with cord blood YKL-40 levels, nor an association between YKL-40 and asthma at 6 years. In the unadjusted analysis, the SNP rs10399805 was associated with asthma at 6 years, even after adjustment for YKL-40 levels (Table 3). The continuous line represents the mean. Data are derived from $n = 135$ children with $n = 15$ with asthma diagnosis

than cord blood YKL-40 levels in unselected infants (Fig. 2). When adjusting for potential risk factors, this association did not reach statistical significance anymore, indicating other risk factors besides rs10399805 to also be relevant for disease development. The protective effect of rs10399931[A] for asthma at 6 years, although non-significant after adjusting for multiple testing (Table 2), provided further evidence for the relevance of genetic variation in *CHI3L1* for asthma development. This SNP was reportedly associated with asthma in Taiwanese adults [27].

We observed a trend of association of increased YKL-40 levels with the OR for asthma at 6 years (Fig. 3). We speculate that the low number of asthmatic subjects in this study of unselected infants resulted in increasingly large confidence intervals and, hence, our findings did not reach formal significance level (P_{trend} = 0.169). An association of increased serum YKL-40 levels with severe asthma in adult [6, 7] and paediatric subjects [5] has been reported. Recent studies regarded YKL-40 as not only a simple biomarker for asthma, but as directly involved in airway remodeling. Increased YKL-40 levels were associated with bronchial wall thickening on computerised tomography in children with severe asthma [5] and subepitehlial basement membrane thickness in adults [4]. Mechanistically, Bara et al. showed that YKL-40 increased bronchial smooth muscle cell proliferation [8]. Despite emerging evidence for a direct involvement of YKL-40 in asthma development, in this study of unselected infants, YKL-40 in cord blood did not serve as a predictor for asthma in early childhood. Future studies will need to investigate if this was due to the low level of asthmatics in the general population and in our cohort.

Atopy

We identified a protective effect of the SNP rs10399931[A] with atopy and positive prick test results, although non-significant after correction for multiple testing. Several studies investigated the association of genetic variation in *CHI3L1* with allergic diseases, but the SNP rs10399931 has not been reported in that context. An association of rs10399805 with atopy, recently reported in Korean children (22), could not be confirmed in our study, possibly due to differing study populations.

While there was no association of YKL-40 with atopy and in two large population studies [4, 6], there was an association of higher serum YKL-40 with allergic rhinitis [28]. In our study, we observed a trend of association of increased YKL-40 levels with the OR for atopy and positive prick (P_{trend} = 0.188, P_{trend} = 0.128). In conclusion, we observed an association of genetic variation in *CHI3L1* and cord blood levels YKL-40 with allergic diseases, but due to low sample size, this association did not reach statistical significance.

Lung function

We did not find an association of genetic variation in *CHI3L1* and YKL-40 levels in cord blood with lung function measures at 5 weeks and 6 years. An association of lung function measures with genetic variation in *CHI3L1* and YKL-40 levels has been well described. Genetic variation in *CHI3L1* was associated with FEV_1, FVC and FEV_1/FVC−ratios in adult European and Taiwanese populations [4, 9, 27] and serum YKL-40 levels were inversely associated with FEV_1 in adult [4, 6, 7], but not paediatric asthmatics [5]. We conclude from these findings that in contrast to previous cross-sectional studies, genetic variation in *CHI3L1* and cord blood YKL-40 levels at birth are not associated with lung function measures in unselected infants.

Relevance

From cross-sectional studies it is well known that both, genetic variation in *CHI3L1* and YKL-40 levels are associated with asthma, atopy, and lung function measures. Our study further explores this association since we assessed genetic variation in *CHI3L1* and YKL-40 in cord blood within the setting of a cohort study before the onset of these diseases. Moreover, our study is the first to study this association in unselected, healthy infants. The limited number of diseased subjects in the general population and in this cohort study might be the cause of the non-significant associations of YKL-40 with asthma, atopy, and prick test results. Studying this association in a much larger cohort might result in significant findings and reveal further insights for the relevance of YKL-40 for these diseases.

Conclusion

In this prospective cohort study we found no robust association between genetic variation in *CHI3L1* and asthma development, but found some indication that rs10399805 might be related to asthma diagnosis at 6 years. In order to replicate findings of this study, and to investigate its robustness, larger studies with a prospective design in an unselected population are warranted. The trend of association between elevated cord blood YKL-40 levels and asthma requires further validation before YKL-40 may be considered an early biomarker for asthma development in unselected infants.

Availability of data

Authors would be pleased to consider requests to share original study data.

Additional file

Additional file 1: Data S1. Methods. **Figure S1.** Flow chart of the study population. **Table S1.** Potential risk factors of the study subjects. **Table S2.** Characteristics and prevalence of the SNPs employed in this study. **Table S3.** Associations between SNPs and cord blood YKL-40 levels. **Table S4.** Associations between SNPs and cord blood YKL-40 levels with weeks with any respiratory symptoms and weeks with severe respiratory symptoms during the first year of life. **Table S5.** Associations between SNPs and cord blood YKL-40 levels with lung function at 5 weeks. **Table S6.** Associations between SNPs and cord blood YKL-40 levels with positive prick test at school age. **Table S7.** Associations between SNPs and cord blood YKL-40 levels with lung function at 6 years. (DOCX 197 kb)

Abbreviations

CHI3L1: chitinase-3-like-1; ELISA: enzyme-linked immunosorbent assay; $FEF_{25-75\%}$: forced expiratory flow at 25-75% of FVC; FEV_1: forced expiratory volume in 1 second; FVC: forced vital capacity; LD: linkage disequilibrium; MAF: minor allele frequency; LOD: logarithm of odds; V'_E: minute ventilation; SNPs: single nucleotide polymorphisms; T_E: expiratory time; T_{PTEF}: time to peak tidal expiratory flow; V_T: tidal volume; YKL-40: A 40 kilodalton chitinase-like protein named after the first three N-terminal amino acids tyrosine (Y), lysine (K) and leucine (L).

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

All authors contributed to the conception and design and to the revisions and final approval of the manuscript. JU, PL, AS, IM, MR and DH performed the data analysis and JU, PL, DH, MR and UF drafted the article. Clinical data was collected and analysed by AS, PL and JU. YKL-40 measurements were done by IM. UF is the primary investigator of the BILD cohort.

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Online supplement

CHI3L1 polymorphisms, cord blood YKL-40 levels and later asthma development

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S1 Methods

Study design and subjects

In this prospective cohort study, healthy term neonates were recruited antenatally from April 1999 to January 2007 in the region of Bern, Switzerland, described in detail elsewhere [1]. Exclusion criteria for the study were pre-term delivery (<37 weeks), major birth defects, respiratory distress after birth, other significant perinatal disease or later diagnosis of airway malformation or specific chronic respiratory disease [1-3]. The Ethics Committee of the region of Bern approved the study and written consent from all parents was acquired at enrolment.

Genotyping and quality control

DNA was extracted from umbilical cord blood. Genome-wide SNP genotyping was performed by Illumina HumanOmniExpress Bead Chips (Illumina Inc., San Diego, USA). Individuals with low genotype rate (<97%), unusual high or low heterozygosity (±3 SD beyond the mean) and discordance between reported and observed sex were excluded (see Figure 1). In total, 142 unrelated individuals of European ancestry with measured YKL-40 levels were considered for the analysis. SNPs were excluded if they had a call rate less than 98%, minor allele frequency (MAF) more than >5%, and Hardy-Weinberg equilibrium p-value less than 10^{-6}. Quality control statistics was calculated using PLINK [4] version 1.07 and R version 3.0.2 [5] (www.r-project.org) using the GenABEL package.

YKL-40 measurement

Cord blood was acquired from the umbilical cord and stored immediately at –80°C. Serum was diluted 1:20 before YKL-40 was measured in duplicates by a sandwich enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, USA) according to the manufacturer´s instructions. The mean of the 2 duplicates was used in the statistical analysis. Minimum detection limit of the assay is 3.55 pg/ml, inter-assay variability is 5.3%, and intra-assay variability is 14%. Data are presented as ng/ml.

Outcomes during the first year

The outcome respiratory symptoms during the first year were assessed by weekly telephone interviews done by research nurses using a standardised symptom score that groups symptoms into four levels according to severity, with a high sensitivity for lower respiratory tract symptoms [3, 6]. The outcomes 'weeks with respiratory symptoms' were defined as total number of weeks a child had any respiratory symptom, independent of type or severity; 'weeks with severe respiratory symptoms' were defined as a symptom score ≥3 e.g repeated sleep disturbances during the night, or general practitioner consultation, as described previously [3]. Lung function was performed according to ERS/ATS standards [7] during unsedated sleep in supine position with the head midline, as previously reported [8]. For infant lung function at 5 weeks, we used the first 100 regular breaths of tidal breathing during non-rapid eye movement (non-REM) sleep from the total recording over 10 min. We calculated mean tidal breathing parameters of flow, volume, and flow-volume loop. Outcome parameters were tidal volume (V_T), mean tidal expiratory flow, time to peak tidal expiratory flow (T_{PTEF})/expiratory time (T_E) ratio and minute ventilation (V'_E). Tidal breathing measurements were done using an ultrasonic flow meter device (Exhalyzer D; Eco Medics AG, Dürnten, Switzerland).

Outcomes at 6 years

At follow-up with 6 years, respiratory health was assessed with questions adapted from the International Study of Asthma and Allergies in Childhood (ISAAC) [9]. The questionnaires have been validated and used in our study since 1999 [1]. Asthma was diagnosed if one on the following was present in the previous year: (1) physician diagnosis of asthma or (2) episodic wheeze. Atopy was defined by allergic rhinitis, allergic asthma or atopic dermatitis. A skin-prick test was done for eight allergens (Dog dander, cat dander, *Dermatophagoides pteronyssinus*, mixed tree pollens, mixed grass pollens, *Alternaria tenuis*, positive control (histamine), negative control (NaCl), Allergomed, Switzerland) positive in case of hives bigger than histamine in any of the tested allergens. Spirometry was performed to measure forced expiratory volume in 1

second (FEV_1), forced vital capacity (FVC), and forced expiratory flow at 25-75% of FVC ($FEF_{25-75\%}$), according to ATS standards [10]. Data are expressed as z-scores using normative data from the Global Lung Function Initiative [11]. Spirometry was done using the JAEGER® Master-Screen Body (CareFusion, Würzburg, Germany).

Risk factors

A standardised questionnaire was employed to assess pre- and post-natal exposure to putative risk factors [3, 8, 9] (e.g. siblings, tobacco smoke exposure, and maternal atopy) on the outcomes respiratory symptoms during the first year, at the 5-week lung function, and at 6 years (asthma, atopy, positive prick test and spirometry) (Table S1). We validated maternal smoking by cotinine levels in the first urine of the newborn (gas-liquid chromatography, IST, Lausanne, Switzerland). Maternal asthma (self-reported or doctor-diagnosed asthma) and maternal atopic disease (history of allergic rhinitis, allergic asthma or atopic dermatitis) was assessed. Parental education was categorised into low (<4 years of apprenticeship) and high (≥4 years of apprenticeship).

Statistical analysis

Anthropometric and clinical outcomes were compared by sex using *t*-test and Mann-Whitney *U*-test. We performed regression analysis with cord blood YKL-40 levels as outcome and SNPs as exposures. Because 44 (21%) of cord blood YKL-40 levels were below detection limit, we used a Tobit regression model with a detection limit of 1 (concentration ng/ml). To minimise the effect of outliers, and to differentiate between different exposure levels, we categorised YKL-40 (ng/ml) into quintiles: YKL-40 non-detects, YKL-40 (7–37.9), YKL-40 (38–49.9), YKL-40 (50–65.9) and YKL-40 (66–98). The YKL-40 non-detects served as reference level. The overall association of different YKL-40 levels with the outcomes was calculated with the Cochran-Armitage trend test, shown as P_{trend}. A trend of association was defined for P_{trend} <0.2.

We examined the association of SNPs and cord blood YKL-40 levels with respiratory symptoms during the first year. The outcome ‘weeks with respiratory symptoms’ showed an over-dispersion, and data was analysed using Poisson regression based on robust variance estimates

[12]. First, univariable regression analysis was performed, and secondly, a multivariable model with adjustment for other exposures associated with respiratory symptoms (2) such as sex, gestational weight, and maternal smoking during pregnancy was used. Then, we analysed with univariable linear regression models the associations of SNPs and YKL-40 levels with natural-log-transformed lung function measures at 5 weeks. In a multivariable model we adjusted for anthropometric data (sex, birth length, age and weight at study) and for sensitivity analysis additionally for potential confounders (e.g. maternal asthma, delivery type).

We assessed the associations of SNPs and cord blood YKL-40 levels with the outcomes (asthma, atopy, prick test results) at 6 years with logistic regression. First, univariable analysis was performed for each outcome variable, and secondly, a multivariable model with adjustment for e.g sex and parental smoking was used. Lung function measures at 6 years were analysed with linear regression analysis. First, univariable analysis was performed and secondly with adjustment for presence or absence of siblings, daycare attendance and asthma of the child. For sensitivity analysis we additionally adjusted for potential confounders (e.g. maternal asthma, delivery type).

The selection of variables for the adjusted models was based on physiological plausibility, potential effects of genetic variation on the outcomes and the best fit based on the Akaike information criterion [13]. An additive genetic model was used with *P*-values adjusted for multiple testing for 7 tests (since 7 SNPs were used in this study) with the Benjamini-Hochberg procedure (false discovery rate <0.1) [14] to balance power and the potential for false-positive results. A *P*–value < 0.05 was considered to be significant. Power was calculated with Quanto [15]. Data was analysed with STATA® (Stata Statistical Software: release 13. STATA® Cooperation, College Station, TX, USA), R version 3.0.2 (www.r-project.org) [5] using the GenABEL package and PLINK [4] version 1.07 (http://pngu.mgh.harvard.edu/purcell/plink/).

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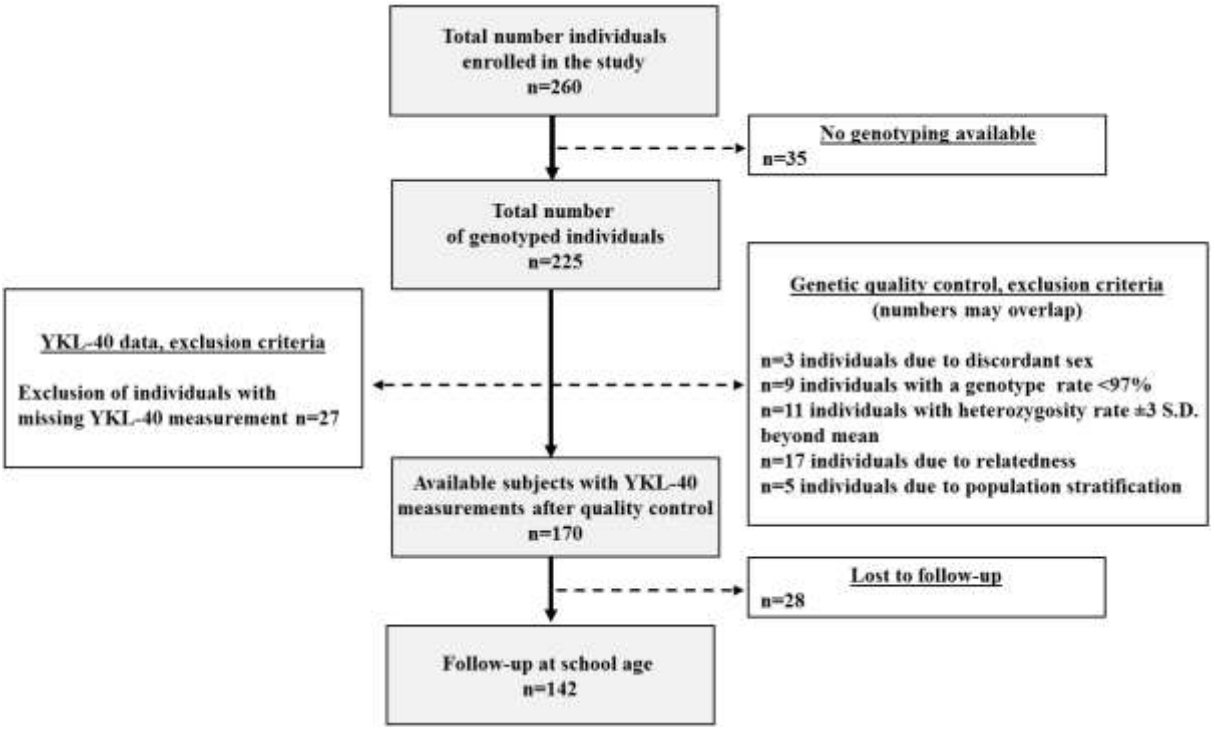

Figure S1 Flow chart of the study population

Table S1 Potential risk factors of the study subjects

Prenatal risk factors	n (%)
Male sex	76 (53.5)
Siblings[*]	73 (51.5)
Caesarean section	17 (11.9)
Maternal asthma[$]	17 (11.9)
Maternal atopy[#]	61 (42.9)
Positive maternal skin prick test[+]	55 (38.7)
Maternal smoking in pregnancy[§]	12 (8.5)
High maternal education[¶]	94 (67.2)
Low maternal education[¶]	46 (32.9)
High paternal education[¶]	112 (80)
Low paternal education[¶]	28 (20)

[*]Defined as presence or absence of siblings; [$]defined as self-reported or doctor-diagnosed asthma; [#]defined if one of the following was present: allergic asthma, allergic rhinitis, atopic dermatitis; [+]positive in case of hives bigger than positive control histamine in any of the tested allergens; [§]maternal active smoking during pregnancy; [¶]parental education was categorised into low (<4 years of apprenticeship) and high (≥4 years of apprenticeship). Data are derived from n=142 subjects.

Table S2 Characteristics and prevalence of the SNPs employed in this study

SNP	Location	Allele	MAF	HW *P*-value	Genotype distribution	
		(major/minor)	(%)		all	prevalence n (%)
rs880633	missense	G/A	46.2	0.564	GG	44 (30.9)
					GA	71 (50)
					AA	27 (19.1)
rs10399931	upstream	G/A	23.3	0.688	GG	85 (59.9)
					GA	52 (36.6)
					AA	5 (3.5)
rs10399805	upstream	C/T	10.5	1	CC	113 (79.6)
					CT	28 (19.7)
					TT	2 (1)
rs1538372	intron	G/A	33.8	1	GG	65 (45.8)
					GA	62 (43.7)
					AA	15 (10.6)
rs10920579[§]	intergenic	C/T	18.2	0.336	CC	92 (64.8)
					CT	49 (34.5)
					TT	1 (1)
rs7542294	intron	C/T	13.5	0.760	CC	108 (76.1)
					CT	31 (21.8)
					TT	3 (2.1)
rs2486064	intergenic	C/T	40.1	0.236	CC	50 (35.2)
					CT	64 (45.1)
					TT	28 (19.7)

HW: Hardy-Weinberg; MAF: minor allele frequency; SNP: single nucleotide polymorphism. [§]Proxy for rs4950928: rs10920579 (r^2=1, D'=1, calculated with HapMap). SNPs were genotyped with Illumina HumanOmniExpress Bead Chips. Data are derived from n=142 subjects.

Table S3 Associations between SNPs and cord blood YKL-40 levels

	Univariable model			Adjusted[#] model		
	Coef	95% CI	*P*-value	Coef	95% CI	*P*-value
[$]SNP						
rs10920579	5.40	-5.07–15.87	0.976	5.91	-4.75–16.57	0.936
rs880633	3.57	-4.13–11.27	0.976	3.64	-4.11–11.38	0.936
rs10399931	7.42	-1.96–16.82	0.839	7.86	-1.61–17.32	0.721
rs10399805	-0.18	-13.05–15.5	0.976	-0.53	-13.56–12.51	0.936
rs1538372	3.38	-4.78–11.57	0.976	3.63	-4.55–11.82	0.936
rs7542294	3.26	-8.01–14.55	0.976	3.02	-8.37–14.21	0.936
rs2486064	3.63	-3.78–11.05	0.976	3.50	-3.51–11.11	0.936

Data is given as the probability of cord blood YKL-40 levels (ng/ml) being above detection per each SNP. Coef: Coefficient; CI: confidence interval; SNP: single nucleotide polymorphism. [#]This model was adjusted for sex, presence or absence of siblings, type of delivery, maternal atopy and parental education. [$]*P*-values for SNPs are shown after correction for multiple testing according to Benjamini-Hochberg. Data are derived from n=142 subjects.

Table S4 Associations between SNPs and cord blood YKL-40 levels with weeks with any respiratory symptoms and weeks with severe respiratory symptoms during the first year of life

	Univariable model			Multivariable[$] model			Full Multivariable[*] model		
	IRR[¶]	95% CI	*P*-value	IRR[¶]	95% CI	*P*-value	IRR[¶]	95% CI	*P*-value
Exposure	Outcome weeks with any respiratory symptoms[#]								
SNP[#]									
rs10920579	0.97	0.73–1.23	0.731	0.96	0.76–1.19	0.963	0.98	0.79–1.19	0.994
rs880633	1.12	0.92–1.35	0.731	1.09	0.91–1.29	0.963	1.09	0.92–1.29	0.994
rs10399931	1.00	0.82–1.30	0.731	1.01	0.83–1.22	0.963	1.03	0.86–1.23	0.994
rs10399805	1.34	0.97–1.75	0.512	1.25	0.96–1.62	0.592	1.25	0.99–1.58	0.364
rs1538372	1.01	0.85–1.25	0.731	1.00	0.84–1.18	0.963	1.00	0.85–1.16	0.994
rs7542294	1.21	0.92–1.53	0.731	1.17	0.92–1.47	0.963	1.21	0.97–1.49	0.428
rs2486064	0.89	0.74–1.02	0.512	0.84	0.84–0.98	0.232	0.85	0.73–0.99	0.267
YKL-40 (ng/ml)									
YKL-40 non-detects	1	reference	P_{trend}	1	reference	P_{trend}	1	reference	P_{trend}
YKL-40 (7–37.9)	1.05	0.5–2.1		1.22	0.6–2.4		1.16	0.5–2.3	
YKL-40 (38–49.9)	1.25	0.5–2.8		1.43	0.6–3.2		1.39	0.6–3.1	
YKL-40 (50–65.9)	1.13	0.6–2.3		1.31	0.6–2.6		1.48	0.7–2.9	
YKL-40 (66–98)	1.42	0.7–2.7	0.131[£]	1.38	0.7–2.5	0.127[£]	1.40	0.7–2.6	0.237[£]
Exposure	Outcome weeks with severe respiratory symptoms[§]								
SNP[#]									
rs10920579	1.23	0.79–1.91	0.571	1.20	0.81–1.77	0.969	1.20	0.82–1.75	0.820
rs880633	1.16	0.76–1.74	0.571	1.07	0.72–1.57	0.969	1.09	0.74–1.59	0.820
rs10399931	1.20	0.81–1.74	0.571	1.16	0.82–1.63	0.969	1.22	0.86–1.71	0.820
rs10399805	1.44	0.89–2.29	0.571	1.31	0.73–2.03	0.969	1.27	0.82–1.93	0.820
rs1538372	1.06	0.74–1.51	0.571	1.01	0.84–1.38	0.969	1.04	0.77–1.38	0.820
rs7542294	1.23	0.76–1.98	0.571	1.19	0.73–1.94	0.969	1.21	0.73–1.96	0.820
rs2486064	0.93	0.65–1.30	0.571	0.88	0.63–1.21	0.969	0.90	0.67–1.21	0.820
YKL-40 (ng/ml)									
YKL-40 non-detects	1	reference	P_{trend}	1	reference	P_{trend}	1	reference	P_{trend}
YKL-40 (7–37.9)	0.93	0.47–1.83		1.13	0.59–2.18		1.06	0.54–2.06	
YKL-40 (38–49.9)	1.13	0.49–2.56		1.36	0.63–2.95		1.28	0.61–2.74	
YKL-40 (50–65.9)	1.08	0.55–2.09		1.25	0.66–2.39		1.41	0.73–2.71	
YKL-40 (66–98)	1.27	0.67–2.41	0.372[£]	1.29	0.67–2.46	0.234[£]	1.32	0.71–2.51	0.190[£]

CI: confidence interval; IRR: incidence risk ratio; SNP: single nucleotide polymorphism. [$]Adjusted for sex, gestational weight, maternal smoking during pregnancy and maternal atopic disease. [*]Adjusted for sex, gestational weight, maternal smoking during pregnancy, maternal atopic disease, presence or absence of siblings, nursery, parental smoking during the first year, parental education. [#]Defined as any (day or night) respiratory symptoms independent of severity. [§]Defined as any (day or night) respiratory symptoms with a symptom score ≥3 [3]. [#]*P*-values for SNPs are shown after correction for multiple testing according to Benjamini-Hochberg. [£]P_{trend} -values were calculated with the Cochran-Armitage trend test. Data are derived from n=142 subjects.

Table S5 Associations between SNPs and cord blood YKL-40 levels with lung function at 5 weeks

	Univariable association			Adjusted* association		
	Coef	95% CI	*P*-value	Coef	95% CI	*P*-value
Exposure	Outcome tidal volume (ml) $					
SNP#						
rs10920579	0.96	0.91–1.01	0.793	0.96	0.91–1.01	0.541
rs880633	0.99	0.94–1.03	0.793	0.98	0.94–1.03	0.920
rs10399931	0.96	0.91–1.01	0.793	0.96	0.91–1.01	0.449
rs10399805	1.01	0.94–1.08	0.793	1.00	0.94–1.08	0.920
rs1538372	0.97	0.93–1.01	0.793	0.98	0.93–1.01	0.920
rs7542294	1.01	0.95–1.07	0.793	1.01	0.95–1.07	0.920
rs2486064	0.99	0.95–1.03	0.793	0.98	0.95–1.03	0.920
YKL-40 (ng/ml)						
YKL-40 non-detects	1	reference	P_{trend}	1	reference	P_{trend}
YKL-40 (7–37.9)	1.05	0.97–1.14		1.02	0.87–1.18	
YKL-40 (38–49.9)	0.95	0.89–1.03		0.98	0.85–-1.13	
YKL-40 (50–65.9)	1.00	0.99–1.08		1.05	0.91–1.21	
YKL-40 (66–98)	1.00	0.92–1.08	0.930£	0.98	0.85–1.13	0.841£
Exposure	Outcome mean tidal expiratory flow$					
SNP#						
rs10920579	1.05	0.97–1.13	0.41	1.05	0.98–1.13	0.478
rs880633	1.03	0.98–1.08	0.41	1.03	0.98–1.08	0.478
rs10399931	0.94	1.01–1.14	0.178	1.07	1.07–1.14	0.233
rs10399805	1.06	0.86–1.03	0.41	0.94	0.86–1.03	0.478
rs1538372	1.07	1.01–1.12	0.112	1.06	1.01–1.12	0.227
rs7542294	0.93	0.86–1.01	0.41	0.93	0.86–1.01	0.431
rs2486064	1.02	0.97–1.07	0.41	1.02	0.97–1.07	0.478
YKL-40 (ng/ml						
YKL-40 non-detects	1	reference	P_{trend}	1	reference	P_{trend}
YKL-40 (7–37.9)	0.99	0.88–1.11		0.983	0.88–1.09	
YKL-40 (38–49.9)	1.05	0.94–1.17		1.06	0.95–1.17	
YKL-40 (50–65.9)	1.05	0.94–1.17		1.06	0.95–1.17	
YKL-40 (66–98)	1.03	0.93–1.15	0.152£	1.03	0.93–1.14	0.194£
Exposure	Outcome ratio T_{PTEF}/T_E%$					
SNP#						
rs10920579	1.10	0.98–1.21	0.549	1.08	0.97–1.19	0.959
rs880633	0.96	0.89–1.03	0.999	0.96	0.89–1.04	0.971
rs10399931	1.04	0.94–1.14	0.999	1.03	0.93–1.13	0.971
rs10399805	0.98	0.86–1.11	0.999	0.99	0.86–1.12	0.971
rs1538372	1.00	0.91–1.07	0.999	0.99	0.91–1.06	0.971
rs7542294	0.93	0.83–1.04	0.999	0.94	0.94–1.05	0.971
rs2486064	1.00	0.93–1.07	0.999	1.00	0.99–1.07	0.971
YKL-40 (ng/ml)						
YKL-40 non-detects	1	reference	P_{trend}	1	reference	P_{trend}
YKL-40 (7–37.9)	1.04	0.89–1.21		1.02	0.89–1.21	
YKL-40 (38–49.9)	1.00	0.86–1.15		0.98	0.86–1.15	
YKL-40 (50–65.9)	1.06	0.91–1.12		1.05	0.91–1.12	
YKL-40 (66–98)	0.99	0.85–1.14	0.866£	0.98	0.85–1.14	0.818£
Exposure	Outcome minute ventilation (ml · min^{-1}) $					
SNP#						
rs10920579	1.03	0.97–1.09	0.409	1.02	0.96–1.08	0.507
rs880633	1.04	0.99–1.08	0.409	1.03	0.98–1.07	0.507
rs10399931	1.05	0.99–1.11	0.395	1.04	0.99–1.09	0.507
rs10399805	0.96	0.89–1.03	0.409	0.96	0.90–1.02	0.507
rs1538372	1.06	1.01–1.11	0.053	1.05	1.01–1.09	0.114
rs7542294	0.95	0.89–1.01	0.409	0.96	0.91–1.01	0.507
rs2486064	1.03	0.96–1.05	0.409	1.01	0.97–1.05	0.507
YKL-40 (ng/ml)						
YKL-40 non-detects	1	reference	P_{trend}	1	reference	P_{trend}

YKL-40 (7–37.9)	1.01	0.93–1.11		1.00	0.92–1.09	
YKL-40 (38–49.9)	1.03	0.95–1.12		1.03	0.94–1.11	
YKL-40 (50–65.9)	1.04	0.96–1.14		1.05	0.96–1.13	
YKL-40 (66–98)	1.05	0.96–1.14	0.174[£]	1.04	0.95–1.12	0.230[£]

Coef: coefficient; CI: confidence interval; T_{PTEF}/T_E ratio of time to reach peak tidal expiratory flow to total expiratory time. All lung function parameters were log converted for analysis and results shown as exponentiated coefficients. [*]Adjusted for the following additional risk factors: sex, gestational length, postnatal age at lung function, weight at lung function. [$]Missing data on n=20; data available for n=122.
[#]*P*-values for SNPs are shown after correction for multiple testing according to Benjamini-Hochberg. [£]P_{trend}-values were calculated with the Cochran-Armitage trend test.

Table S6 Associations between SNPs and cord blood YKL-40 levels with positive prick test at school age

	Univariable association			Multivariable* association		
	OR	95% CI	*P*-value	OR	95% CI	*P*-value
Exposure	Outcome positive prick test+					
SNP#						
rs10920579	0.78	0.27–2.19	0.921	0.65	0.22–1.90	0.983
rs880633	0.96	0.46–1.99	0.921	0.87	0.39–1.88	0.983
rs10399931	0.56	0.21–1.14	0.921	0.46	0.17–1.12	0.945
rs10399805	0.89	0.24–3.19	0.921	0.99	0.26–3.36	0.983
rs1538372	1.06	0.49–2.23	0.921	0.93	0.42–2.01	0.983
rs7542294	0.91	0.31–2.64	0.921	0.92	0.32–2.69	0.983
rs2486064	0.93	0.45–1.91	0.921	0.78	0.36–1.63	0.983
YKL-40 (ng/ml)						
YKL-40 non-detects	1	reference	P_{trend}	1	reference	P_{trend}
YKL-40 (7–37.9)	1.01	0.24–4.24		0.95	0.22–4.14	
YKL-40 (38–49.9)	1.24	0.29–5.26		1.05	0.23–4.73	
YKL-40 (50–65.9)	1.17	0.27–4.96		1.07	0.25–4.64	
YKL-40 (66–98)	2.8	0.75–10.42	0.128£	2.56	0.66–9.88	0.169£

CI: confidence interval; OR: odds ratio; SNP: single nucleotide polymorphism. *Adjusted for the following additional risk factors: sex, parental smoking during childhood, maternal atopy, parental education. +Positive in case of hives bigger than positive control histamine in any of the tested allergens. Missing data on n=38; data available for n=104. #*P*-values for SNPs are shown after correction for multiple testing according to Benjamini-Hochberg. £P_{trend}-values were calculated with the Cochran-Armitage trend test.

Table S7 Associations between SNPs and cord blood YKL-40 levels with lung function at 6 years

	Univariable association			[§]Adjusted association		
	Coef	95% CI	p-value	Coef	95% CI	p-value
Exposure	Outcome FVC (z-score)[$]					
SNP[#]						
rs10920579	0.27	-0.29–0.47	0.803	0.10	-0.31–0.51	0.968
rs880633	0.18	-0.07–0.44	0.609	0.17	-0.10–0.44	0.968
rs10399931	0.05	-0.29–0.38	0.803	0.05	-0.29–0.49	0.968
rs10399805	0.44	-0.06–0.95	0.526	0.44	-0.11–1.02	0.714
rs1538372	0.01	-0.26 –0.29	0.803	-0.01	-0.30–0.29	0.968
rs7542294	0.43	0.01–0.85	0.332	0.43	-0.01–0.84	0.336
rs2486064	-0.14	-0.44–0.15	0.803	-0.15	-0.44–0.13	0.968
YKL-40 (ng/ml)						
YKL-40 non-detects	1	reference	P_{trend}	1	reference	P_{trend}
YKL-40 (7–37.9)	0.14	-0.44–0.72		0.20	-0.40–0.81	
YKL-40 (38–49.9)	0.16	-0.43–0.74		0.21	-0.41–0.82	
YKL-40 (50–65.9)	0.10	-0.45–0.65		0.16	-0.43–0.75	
YKL-40 (66–98)	0.34	-0.22–0.91	0.280[£]	0.39	-0.21–0.99	0.326[£]
Exposure	Outcome FEV_1 (z-score)[#]					
SNP[#]						
rs10920579	-0.07	-0.47–0.33	0.743	-0.09	-0.52–0.33	0.863
rs880633	0.05	-0.24–0.33	0.743	0.03	-0.22–0.25	0.863
rs10399931	-0.08	-0.44–0.28	0.743	-0.09	-0.61–0.25	0.863
rs10399805	0.36	-0.15–0.29	0.743	0.36	-0.14–0.22	0.863
rs1538372	-0.14	0.45–0.86	0.743	-0.17	0.44–0.19	0.863
rs7542294	0.26	-0.41–0.17	0.743	0.27	-0.15–0.67	0.863
rs2486064	-0.23	-0.52–0.67	0.743	-0.26	-0.53–0.07	0.721
YKL-40 (ng/ml)						
YKL-40 non-detects	1	reference	P_{trend}	1	reference	P_{trend}
YKL-40 (7–37.9)	0.01	-0.59–0.61		0.09	-0.53–0.71	
YKL-40 (38–49.9)	-0.14	-0.73–0.45		-0.04	-0.67–0.61	
YKL-40 (50–65.9)	0.03	-0.54–0.61		0.11	-0.51–0.73	
YKL-40 (66–98)	0.28	-0.31–0.88	0.428[£]	0.43	-0.21–1.11	0.239[£]
Exposure	Outcome $FEF_{25-75\%}$ (z-score)[&]					
SNP[#]						
rs10920579	-0.10	-0.51–0.31	0.963	-0.18	-0.61–0.23	0.709
rs 880633	-0.30	-0.58–0.27	0.834	-0.30	-0.57–0.01	0.276
rs10399931	-0.17	-0.52–0.19	0.963	-0.22	-0.59–0.13	0.709
rs10399805	-0.18	-0.72–0.36	0.963	-0.11	-0.66–0.43	0.709
rs1538372	-0.29	-0.58–0.01	0.963	-0.31	-0.58–0.01	0.332
rs7542294	-0.16	-0.61–0.29	0.963	-0.15	-0.62–0.28	0.709
rs2486064	-0.13	-0.44–0.18	0.963	-0.14	-0.46–0.17	0.709
YKL-40 (ng/ml)						
YKL-40 non-detects	1	reference	P_{trend}	1	reference	P_{trend}
YKL-40 (7–37.9)	0.25	-0.31–0.81		0.27	-0.31–0.84	
YKL-40 (38–49.9)	-0.26	-0.83–0.31		-0.17	-0.77–0.43	
YKL-40 (50–65.9)	-0.08	-0.61–0.45		-0.05	-0.61–0.52	
YKL-40 (66–98)	-0.07	-0.61–0.47	0.610[£]	0.01	-0.56–0.59	0.836[£]

Coef: Coefficient; CI: confidence interval; SNP: single nucleotide polymorphism; FVC: forced vital capacity; FEV_1: forced expiratory volume in one second; FEF: forced expiratory flow from 25-75% of exhalation. [$]Missing data on n=51; data available for n=91. [#]Missing data on n=66; data available for n=76. [&]Missing data on n=62; data available for n=80. [§]Adjusted for presence or absence of siblings, daycare attendance and asthma of the child. [#]*P*-values for SNPs are shown after correction for multiple testing according to Benjamini-Hochberg.
[£] P_{trend} -values were calculated with the Cochran-Armitage trend test.

3.4.2 Time series approach: characterization of respiratory symptoms

Dynamics of respiratory symptoms during infancy and associations with wheezing at school age

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Manuscript ready for submission

Dynamics of respiratory symptoms during infancy and associations with wheezing at school age

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Results - Time series approach: characterization of respiratory symptoms

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Key messages

- In this study, we developed a method to objectively characterize the dynamic symptom pattern of prospectively assessed respiratory symptoms during infancy.
- Utilizing this method, we identified a group of infants exposed to host factors and environmental exposures, which were also at increased risk for wheezing and atopy at school age.
- This suggests that the assessment of dynamic symptom patterns may help to better characterize subjects susceptible for later disease.

Abstract

Background: Children with frequent respiratory symptoms in infancy have an increased risk for later asthma, but the association with the dynamic symptom pattern is unknown. We developed an observer-independent method to characterize the dynamics of symptoms and tested its association with respiratory morbidly at school age.

Methods: In this birth-cohort of healthy neonates, we prospectively assessed weekly respiratory symptoms during infancy, resulting in a time series of 52 symptom scores. For each infant, we calculated the probability of transition between two consecutive symptom scores. These transition probabilities were used to construct a Markov matrix, which characterized the dynamic symptom pattern quantitatively using a single entropy parameter. Based on this parameter we determined 4 phenotypes. Using logistic regression, we then determined the association with wheezing and atopy at school age.

Results: From 369 eligible neonates, 322 (87%) attended follow-up at 6 years and had complete data for >48 weeks of respiratory symptom scores during infancy (16864 observations). Compared to the healthy reference phenotype, the high-risk phenotype, defined by the highest irregularity (and thus entropy parameter) of the Markov matrix, had an (adjusted odds ratio; 95% CI) for wheezing of (OR 3.85; 1.11–13.43), and for atopy of (OR 3.45; 1.09–10.87) at school age. The high-risk phenotype was predominantly male (82%), and contained more infants exposed to maternal asthma (23%), and environmental tobacco smoke (41%).

Conclusion: Our study describes a novel method to characterize dynamics of respiratory symptoms at infancy, which may reflect susceptibility and recovery patterns of the airways.

Introduction

Wheezing disorders in early childhood have a high prevalence [1], with a major health issue [2], and methods to identify infants at risk for subsequent asthma are needed. Exposure to host factors (e.g. sex, maternal atopy), and environmental risk factors (e.g. childcare, siblings, environmental tobacco smoke (ETS) exposure, air pollution) influence the incidence and duration of respiratory symptoms during infancy [3, 4], and are associated with wheezing episodes during childhood [5]. In clinical practice, assessing risk factors, estimating the frequency of respiratory symptoms, and also the symptom pattern (e.g. episodic *versus* persistent symptoms) [6], may help to identify infants at risk for later asthma.

Especially the pattern of symptom deterioration and recovery (i.e. progression from a given symptoms state to another) may be informative, as it is determined by the dynamic symptom pattern that a subject undergoes during a given time window. While it is known that persistent wheeze in infants is more closely associated with later asthma and reduced lung function than episodic wheeze [6, 7], it is difficult to characterize the dynamic symptom pattern observer-independently, and to estimate its predictive value for persistence of respiratory symptoms during preschool age.

We hypothesize that the dynamic symptom pattern may not only be determined by exposure to infectious risk factors (siblings, childcare), but also by host factors and exposure to ETS or air pollution. The dynamic symptom pattern may thus contain information on susceptibility of the airways to infectious triggers. This hypothesis was supported by our previous study (Stern et al. [8]), in which infants exposed to higher air pollution levels recovered more slowly from viral infections than those with lower exposure levels. Similarly, infants of allergic mothers [9], or those exposed to ETS [9], are more likely to suffer from persistent wheeze during childhood.

Previous studies used a Markov model the trajectory of asthma severity [10], and to model asthma control [11]. The dynamic pattern of subsequently assessed respiratory symptoms could be mathematically represented using this Markov model (also known as Markov matrix) [12].

Each row of this matrix encodes a conditional probability distribution, which can be measured using the Shannon entropy [13]. Consequently, by calculating entropy of the probability distributions encoded in the Markov matrix, we could objectively characterize the pattern of symptom deterioration and recovery.

The aim was to develop a method to characterize the dynamic pattern of weekly assessed respiratory symptom scores during infancy with a Markov matrix for each infant. First, we tested if we could identify specific dynamic phenotypes using these Markov matrices. Next, we tested whether these dynamic phenotypes predicted wheezing and atopy at school age. Lastly, we determined if environmental risk factors were more common in specific dynamic phenotypes, to explore if specific dynamic symptom patterns are influenced by host factors and environmental exposures.

Methods

Details are outlined in the appendix.

Study design

In the Basel-Bern infant lung development (BILD) birth-cohort study, we prospectively assessed weekly respiratory symptom scores (states 0 to 4) [14] during infancy, resulting in 52 consecutive observations. We used these symptom scores to develop a method which summarizes the dynamic symptom pattern with a Markov matrix for each infant. These Markov matrices were characterized using one single quantitative measure, namely entropy. First, we tested if we could identify specific dynamic phenotypes based on this entropy parameter. Next, we tested the association between dynamic phenotypes with wheezing and atopy at school age (primary outcomes). Allergic sensitization, upper respiratory tract infection (URTI), lung function and FeNO measurements at school age were secondary outcomes. Lastly, we compared the distribution of risk factors across dynamic phenotypes.

Study participants

This study comprised a group of unselected, healthy term born neonates recruited antenatally in two centers (Bern and Basel) in Switzerland [15]. From 1999 to 2015, 369 children from Bern were invited for a follow up at 6 years. The Ethics Committees of Bern and Basel, Switzerland approved the study. Written informed consent was obtained from parents before enrolment.

Exposures: **respiratory symptoms during infancy**

During the first year of life, research nurses called the parents weekly to assess the child's health and respiratory symptoms using a standardized symptom score that groups symptoms into 4 levels according to severity [4, 14]. Weeks with respiratory symptoms were defined as the total number of weeks a child had any respiratory symptom, independent of type or severity; weeks with severe respiratory symptoms were defined as a symptom score of ≥3 (e.g repeated sleep disturbances during the night, or general practitioner consultation), as described previously [4, 14] (Table S1).

Markov matrix to assess dynamics of respiratory symptoms

We used a Markov model approach to examine trajectories between subsequently assessed symptom scores. We assessed transitions between different levels of the symptom scores: state zero: healthy, symptom score 0; states 1-4: symptomatic states, symptom scores 1-4. For each symptom state, we counted how often a transition to any other state, assessed in the subsequent week occurred. For each child, this count information can be displayed in a 5×5 matrix (vertical axis initial state, horizontal-axis target state). These counts are absolute frequencies, which were used to calculate relative frequencies for each transition (Figure 1 A-C). This matrix is called Markov matrix [12], which was then graphically represented using a three-dimensional landscape. Finally, the landscape's irregularity was quantified using a single entropy value [16] (Figure 2, supplementary methods).

Risk factors

At baseline, we used a standardized questionnaire to assess pre-and postnatal exposure to risk factors for respiratory symptoms during infancy [4] or asthma development (e.g. number of siblings, maternal asthma). Parental atopic disease was defined as self-reported, doctor-diagnosed asthma, hay fever, or eczema. Maternal education was categorized as low (3 years of secondary education) and high (≥4 years of secondary education). Duration of breastfeeding (exclusive or non-exclusive) was weekly assessed and binary coded (as <26 and ≥26 weeks). All risk factors were categorized as binary variables in order to compare under-or overrepresentation across phenotypes.

Outcomes: respiratory outcomes and atopic sensitization at school age

At 6 years, asthma and allergy were assessed by an adapted International Study of Asthma and Allergies in Childhood (ISAAC) questionnaire [17]. We choose the following outcomes: "any wheezing between 1-6 years"; "current wheezing", defined by wheezing over the past 12 months; upper respiratory tract infection (URTI), defined by ear infection, throat infection, or serious cold (defined by symptoms ≥2 weeks) over the past 12 months. Atopy was defined as

allergic rhinitis, allergic asthma, or atopic dermatitis. A skin-prick test (SPT) was determined positive if a wheal diameter of any of the seven tested aeroallergens was greater than positive control [15].

Spirometry was performed using the MasterLab setup (Jaeger, Wuerzburg, Germany) according to standard guidelines [18]. Forced expiratory volume in 1 s (FEV_1), forced vital capacity (FVC), FEV_1/FVC ratio, and forced expiratory flow at 25-75% of FVC ($FEF_{25-75\%}$) were expressed as z-scores [19]. We measured the fraction of exhaled nitric oxide (FeNO) using a commercially available analyzer (CLD 77 AM; Eco Medics AG, Duernten, Switzerland) according to current guidelines [20].

Statistical analysis

Entropy is a measure for disorder within a dynamic system, and higher entropy values correspond to more irregularity [13]. For each infant, we calculated entropy [21] of the Markov matrix, which provides a quantitative measure of the irregularity patterns of symptom deterioration and recovery. The frequency distribution of entropy visually appeared multimodal, with 4 modi (Figure 3A). Therefore, we defined 4 dynamic phenotypes based on the slope of the cumulative distribution function curve, and a slope of zero was used for cutoff. We defined 4 similarly sized reference phenotypes by the frequency distribution of total number of weeks with respiratory symptoms during infancy (Figure 3B).

Using logistic regression, we studied the association of the 4 dynamic phenotypes, the 4 reference phenotypes, and asthma risk factors, with the child's outcomes. For "any wheezing", "current wheezing", atopy and positive prick test of the child, analyses were adjusted for sex, maternal education and asthma, ETS exposure, childcare, and siblings. For FeNO, we additionally adjusted for asthma of the child and inhaled corticosteroid use. For lung function, we adjusted for maternal education, maternal asthma, ETS exposure, childcare, and siblings. Chi^2 and Kruskal–Wallis tests were used to compare characteristics across phenotypes. A Bonferroni-corrected significance level was used to account for multiple pair-wise testing. We

used the weighted kappa-statistic [22] to compare agreement between dynamic and reference phenotypes.

For sensitivity analyses, we repeated the analysis in infants having ≥1 episode of symptom score ≥3, and within an additional, independent sample from our cohort of 242 infants. To explore that the entropy distribution was not an artifact of our analysis, we re-categorized the symptom states (0, 1, 2, (3+4)→3), we simulated data, and perturbed the existing data. Furthermore, we corrected our findings for potentially unobserved events.

Results

From 369 eligible subjects, 322 (87%) were studied (Figure S1), having >48 weeks of symptom series during infancy and complete data on risk factors and outcomes (Table 1). Demographic data and distribution of respiratory symptoms did not differ between infants followed-up and those lost to follow-up, but exposure to risk factors did (Table S2).

Distribution of respiratory symptoms

In infants followed-up, we had information for 16864 person–weeks. On average, the number of weeks with any respiratory symptom was (median; range) (4; 0-23 weeks). In contrast, severe symptoms were rare (0; range 0-6 weeks). Figure 1D shows the distribution of all symptom states.

Dynamics of respiratory symptoms assessed by the Markov matrix

Figure 2 shows the dynamic pattern of respiratory symptoms for 2 infants. Both had the same number of symptom weeks, but different patterns of the Markov matrix landscape. The shape of the matrix landscape, characterized by a single entropy parameter differed as well.

Outcomes at school age

From 322 children at follow-up, 105 (32.9%) had any wheezing, and 38 (11.7%) had current wheezing. There were 120 (37.5%) children with allergic diseases, and a subgroup of 270 completed a SPT, 37 (13.6%) of which were positive. Lung function was completed in 222 (68.7%) children, and 231 (71.7%) had FeNO measurements.

Associations of dynamic phenotypes, reference phenotypes and risk factors with outcomes at school age

We defined 4 dynamic phenotypes using the Markov matrix and 4 corresponding reference phenotypes by the distribution of respiratory symptom weeks (Figure 3). Compared to baseline phenotpye one, in the adjusted logistic regression model, male gender, maternal asthma, dynamic phenotype 4 and reference phenotypes 2 and 3 were associated with any wheezing during childhood (Table 2). Dynamic phenotype 4 and male gender were also associated with current

wheezing and atopy (Table 2). Similar associations were obtained for the outcome positive SPT, but only reference phenotype 3 was associated with URTI (Table S3). Compared to the baseline phenotype, lung function and FeNO measurements did not differ between phenotypes 2-4 (Table S4). We obtained similar results in infants with ≥1 episode of symptom score ≥3 (data not shown).

Over-and underrepresentation of risk factors across phenotypes

Dynamic phenotypes

These 4 phenotypes included 141, 102, 62 and 17 infants (Table 3, Figure 3). These differed with regards to the number of weeks with any and severe symptoms: phenotype 1 had severe symptoms for 0.21 weeks, phenotype 2 for 0.57 weeks, phenotype 3 for 1.53 weeks, and phenotype 4 for 3.06 weeks. Also, entropy differed across phenotypes: phenotype 1 had entropy of 0.012, phenotype 2 of 0.15, phenotype 3 of 0.29, and phenotype 4 of 0.44.

Furthermore, risk factors differed across phenotypes: phenotype 1 had an underrepresentation of children with siblings, or attended childcare and were more likely born via Cesarean section. Phenotype 2 had an underrepresentation of males, and more infants had siblings. Phenotype 3 had more males, and infants were less likely born via Cesarean section. Phenotype 4, the smallest group, had an overrepresentation of males, and infants attending childcare. In this phenotype, more infants were born to asthmatic mothers, or to mothers who smoked during pregnancy, and were also more likely exposed to tobacco during infancy. We considered this as high-risk dynamic phenotype, since associations with later wheezing and atopy were strongest.

Reference phenotypes

Phenotypes included 147, 98, 57, and 20 infants (Table 3), respectively. Phenotypes 1, 2, 3, and 4 had 0.14, 0.70, 1.81, and 2.05 weeks with severe symptoms; entropy was 0.05, 0.16, 0.29, and 0.32, respectively. Risk factors differed across phenotypes. Phenotype 1 had an underrepresentation of males; fewer infants had siblings and were more likely born via Cesarean section. In phenotype 2, infants were less likely born via Cesarean section. Phenotype 3 had

more males, more infants with siblings and childcare attendance, and fewer infants born via Cesarean section. Phenotype 4 had an overrepresentation of males and more infants had siblings, and were more likely born to asthmatic mothers.

Comparison between temporal and reference phenotypes

There was high overlap between the dynamic and reference phenotypes. Overlap was strongest between phenotype 1, with a low number of symptoms and weakest between phenotype 4, with a high number of symptoms (Figure 4). This indicates that phenotyping based on dynamic symptom patterns identifies infants with different characteristics compared to the reference method based on number of symptom weeks. In the high-risk dynamic phenotype 4, we found an overrepresentation of infants from smoking and asthmatic mothers, whereas in the reference phenotype 4, e.g. presence of siblings was strongly overrepresented. Although symptoms were frequent in reference phenotype 4, a much small proportion compared to dynamic phenotype 4 developed wheezing and atopy.

Validation of the phenotypes, sensitivity analysis

Within the validation dataset, there were differences in anthropometric data and risk factors between centers (Table S5), but not in the distribution of respiratory symptoms. Phenotypes characteristics were comparable to the main analysis (Table S6). Several sensitivity analyses ruled out that the multimodal distribution of entropy was an artifact due to our methodology. After correcting our findings for potentially unobserved events, we observed three dynamic phenotypes, as detailed in the online supplement, and Figure S2 and Figure S3.

Discussion

Based on times series of weekly symptom scores during infancy from healthy infants, we developed a novel method, which characterizes the dynamic symptom pattern in an observer-independent manner. With this method, we calculated the transition probability between two consecutive symptom scores. These transition probabilities were used to construct a Markov matrix which was characterized using a single entropy parameter for each infant. Based on entropy, we determined 4 phenotypes. The phenotype with the highest entropy value (high risk dynamic phenotype) had more infants attending childcare, and exposure to maternal asthma a prenatal ETS, and had the highest prevalence of wheezing (35%) and atopy (64%) at school age. Although defined by entropy, this phenotype had also the highest total number of severe respiratory symptom weeks. In comparison, in the reference phenotype defined by the total number of any respiratory symptom weeks, infants with a high number of symptom weeks had more likely siblings or asthmatic mothers. Only 20% had current wheezing and 35% atopy at school age. These findings are consistent with the hypothesis that host factors and exposure to ETS may play a role for the dynamic symptom pattern, and are likely relevant for future disease, although we observed on overlap of infectious risk factors between the dynamic and reference phenotypes.

Comparison with literature

Similarly to other studies [10, 23], we observed an association between male gender and childhood asthma. The association between low maternal education and the risk for atopy should be interpreted with caution since mothers in our study were all relatively well educated. The "low" maternal education in our study is therefore only partially comparable to previous studies.

Phenotypes from the dynamic symptom pattern as well as total number of symptom weeks during infancy were not associated with lung function or FeNO at school age. These findings differ to results from a large European birth cohort (ALSPAC study) describing an association between different wheezing phenotypes (early transient, persistent wheeze, etc.) with lung

function at 8-9 years of age [7, 24]. Differences could be due to the small number of lung function measurements at school age in our study, and because only few children had a severely reduced lung function in this unselected study population. Also, while our method assessed any respiratory symptoms (mostly cough and rhinitis), ALSPAC measures 'wheezing' [7, 24], which may reflect other airway properties. Nevertheless, future studies may profit from the use of our observer-independent method for the analysis of wheezing symptoms.

Potential mechanism, interplay between host and environmental risk factors

Interplay between an exposure (e.g. virus) and host predisposition (e.g. infant exposed to maternal asthma) is thought to determine the response to a respiratory infection. Studies further suggest that the severity of respiratory infections in children does not only depend on the virus type [25], but is modified by microbial composition [26] and environmental factors [4, 9]. Interplay between viral stimuli, microbial composition and genetic predisposition are suggested to affect airway epithelial function [27, 28]. We speculate that infants with a higher fluctuation between respiratory symptom scores (and thus higher average entropy of the Markov matrix) are those having an epithelial dysfunction and are at increased risk for developing asthma and atopy. Markers associated with asthma exacerbations (e.g cytokine expression, impaired interferon response) were identified previously [27]. In order to test our hypothesis of an epithelial dysfunction during infancy relevant for future disease, prospective studies assessing these markers during respiratory infections are needed.

Methodological aspects, limitations of the method

While we did not define phenotypes based on "classical" data driven methods (e.g. Ward's hierarchical clustering [29] or latent class analysis [7, 24, 30, 31]), our phenotype identification was still data driven, as they were exclusively defined by the data structure of transition patterns. Previous studies using unsupervised methods assessed symptoms at 2 [30] up to 14 times [32]. The resolution of the respiratory symptoms of this study is unique, since we had a minimum of 49 observation time points. Comparison of phenotypes in this and previous studies is limited

since previous studies phenotyped based on wheeze during childhood [7, 29-31, 33], while this study focused on any respiratory symptom during infancy. Most previous studies investigated the association of phenotypes with the child's risk factors, (e.g. allergic sensitization), while our study assessed prenatal (e.g. maternal smoking during pregnancy), and early postnatal (e.g. breastfeeding, childcare attendance) risk factors.

It is striking that entropy of the Markov matrix in our study showed a multimodal distribution with 4 peaks, suggesting we can use entropy to define 4 phenotypes. Sensitivity analysis ruled out the possibility that this distribution is an artifact due to our methodology. For example, we tested if this distribution could be a mathematical phenomenon related to the scoring system (5 states). However, re-categorization of the states (0, 1, 2 (3+4)→3), did not systematically change the identified phenotypes. We further challenged our method and corrected our analysis for potentially unobserved events, resulting in higher average entropy values of the Markov matrix. We defined 3 phenotypes, indicating that applying our method in other populations with different symptom patterns may result in different entropy distributions.

Strengths and limitations

We prospectively assessed respiratory symptoms weekly by telephone interviews, obtaining reliable data on respiratory morbidity [14] and reducing a potential recall bias compared to retrospectively reported symptoms. We had only a few missing data points; 2 infants out of the eligible participants had ≤48 weeks of observations and were excluded. We used the same questionnaire to assess risk factors and outcome data throughout the entire study period in both centers, reducing potential inter-center differences. Since this study was conducted in a healthy population, there were only 38 subjects with current wheezing (38) at school age. Furthermore, the high risk-phenotypes incorporated only small numbers and misclassification of just one subject could have a major influence on our findings. Very few cohort studies prospectively assessed respiratory symptoms during infancy (e.g. for high risk infants [34], overview for unselected infants [4]). Due to lack of data, we could not validate our method in any of those, but

we performed an external validation in 242 infants from our cohort not seen for follow-up. This was an unselected study population, and results are therefore not directly applicable to high-risk populations. Performing a similar study within a larger cohort at increased risk for asthma and allergic diseases could further validate the developed method, and may contribute to our understanding of the predictive value for future disease.

Clinical relevance and research application

Our results indicate that specific dynamic symptom patterns during infancy are associated with wheezing at school age. This may guide pediatricians to identify those at risk among the large number of symptomatic subjects. Our method could be used in ongoing research on asthma control, as it objectively quantifies symptom patterns, and could be considered complementary to routine asthma biomarkers (e.g. FeNO, peak expiratory flow). Furthermore, assessment of symptom patterns is non-invasive, does not require laboratory equipment, and thus could be used in telemonitoring [35].

Conclusion

In this study, we developed a method to characterize the transition probabilities between respiratory symptoms and found that they were associated with wheezing and atopy at school age. Furthermore, these transitions probabilities enabled us to identify four distinct phenotypes of which one consisted of predominantly male subjects of asthmatic mothers and ETS exposure. This indicates that symptom patterns contain relevant information on risk factors for asthma development. The developed method has potential in various research settings, since it is based only on respiratory symptom scores, which can be assessed without laboratory equipment or specially trained experts.

Contributors

JU, PL, MR, CK and UF were investigators in this study and contributed to the study design. IK, AS, PA, EP and OG are members of the BILD study team and contributed to data collection and analysis. JU, BX, MR, CK, PL, EDE and UF contributed to the data interpretation. EDE and BX conceived the computational approach. All authors contributed to the writing, review of the report, and approved the final version of the manuscript. AS and EP are part of the BILD study group.

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Conflict of interest

The authors declare that they have no relevant conflicts of interest.

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Table 1 Characteristics of the study population

Anthropometric data at birth	Mean (SD)	Median (IQR)	Range	n (%)
Gestational age, weeks	39.6 (1.2)	39.8 (38.8–40.5)	36.7–41.8	
Birth weight, kg	3.3 (0.4)	3.3 (3.0–3.6)	2.1–4.9	
Length, cm	49.5 (1.9)	50.0 (48–51)	44–57	
Respiratory symptoms in the first year of life				
Weeks with symptoms	5.3 (4.6)	4 (0–20)	0–23	
Weeks with severe symptoms	0.71 (1.1)	0 (0–1)	0–6	
Risk factors				
Male sex				167 (51.8)
Siblings				159 (49.4)
Caesarean section				54 (16.7)
Maternal asthma				34 (10.5)
Maternal atopy				116 (36.1)
Childcare				62 (19.5)
Maternal smoking in pregnancy				27 (8.4)
Parental smoking during infancy				70 (21.7)
Breastfeeding >26 weeks				252 (78.3)
Low maternal education				202 (62.7)
Season of birth				
Spring				87 (27.4)
Summer				83 (25.7)
Autumn				80 (24.8)
Winter				72 (22.6)
Outcomes during childhood				
Any wheezing				105 (32.9)
Current wheezing				38 (11.7)
Atopy				120 (37.5)
Skin prick test positive*				37 (13.6)
URTI+				122 (37.9)

Values are means (SD), median (IQR) or number (percentage). IQR: interquartile range; n: number; SD: standard deviation; URTI: Upper respiratory tract infection. Data are derived from 322 infants with a total of 16864 observed symptom weeks. *A skin prick test was completed in a subset of 270 children.

Table 2 Association of symptom dynamic phenotypes, reference phenotypes, and risk factors with outcomes during childhood

	Univariable association			Multivariable association[#]		
	OR	95% CI	P-value	OR	95% CI	P-value
Exposure	Outcome any wheezing between 1-6 years[+] (105/322)					
Respiratory symptom, weeks (Reference Phenotypes)						
Phenotype 1 (baseline) (147)	1	reference		1	reference	
Phenotype 2 (98)	2.09	1.19 –3.67	0.010	1.88	1.04–3.40	0.036
Phenotype 3 (57)	3.10	1.62–5.94	0.001	2.62	1.31–5.23	0.006
Phenotype 4 (20)	2.82	1.07–7.39	0.034	1.99	0.70–5.61	0.192
Transition states, entropy (Dynamic Phenotypes)						
Phenotype 1 (baseline) (141)	1	reference		1	reference	
Phenotype 2 (102)	1.02	0.57–1.81	0.931	1.05	0.57–1.93	0.855
Phenotype 3 (62)	1.95	1.04–3.66	0.036	1.80	0.92–3.50	0.081
Phenotype 4 (17)	12.64	3.44–46.47	<0.001	9.14	2.35–35.46	0.001
Risk factors						
Male sex	3.05	1.86–5.01	<0.001	2.90	1.75–4.81	<0.001
Siblings	1.22	0.77–1.95	0.386	1.27	0.78–2.09	0.328
Maternal asthma	2.23	1.09–4.58	0.028	2.16	1.03–4.55	0.041
Childcare	1.49	0.84–2.64	0.169	1.40	0.75–2.59	0.284
Parental smoking during infancy	1.08	0.61–1.89	0.783	0.99	0.55–1.81	0.995
Low maternal education	1.23	0.76–2.01	0.391	1.13	0.66–1.93	0.644
Exposure	Outcome current wheezing at 6 years[¶] (38/322)					
Respiratory symptom, weeks (Reference Phenotypes)						
Phenotype 1 (baseline) (147)	1	reference		1	reference	
Phenotype 2 (98)	1.04	0.42–2.54	0.927	0.92	0.36–2.31	0.865
Phenotype 3 (57)	2.74	1.16–6.45	0.020	2.41	0.97–5.98	0.056
Phenotype 4 (20)	2.57	0.74–8.85	0.133	1.89	0.51–7.06	0.340
Transition states, entropy (Dynamic Phenotypes)						
Phenotype 1 (baseline) (141)	1	reference		1	reference	
Phenotype 2 (102)	1.07	0.44–2.54	0.878	1.12	0.46–2.75	0.792
Phenotype 3 (62)	1.67	0.67–4.14	0.267	1.61	0.62–4.11	0.327
Phenotype 4 (17)	5.37	1.71–16.91	0.004	3.85	1.11–13.43	0.034
Risk factors						
Male sex	2.52	1.21–5.28	0.014	2.26	1.06–4.81	0.033
Siblings	1.15	0.58–2.28	0.670	1.19	0.61–2.42	0.616
Maternal asthma	1.71	0.65– 4.45	0.141	1.66	0.62–4.41	0.306

Childcare	1.35	0.61–3.02	0.462	1.38	0.58–3.27	0.457
Parental smoking during infancy	1.33	0.61–2.89	0.468	1.15	0.51–2.59	0.723
Low maternal education	1.77	0.82–3.78	0.141	1.63	0.73–3.66	0.229

Exposure	Outcome atopy at 6 years* (120/320)					
Respiratory symptom, weeks (Reference Phenotypes)						
Phenotype 1 (baseline) (147)	1	reference		1	reference	
Phenotype 2 (98)	0.93	0.54–1.59	0.797	0.86	0.49–1.53	0.630
Phenotype 3 (57)	1.52	0.81–2.83	0.188	1.51	0.77–2.96	0.228
Phenotype 4 (20)	0.94	0.35–2.51	0.910	0.73	0.25–2.10	0.562
Transition states, entropy (Dynamic Phenotypes)						
Phenotype 1 (baseline) (141)	1	reference		1	reference	
Phenotype 2 (102)	1.13	0.66–1.93	0.643	1.26	0.72–2.22	0.413
Phenotype 3 (62)	1.16	0.62–2.16	0.641	1.20	0.62–2.34	0.573
Phenotype 4 (17)	3.51	1.22–10.08	0.019	3.45	1.09–10.87	0.034
Risk factors						
Male sex	2.15	1.35–3.42	0.001	2.10	1.29 –3.40	0.003
Siblings	1.09	0.69–1.72	0.686	1.01	0.63–1.63	0.944
Maternal asthma	1.55	0.76–3.18	0.226	1.51	0.72–3.17	0.274
Childcare	0.62	0.34–1.14	0.127	0.63	0.33–1.21	0.168
Parental smoking during infancy	1.09	0.63–1.88	0.752	0.92	0.5–1.64	0.786
Low maternal education	2.30	1.40–3.77	0.001	2.03	1.20–3.41	0.008

CI: confidence interval; OR: odds ratio. Logistic regression for the outcomes any wheezing, current wheezing and atopic disease. Symptom dynamic phenotypes were defined by entropy of transition states, reference phenotypes by weeks with any respiratory symptom. When considering phenotypes as exposure, phenotype one from the reference phenotype, or symptom dynamic phenotype served as baseline, respectively. Numbers in brackets indicate group sizes. [#]Adjusted for the following binary variables: male gender, low maternal education, maternal asthma, maternal smoking during pregnancy, childcare attendance during infancy, and presence of siblings. [+]Defined as any wheezing episode between one and six years of age. [¶]Defined by wheezing over the past 12 months before follow-up. [*]Atopic disease was defined as allergic rhinitis, allergic asthma, or atopic dermatitis.

Table 3 Characteristics of symptom dynamic phenotypes and reference phenotypes

	Phenotype 1		Phenotype 2		Phenotype 3		Phenotype 4		P-value	
	Dyn.	Ref.	Dyn.	Ref.	Dyn.	Ref.	Dyn.	Ref.	Dyn.	Ref.
	141	147	102	98	62	57	17	20		
Respiratory symptoms										
Weeks with severe symptoms	0.21 (0.41)	0.14 (0.37)	0.57 (0.78)	0.70 (0.75)	1.53 (1.40)	1.81 (1.42)	3.06 (1.20)	2.05 (1.79)	<0.001	<0.001
Weeks with symptoms	1.74 (1.52)	1.56 (1.09)	6.07 (3.32)	5.70 (1.44)	10.13 (3.56)	10.51 (1.54)	13.18 (3.94)	16.50 (2.93)	<0.001	<0.001
Respiratory symptoms transition states										
Entropy of transition states	0.012 (0.01)	0.05 (0.03)	0.15 (0.02)	0.16 (0.09)	0.29 (0.03)	0.29 (0.09)	0.44 (0.03)	0.32 (0.09)	<0.001	<0.001
Risk factors										
Male sex	49.6	43.5	45.1	54.0	59.6	63.1	82.3	70.0	0.076	0.08
Siblings	36.8	38.1	57.8	53.0	59.6	63.1	64.7	75.0	0.004	0.004
Caesarean section	24.1	23.8	13.7	10.2	4.4	10.5	17.6	5.0	0.024	0.08
Maternal asthma	9.9	8.8	9.8	11.2	9.6	10.5	23.5	20.0	1	1
Maternal atopy	37.5	33.3	30.3	37.7	41.9	38.6	35.2	40.0	1	1
Childcare	13.4	11.5	19.6	23.4	24.1	28.0	47.0	30.0	0.024	0.044
Maternal smoking during pregnancy	7.8	6.8	7.8	8.1	6.4	12.2	23.5	10.0	0.56	1
Parental smoking during infancy	24.1	23.1	17.6	19.3	17.7	22.8	41.1	20.0	0.48	1
Breastfeeding ≤26 weeks	21.3	20.5	20.6	24.5	20.9	24.6	29.5	5.0	1	1
Low maternal education	64.5	61.9	59..8	65.3	59.7.3	57.9	76.4	70.0	1	1
Seasons at birth										
Spring	24.8	26.5	33.3	28.5	28.5	24.5	24.8	30.0	1	1
Summer	22.7	18.3	24.5	37.7	37.7	26.3	22.7	20.0	1	0.032
Autumn	36.1	35.3	19.6	17.3	17.3	12.2	36.1	20.0	<0.001	0.004
Winter	16.1	19.7	22.5	16.3	16.3	36.8	16.3	30.0	0.112	0.068
Outcomes during childhood										
Any wheezing[+]	26.8	22.2	27.7	37.7	41.9	47.3	82.3	45.0	<0.001	0.008
Current wheezing[$]	9.2	8.8	9.8	9.2	14.5	21.1	35.3	20.0	0.112	0.196
Atopy	34.3	36.3	37.3	34.7	37.7	46.4	64.7	35.0	<0.001	1
Skin prick test positive[#]	12.8	14.5	13.7	12.2	12.3	12.3	25.0	21.4	0.052	1
URTI[¶]	36.8	34.1	41.2	37.7	35.5	50.9	35.3	30.0	0.448	0.54

Values are percent or mean (SD). Symptom dynamic phenotypes (Dyn.) are shown on white background, and reference phenotypes (Ref.) are shown on grey shading background· Symptom dynamic phenotypes were defined by average entropy of transition states, reference phenotypes by weeks with any respiratory symptom. Differences in the distribution of characteristics across phenotypes were assessed using Chi-squared tests for categorical variables, and Kruskal–Wallis for continuous variables. P-values are Bonferroni corrected, accounting for multiple testing. [+]Defined as any wheezing episode between one and six years of age. [$]Defined by wheezing over the past 12 months before follow-up. [*]Atopic disease was defined as allergic rhinitis, allergic asthma, or atopic dermatitis. [#]A skin prick test was completed in a subset of 270 children. [¶]Upper respiratory tract infection (URTI) was defined by ear infection, throat infection, or serious cold (defined by symptoms ≥two weeks) over the past 12 months before follow-up.

Figures

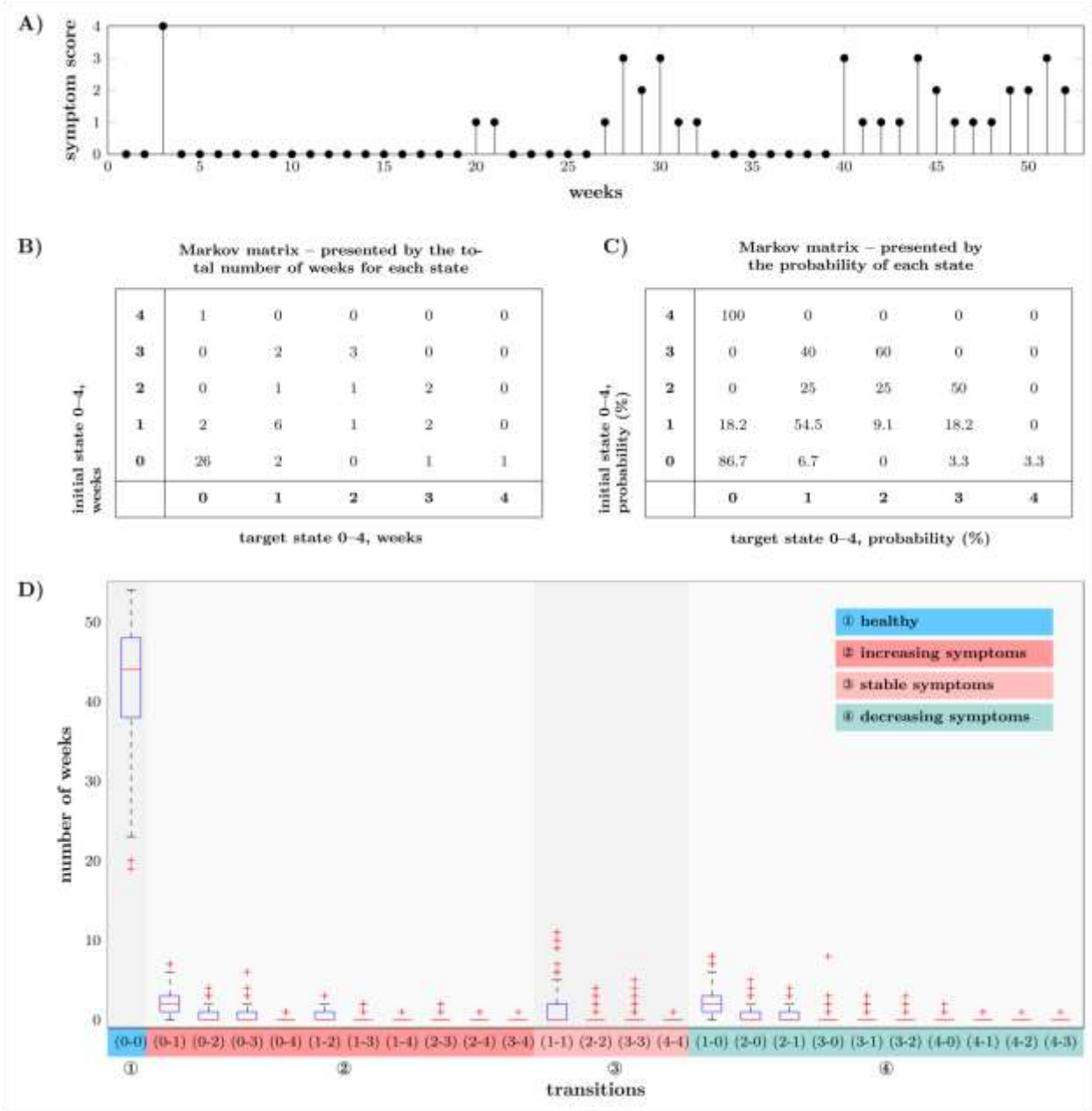

Figure 1 Representative Markov matrix of one infant and distribution of respiratory symptoms states during infancy among all study participants. A) Temporal pattern of respiratory symptoms scores during the first year of life for one infant. We assessed transitions between different levels of the symptom scores: state zero: healthy, symptom score 0; states 1-4: symptomatic states, symptom scores 1-4. For each symptom state (i.e. initial state, 1st time point), we counted how often a transition to any other state (i.e. target state, 2nd time point), as assessed during the subsequent week, occurred. B) This count information is displayed in a 5×5 matrix. C) These

counts are absolute frequencies, which were used to calculate relative frequencies. D) Transitions of all study participants. Vertical-axis represents the total number of weeks of each transition state. Horizontal-axis represents all possible transitions. Type1: healthy state; Type 2: increasing symptoms; Type 3: stable symptoms; Type 4: decreasing symptoms. Data are shown as box plots. Numbers in brackets indicate initial symptom score (initial state) and target symptom score (target state) assessed in the subsequent week.

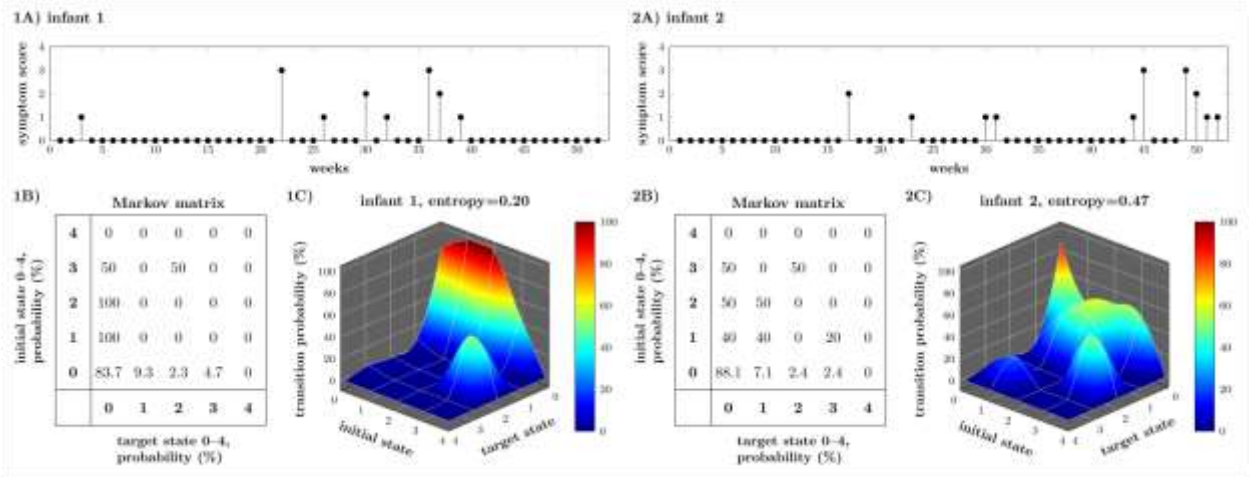

Figure 2 Representative Markov matrix patterns for two infants. Figure 1A (infant 1) and 2A (infant 2) show the respiratory symptoms states (scores 0-4) over the first year of life for each week. The total number of "weeks with respiratory symptoms" during the first year for both infants was the same (44 weeks symptom score 0, 4 weeks symptom score 1, 2 weeks symptom score 2 and 2 weeks symptom score 3). The Markov matrices in figure 1B and 2B show the empirical probability of each transition (described in the methods and Figure 1). 1C, 2C: Colors of the peaks' heights correspond to the empirical probability of each transition. The pattern of the Markov matrix landscape was expressed using a single average entropy parameter16 (described in online supplement methods), which was 0.2 for infant 1, and 0.47 for infant 2.

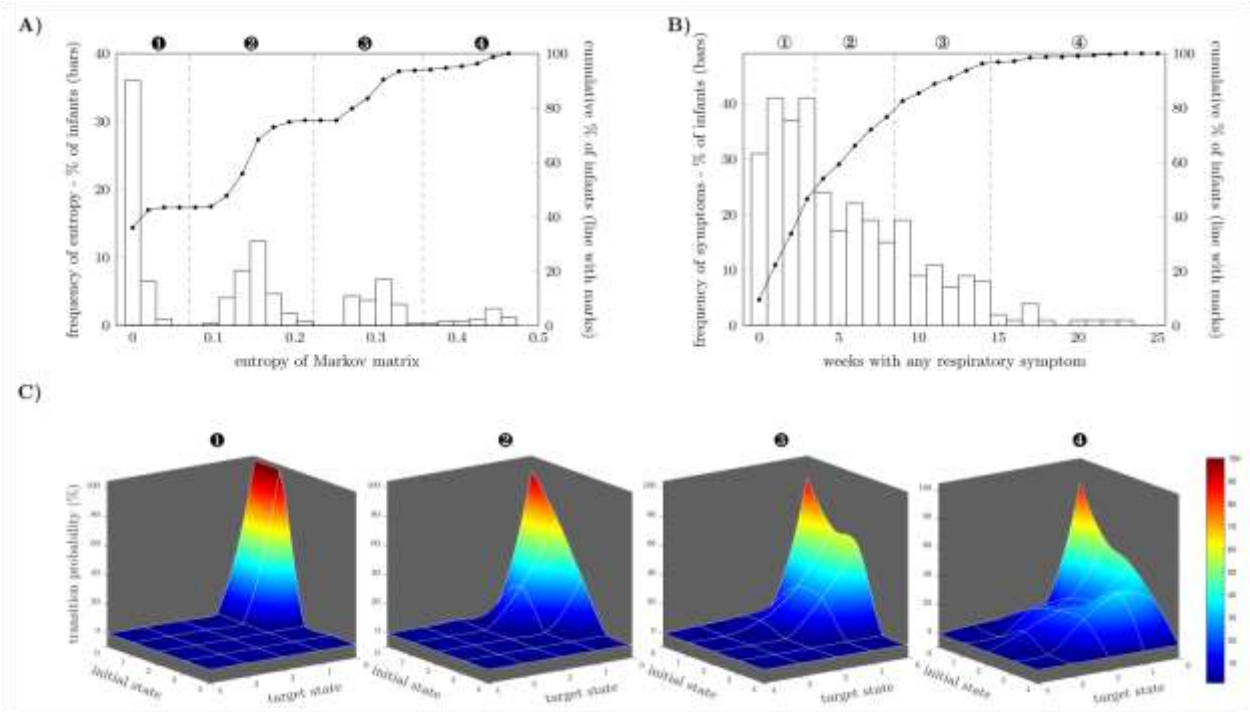

Figure 3 Frequency distribution of entropy of the Markov matrices and "weeks with any respiratory symptom" among the 322 study participants. Left side scaling axis indicate number of infants. Continuous line indicates cumulative probability of infants (right side scaling axis) dotted lines indicate cut-off between phenotypes. A) We used the frequency distribution of average entropy from the Markov matrices to define 4 phenotypes including 141, 102, 62 and 17 infants. B) We used the frequency distribution of the total number of weeks with any respiratory symptom to define phenotypes with sizes similar to those defined in panel A), including 147, 98, 57 and 20 infants. C) Three-dimensional representation of the average transition patterns from all infants within one phenotype, as defined in panel A). Colors correspond to the empirical probability of each transition. Numbers 0-4 represent the respiratory symptom scores.

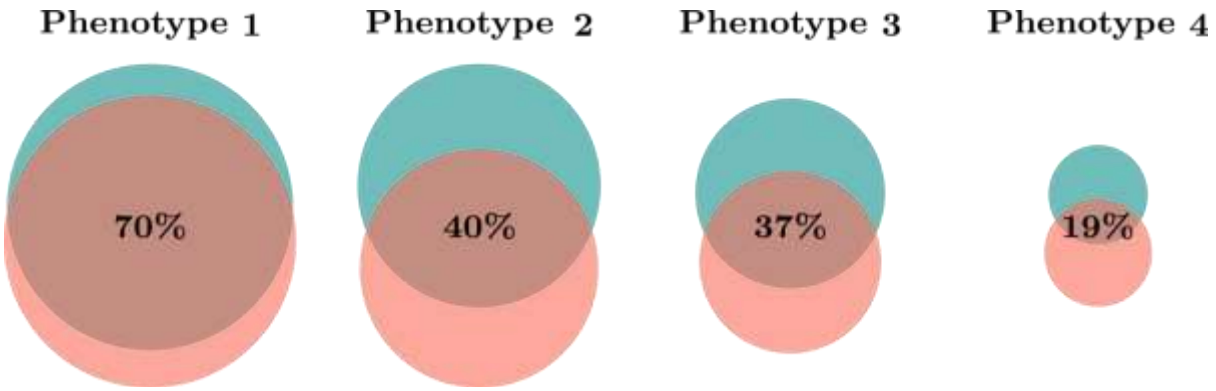

Figure 4 Overlap of infants allocated to the same phenotype compared between the two phenotyping methods. Individual circle size is proportional to the number of infants in each phenotype. Green indicates dynamic phenotypes and red reference phenotypes. The overlap of infants allocated to phenotype 1 was 122 (70%), to phenotype 2 was 57 (40%), to phenotype 3 was 32 (37%) and to phenotype 4 was 6 (19%). There was high overall agreement between the two clustering methods, indicated by a weighted kappa-statistic value of 0.64 (range 0-1).

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Supplementary Information

Additional methods for study participants

This ongoing prospective birth cohort study comprised a group of unselected, healthy term-born neonates recruited antenatally [1]. Infants were enrolled in two centers in Switzerland (in Bern between 1999 and 2015, and Basel between 2011 and 2015). Exclusion criteria for the study were preterm delivery (< 37 week gestational age), major birth defects, respiratory distress after birth, other significant perinatal disease or a later diagnosis of airway malformation, or specific chronic respiratory disease.

Additional methods on skin prick testing

Skin prick testing was performed in a subgroup of 270 children to the following allergens: dog and cat dander, two tree mixtures, grass mixture, *Alternaria alternate*, *Dermatophagoides pteronyssinus*, and *Dermatophagoides farinae* (Allergomed AG, Therwil, Switzerland). Positive control was histamine; negative control was the solution in which allergens were dissolved. The reaction was assessed 15 minutes after skin prick testing. Response was determined positive if a wheal diameter of any of the tested allergen was greater than the positive control.

Additional methods on lung function and fractional exhaled nitric oxide measurement

Spirometry was performed with the child in a seated position with the nose clipped using the MasterLab setup (Jaeger, Wuerzburg, Germany) according to standard guidelines. The best of at least three repeatable forced expiratory maneuvers (within 100 ml) was recorded [2]. Forced expiratory volume in 1 s (FEV_1), forced vital capacity (FVC), FEV_1/FVC ratio and forced expiratory flow at 25-75% of FVC ($FEF_{25-75\%}$) were expressed as sex- and height-standardized z-scores [3]. We measured the fraction of exhaled nitric oxide (FeNO) by the single-breath method with a rapid-response chemoluminescence analyzer (CLD 88 sp; EcoMedics, Duernten,

Switzerland). Flow was recorded using an ultrasonic flow meter (Spiroson; EcoMedics) according to current guidelines [4].

Additional methods for the Markov matrix

Respiratory tract infections during infancy were prospectively assessed using the following scores: Score zero for the healthy state and scores 1-4 (in ascending order of severity) for the symptomatic states (Table S1). For each possible symptom state *x* out of the five states 0,1,2,3,4, we counted how often, during the time window of 52 weeks observation, a transition to any other state *y* (in symbols $x \rightarrow y$) took place within one week's time. For example, an infant is healthy (symptom score=0) for three consecutive weeks (transition: 0→0→0); develops a severe infection during the fourth week with symptom score 3 (transitions: 0→0→0→3); this infection (symptom score=3) lasts another week (transitions: 0→0→0→3→3); and finally, the infant fully recovers during the subsequent week (symptom score=0) (transitions: 0→0→0→3→3→0). Hence, this 6 weeks observation period can be described by the following transitions (momentary state → target state): 0→0, 0→0, 0→3, 3→3, 3→0. Therefore, during the time window of observation, the transition 0→0 was observed two times, and the transitions 0→3, 3→3, 3→0 once, respectively. All the other remaining possible transitions (there is a total of 5×5=25 possible transitions among the five states 0, 1, 2, 3, and 4), were not observed during this 6 weeks observation period. All these counts, or absolute frequencies, can be displayed in a 5×5-table in which the rows and columns are labeled using the symptom states (0, 1, 2, 3, and 4). Such a table is displayed in Figure 1B for one patient of the cohort.

The absolute frequencies from this table were then replaced by the corresponding relative frequencies (empirical probabilities). Thereby, the entries in the table can be interpreted as conditional probabilities. For example, a given patient is currently displaying symptoms corresponding to state *x*, and the probability that the symptoms will progress to symptom state *y* can be calculated (Figure 1C). Such a table of conditional probabilities is referred to in

mathematicians as a Markov matrix [5]. Each row in this matrix encodes a discrete probability distribution, namely the probability to progress from a given state to any other of the possible five states. We calculated the average estimated entropy [6] of all the conditional probability distributions defined by the rows of the participant's Markov matrix. This average estimated entropy gives a quantitative measure for the irregularity of the patterns of symptom deterioration and recovery of a given patient.

The entropy was estimated using the maximum likelihood estimator, which results from the substitution of the empirical probabilities (i.e., the entries in a given row of a Markov matrix) into the Shannon entropy formula:

$$Entropy = -\sum_{i=0}^{4} p_i \log(p_i)$$

$P_{i,j}$ denotes the probability coefficient of each states transition at i_{th} row and j_{th} column of the Markov matrix.

When a row in a given Markov matrix only contained zeros, the value of the entropy was not calculated. Such rows did not contribute to the calculation of the average entropy over all rows of the Markov matrix. A row containing only zeros corresponds to a lack of empirical knowledge about those symptom transitions that were not reported. For the sake of consistency, this "policy" was also applied when we estimated the entropy using an estimator that addresses the issue of potentially unobserved events within the framework of our sensitivity analysis (see next subsection below).

Additional methods for statistical analysis

The Shannon entropy is defined as the average amount of information needed to specify the state of a random variable governed by a given probability distribution. In the context of statistical physics in can be interpreted as measure of disorder within a system; higher entropy values

correspond to more irregularity [7]. For each infant, we calculated the average estimated entropy [6] of all the conditional probability distributions defined by the rows of the participant's Markov matrix. This average estimated entropy gives a quantitative measure of the irregularity in the patterns of symptom deterioration and recovery of a given patient. The frequency distribution of entropy visually appeared multimodal, with 4 clearly distinguishable modi (Figure 3a). Therefore, we defined 4 dynamic phenotypes based on the slope of the cumulative distribution function curve, and a slope of zero was used for cutoff. We defined 4 similarly sized reference phenotypes by the frequency distribution of total number of weeks with respiratory symptoms during infancy (Figure 3b).

Using logistic regression analysis, we studied the association of the 4 dynamic phenotypes, the 4 reference phenotypes, and known asthma risk factors (e.g. sex, maternal asthma), with the child's outcomes at 6 years. For "any wheezing", "current wheezing", atopy and positive prick test of the child, we adjusted the analyses for sex, maternal education, maternal asthma, ETS exposure, childcare, and presence of siblings. For the outcome FeNO, we additionally adjusted for asthma of the child and inhaled corticosteroid use. For lung function, analyses were adjusted for maternal education, maternal asthma, ETS exposure, childcare, and presence of siblings. Differences in the distribution of characteristics across phenotypes were assessed using Chi^2 tests for categorical variables, and Kruskal–Wallis for continuous variables. A Bonferroni-corrected significance level was used to account for multiple pair-wise testing. We used the weighted kappa-statistic [8] to compare agreement between the dynamic and reference phenotypes. Analyses were done using Stata 11.2 software (StataCorp, College Station, Tex), Matlab R2015b (The MathWorks, Inc., Natick, MA, USA) and R, Version 3.0.2 [9].

Additional methods for sensitivity analysis

We performed several sensitivity analyses:

1) We repeated the analysis in infants having ≥1 episode of symptom score ≥3.

2) Robustness of the identified phenotypes was investigated within an additional, independent sample from our cohort. That sample, included infants not seen for follow up, and hence, these infants were not included in the main analysis. This sample served as an independent data set for an external validation of the method.

3) To explore that the distribution of entropy was not a statistical artifact of our analysis approach, we re-categorized the symptom states (0, 1, 2, (3+4)→3), we simulated different data sets and perturbed the existing data sets.

4) As revealed by our data, within this healthy cohort some symptoms transitions were hardly ever reported, especially transitions between severe respiratory symptoms. Many participants did not report certain transitions at all during the time window of observation. Assigning probability zero to a transition just because it was not reported during the time window of observation may be a precipitate conclusion. Bayesian inference provides a rigorous mathematical framework for dealing with unobserved events [10]. Therefore, we estimated the entropy using an estimator that takes into account the issue of unobserved events (Schuerman and Grassberger [11]) and thereby, using a Bayesian approach, compensating for the underestimation of the entropy that may arise from the use of the maximum likelihood estimator in the presence of unobserved events.

We applied the suggested approach by Schuerman and Grassberger [11], and conducted also a simulation- and perturbation-based sensitivity analysis to rule out a potential artifact behind the observed multimodal distribution of the average estimated entropy.

Additional results for sensitivity analysis

1) We obtained similar results in infants with ≥1 episode of symptom score ≥3 (data not shown).

2) In the validation dataset of 242 infants, dynamic phenotypes included 83, 101, 46 and 12 infants. Phenotypes 1, 2, 3 and 4 had 0.23, 0.65, 1.41, and 2.92 weeks with severe symptoms, respectively. Risk factors differed across phenotypes Risk factors differed across phenotypes:

phenotype 1 had an underrepresentation of children attended childcare. Phenotype 2 had an underrepresentation of males. Phenotype 3 had more males, and more infants had siblings. Phenotype 4, the smallest group, had an overrepresentation infants attended childcare, and were more breastfed. Reference phenotypes included 83, 131, 41 and 15 infants, respectively. Phenotypes 1, 2, 3 and 4 had 0.16, 0.66, 1.34, and 3.2 weeks with severe symptoms, respectively. Phenotype 1 had an underrepresentation of males; fewer infants had siblings and attended less childcare. In phenotype 2, infants were more likely born via Cesarean section. Phenotype 3 had more males, more infants with siblings and childcare attendance, and fewer infants born via Cesarean section. Phenotype 4 had an overrepresentation of males, and more infants had siblings, and were more likely born to atopic mothers (Table S6).

3) The outcomes of the following sensitivity analysis ruled out the possibility of an artifact in our methodology. Re-categorization of the states (0, 1, 2 (3+4)→3), did not systematically change the entropy distribution and the identified phenotypes (data not shown). The striking observed multimodality of the distribution of the average estimated entropy (4 dominant peaks) did not show up in a simulated dataset in which all possible symptom transitions were assumed to be equally likely (Figure S2B). We perturbed the existing data by shuffling and thereby randomly altering the chronological order of each patient's reported symptoms. The resulting distribution of the average entropy remained qualitatively very similar to the one observed in the unperturbed data (Figure S2C). A multimodal distribution was observed when the data was simulated in such a way, that the probability of any given symptom transition was determined by the pooled, existing data of the cohort. However, the shape of the distribution changed considerably in this simulated dataset (Figure S2D). This is not surprising, as our method captures the patterns of symptom deterioration and recovery, and not necessarily the *temporal* symptom pattern.

4) We repeated the whole analysis to define dynamic phenotypes, taking into account the issue of potentially unobserved events [11]. Based on this method, we defined three phenotypes. These phenotypes included 122, 117, and 12 infants, respectively. Phenotypes 1, 2, 3 and 4 had 0.23,

0.03, 1 and 2.0 weeks with severe symptoms, respectively. Risk factors differed across phenotypes: phenotype 1 had an overrepresentation of children attended childcare and less infants were exposed to tobacco smoke during pregnancy or during the first year of life. Phenotype 2 had an underrepresentation of males and more infants were born via Cesarean section. Phenotype 3 had more males, and more infants had siblings. More mothers had asthma and infants were more exposed to tobacco smoke during pregnancy and thereafter. Dynamic phenotype 3 had the highest prevalence of current wheezing (20.5%) and atopy (49.4%) at school age.

Analogously, we performed the simulation- and perturbation-based sensitivity analysis, which ruled out an artifact behind the observed multimodal distribution of the average estimated entropy (Figure S3).

Results

Table S1 Respiratory symptom score*

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

*Published with permission from the BMJ publishing group. GP: general practitioner.

Table S2 Characteristics of the cohort

	Follow-up study 322	Lost to follow-up 47	P-value
Anthropometrics at birth			
Gestational age, weeks	39.6 ± 1.2	39.8 ± 1.2	0.139
Birth weight, kg	3.3 ± 0.4	3.3 ± 0.4	0.622
Length, cm	49.5 ± 1.9	49.4 ± 2.2	0.821
Respiratory symptoms in the first year of life			
weeks with severe symptoms	0.72 ± 1.1	1.0 ± 2.04	0.169
weeks with symptoms	5.33 ± 4.61	5.74 ± 4.23	0.568
Risk factors			
Male sex	167 (51.8)	29 (61.7)	0.207
Siblings	159 (49.4)	28 (59.5)	0.192
Caesarean section	54 (16.7)	4 (14.8)	0.146
Maternal asthma	34 (10.5)	9 (19.2)	0.086
Maternal atopy	116 (36.1)	17 (36.2)	0.985
Childcare	62 (19.2)	7 (13.5)	0.474
Maternal smoking during pregnancy	27 (8.4)	9 (19.2)	0.020
Parental smoking during infancy	70 (21.7)	16 (34.1)	0.062
Breastfeeding >26 weeks	252 (78.3)	23 (65.2)	<0.001
Low maternal education	202 (62.7)	30 (65.2)	0.744
Season of birth			
Spring	87 (27.1)	10 (21.3)	0.404
Summer	83 (25.7)	27 (27.6)	0.783
Autumn	80 (24.8)	14 (29.8)	0.468
Winter	72 (22.4)	10 (21.3)	0.867

Data are given as mean ± standard deviation or absolute numbers (percent). Children younger than 5 years were not enrolled for pre-school follow-up. P-values are for comparison between subjects lost-to follow-up, and those who were followed up. We used the Chi-squared tests for categorical variables, and Kruskal–Wallis for continuous variables.

Table S3 Associations between phenotypes defined by different dimension of respiratory symptoms during infancy and current wheezing and positive prick test

	Univariable association			Multivariable association*		
	OR	95% CI	P-value	OR	95% CI	P-value
Exposure	Outcome upper respiratory tract infection¶ (122/322)					
Respiratory symptom, weeks (Reference Phenotypes)						
Phenotype 1 (baseline) (147)	1	reference		1	reference	
Phenotype 2 (98)	1.17	0.69 – 2.01	0.549	1.22	0.71 – 2.12	0.466
Phenotype 3 (57)	2.01	1.07 – 3.74	0.028	2.16	1.12 – 4.15	0.021
Phenotype 4 (20)	0.89	0.31 – 2.29	0.722	0.92	0.32 – 2.64	0.882
Transitions, entropy (Dynamic Phenotypes)						
Phenotype 1 (baseline) (141)	1	reference		1	reference	
Phenotype 2 (102)	1.19	0.71 – 2.02	0.497	1.25	0.73 – 2.14	0.411
Phenotype 3 (62)	0.94	0.51 – 1.75	0.849	0.97	0.51 – 1.84	0.929
Phenotype 4 (17)	0.93	0.32 – 2.67	0.898	0.91	0.31 – 2.74	0.865
Risk factors						
Male sex	1.03	0.66 – 1.63	0.867	1.02	0.64 – 1.63	0.901
Siblings	0.79	0.51 – 1.25	0.330	0.81	0.51 – 1.27	0.367
Maternal asthma	1.01	0.48 – 2.21	0.965	1.01	0.48 – 2.09	0.996
Childcare	1.23	0.71 – 2.16	0.465	1.18	0.66 – 2.13	0.556
Parental smoking during infancy	1.03	0.61 – 1.78	0.894	1.04	0.59 – 1.81	0.886
Low maternal education	0.97	0.61 – 1.54	0.899	0.99	0.61 – 1.61	0.970
Exposure	Outcome positive prick test (37/270)					
Respiratory symptom, weeks (Reference Phenotypes)						
Phenotype 1 (baseline) (147)	1	reference		1	reference	
Phenotype 2 (98)	0.81	0.36 – 1.84	0.631	0.79	0.33 – 1.85	0.591
Phenotype 3 (57)	0.82	0.30 – 2.22	0.698	0.82	0.28 – 2.35	0.714
Phenotype 4 (20)	1.60	0.40 – 6.35	0.501	1.88	0.41 – 8.60	0.412
Transitions, entropy (Dynamic Phenotypes)						
Phenotype 1 (baseline) (141)	1	reference		1	reference	
Phenotype 2 (102)	1.07	0.47 – 2.42	0.864	1.22	0.52 – 2.88	0.638
Phenotype 3 (62)	0.94	0.34 – 2.60	0.919	0.98	0.34 – 2.83	0.977
Phenotype 4 (17)	2.26	0.64 – 7.94	0.201	2.63	0.64 – 10.67	0.175
Risk factors						
Male sex	2.63	1.21 – 5.68	0.014	2.57	1.16 – 5.67	0.019
Siblings	0.99	0.49 – 1.98	0.986	0.92	0.44 – 1.92	0.841
Maternal asthma	0.43	0.99 – 1.91	0.272	0.41	0.09 – 1.86	0.252

Childcare	0.76	0.30 – 1.94	0.575	0.78	0.29 – 2.11	0.634
Parental smoking during infancy	0.96	0.41 – 2.22	0.925	0.77	0.322 – 1.87	0.577
Low maternal education	2.16	0.94 – 4.93	0.068	1.95	0.82 – 4.64	0.131

CI: confidence interval; OR: odds ratio. Logistic regression for the outcomes any wheezing, current wheezing and atopic disease. Symptom dynamic phenotypes were defined by entropy of transition states, reference phenotypes by weeks with any respiratory symptom. When considering phenotypes as exposure, phenotype one from the reference phenotype, or dynamic phenotype served as baseline, respectively. Numbers in brackets indicates group sizes. *Adjusted for the following variables: male gender, low maternal education, maternal asthma, maternal smoking during pregnancy, childcare attendance during infancy and presence of siblings• ¶Upper respiratory tract infection (URTI) was defined by ear infection, throat infection, or serious cold (defined by symptoms ≥two weeks) over the past 12 months before follow-up.

Table S4 Associations between phenotypes defined by different dimension of respiratory symptoms during infancy and lung function at school age

	Univariable association			Multivariable association*		
	OR	95% CI	P-value	OR	95% CI	P-value
Exposure	Outcome $zFEV_1$					
Respiratory symptom, weeks (Reference Phenotypes)						
Phenotype 1 (baseline) (105)	1	reference		1	reference	
Phenotype 2 (61)	0.09	-0.19 – 0.38	0.516	0.7	-0.22 – 0.36	0.645
Phenotype 3 (42)	-0.10	-0.42 – 0.21	0.524	-0.10	-0.45 – 0.24	0.546
Phenotype 4 (14)	-0.11	-0.62 – 0.38	0.649	-0.07	-0.60 – 0.45	0.772
Transitions, entropy (Dynamic Phenotypes)						
Phenotype 1 (baseline) (100)	1	reference		1	reference	
Phenotype 2 (68)	0.04	-0.23 – 0.32	0.731	0.06	-0.22 – 0.35	0.662
Phenotype 3 (45)	0.15	- 0.16 – 0.47	0.332	0.18	-0.14 – 0.51	0.265
Phenotype 4 (9)	0.05	-0.56 – 0.67	0.867	0.06	-0.59 – 0.71	0.853
Exposure	Outcome zFVC					
Respiratory symptom, weeks (Reference Phenotypes)						
Phenotype 1 (baseline) (105)	1	reference		1	reference	
Phenotype 2 (61)	0.01	-0.05 – 0.08	0.676	0.00	-0.06 – 0.07	0.859
Phenotype 3 (42)	0.02	-0.05 – 0.11	0.490	0.01	-0.07 – 0.09	0.744
Phenotype 4 (14)	0.01	-0.11 – 0.14	0.819	0.00	-0.12 – 0.13	0.952
Transitions, entropy (Dynamic Phenotypes)						
Phenotype 1 (baseline) (100)	1	reference		1	reference	
Phenotype 2 (68)	0.01	-0.05 – 0.08	0.618	0.02	-0.04 – 0.09	0.525
Phenotype 3 (45)	0.01	-0.06 – 0.09	0.764	0.00	-0.07 – 0.88	0.873
Phenotype 4 (9)	-0.03	-0.17 – 0.11	0.665	-0.05	-0.20 – 0.08	0.434
Exposure	Outcome $zFEV_1/FVC$					
Respiratory symptom, weeks (Reference Phenotypes)						
Phenotype 1 (baseline) (105)	1	reference		1	reference	
Phenotype 2 (61)	0.01	-0.29 – 0.31	0.944	0.05	-0.25 – 0.36	0.718
Phenotype 3 (42)	-0.06	-0.40 – 0.28	0.723	0.02	-0.33 – 0.37	0.907
Phenotype 4 (14)	-0.15	-0.69 – 0.37	0.558	-0.11	-0.66 – 0.43	0.679
Transitions, entropy (Dynamic Phenotypes)						
Phenotype 1 (baseline) (100)	1	reference		1	reference	
Phenotype 2 (68)	-0.01	-0.31 – 0.27	0.904	0.06	-0.24 – 0.36	0.696
Phenotype 3 (45)	-0.01	-0.35 – 0.32	0.924	0.05	-0.28 – 0.39	0.758

Phenotype 4 (9)	-0.19	-0.85 – 0.45	0.549	-0.15	-0.83 – 0.52	0.656
Exposure			Outcome $zFEF_{25-75\%}$			
Respiratory symptom, weeks (Reference Phenotypes)						
Phenotype 1 (baseline) (105)						
Phenotype 2 (61)	0.06	-0.20 – 0.33	0.641	0.10	-0.18 – 0.39	0.468
Phenotype 3 (42)	0.01	-0.30 – 0.33	0.916	0.08	-0.24 – 0.41	0.616
Phenotype 4 (14)	-0.23	-0.72 – 0.25	0.346	-0.13	-0.64 – 0.37	0.611
Transitions, entropy (Dynamic Phenotypes)						
Phenotype 1 (baseline) (100)						
Phenotype 2 (68)	-0.03	-0.30 – 0.23	0.781	0.01	-0.25 – 0.29	0.889
Phenotype 3 (45)	0.21	-0.09 – 0.52	0.171	0.29	-0.03 – 0.61	0.076
Phenotype 4 (9)	-0.10	-0.64 – 0.43	0.697	0.01	-0.54 – 0.58	0.948
Exposure			[+]Outcome FeNO ppb			
Respiratory symptom, weeks (Reference Phenotypes)						
Phenotype 1 (baseline) (101)						
Phenotype 2 (74)	0.96	0.80 – 1.16	0.715	0.98	0.81 – 1.18	0.860
Phenotype 3 (40)	0.90	0.72 – 1.13	0.386	0.88	0.69 – 1.11	0.301
Phenotype 4 (16)	1.15	0.83 – 1.59	0.390	1.20	0.86 – 1.69	0.270
Transitions, entropy (Dynamic Phenotypes)						
Phenotype 1 (baseline) (99)						
Phenotype 2 (77)	1.05	0.87 – 1.26	0.595	1.03	0.85 – 1.24	0.751
Phenotype 3 (46)	0.97	0.78 – 1.20	0.804	0.96	0.76 – 1.20	0.740
Phenotype 4 (9)	0.75	0.49 – 1.14	0.184	0.69	0.44 – 1.07	0.102

Coef: coefficient CI: confidence interval; FeNO: fractional exhaled nitric oxide; FVC: forced vital capacity; FEV_1: forced expiratory volume in one second; FEF: forced expiratory flow from 25-75% of exhalation. Lung function parameters are expressed as z-scores according to Qanjer et al.[33], FeNO was log-transformed before analyses and results are shown for exponentiated coefficients. Symptom dynamic phenotypes were defined by entropy of transition states, reference phenotypes by weeks with any respiratory symptom. Phenotype one from the reference phenotype, or symptom dynamic phenotype served as baseline, respectively. Numbers in brackets indicates group sizes. [*]Adjusted for the following binary variables: maternal education, maternal asthma, maternal smoking during pregnancy, childcare attendance during infancy and presence of siblings. [+]Additionally adjusted for the following binary variables: sex, hay fever of the child, inhaled corticosteroid use.

Table S5 Comparison of characteristics between the 242 infants enrolled in the two study centers not seen for follow up

	Basel 75	Bern 167	P-value
Anthropometric data at birth			
Gestational age, wks	39.31 ± 1.32	39.75 ± 1.11	0.007
Birth weight, kg	3.2 ± 0.55	3.4 ± 0.44	0.016
Length, cm	49.14 ± 2.51	49.61 ± 2.09	0.139
Respiratory symptoms in the first year of life			
weeks with severe symptoms	0.77 ± 1.19	0.76 ± 1.19	0.861
weeks with symptoms	6.14 ± 4.54	6.43 ± 4.72	0.753
Risk factors			
Male sex	36 (48.0)	92 (54.9)	0.307
Siblings	30 (40.0)	117 (70.1)	<0.001
Caesarean section	31 (41.3)	30 (17.9)	<0.001
Maternal asthma	12 (9.0)	15 (8.9)	0.468
Maternal atopy	36 (48.0)	52 (31.1)	0.012
Childcare	46 (61.3)	109 (22.3)	0.001
Maternal smoking in pregnancy	3 (4.0)	16 (9.6)	0.146
Parental smoking during infancy	15 (20.0)	38 (22.7)	0.632
Breastfeeding ≤26 weeks	27 (36.0)	108 (22.7)	0.920
Low maternal education	21 (28.0)	82 (50.3)	0.229
Seasons at birth			
Spring	22 (29.3)	37 (22.6)	0.464
Summer	15 (20.0)	46 (27.5)	0.238
Autumn	21 (28.0)	51 (30.5)	0.826
Winter	17 (22.6)	33 (19.7)	0.606

Values are means ± standard deviation or number (percentage). Differences in the distribution of characteristics between centers were assessed using Chi^2 tests for categorical variables, and Kruskal–Wallis for continuous variables.

Table S6 Characteristics of phenotypes defined by transition probabilities among respiratory symptoms states and by respiratory symptoms weeks during infancy from the 242 study participants not seen for follow-up

	Phenotype 1		Phenotype 2		Phenotype 3		Phenotype 4		P-value	
	Dyn.	Ref.	Dyn.	Ref.	Dyn.	Ref.	Dyn.	Ref.	Dyn.	Ref.
	83	83	101	103	46	41	12	15		
Respiratory symptoms										
Weeks with severe symptoms	0.23 (0.45)	0.16 (0.41)	0.65 (0.96)	0.66 (0.85)	1.41 (2.02)	1.34 (1.35)	2.92 (1.56)	3.2 (2.98)	<0.001	<0.001
Weeks with symptoms	1.74 (1.52)	1.56 (1.09)	6.65 (3.05)	5.70 (1.44)	10.46 (3.33)	10.51 (1.54)	16.42 (2.84)	16.50 (2.93)	<0.001	<0.001
Respiratory symptoms transition states										
Entropy of transition states	0.02 (0.02)	0.03 (0.04)	0.16 (0.02)	0.16 (0.07)	0.29 (0.03)	0.26 (0.08)	0.4 (0.04)	0.4 (0.09)	<0.001	<0.001
Risk factors										
Male sex	55.4	53.1	45.5	50.1	67.4	63.4	41.6	40.0	0.304	1
Siblings	53.1	53.1	63.4	65.1	73.9	63.4	41.6	66.6	0.224	1
Caesarean section	20.5	18.1	26.7	32.1	28.2	2.9	33.3	26.6	1	0.676
Maternal asthma	12.1	13.3	7.8	7.8	8.7	9.8	16.7	6.7	1	1
Maternal atopy	42.2	38.5	31.7	38.8	36.9	24.4	33.3	40.0	1	1
Childcare	25.3	21.7	46.5	47.6	34.8	41.4	75	60.0	0.004	0.004
Maternal smoking during pregnancy	8.4	8.4	8.9	8.7	4.4	4.8	8.3	6.7	1	1
Parental smoking during infancy	21.7	20.5	24.2	21.4	17.4	24.4	16.7	26.7	1	1
Breastfeeding ≤26 weeks	62.6	59.1	67.3	70.8	54.4	58.5	91.7	66.6	0.372	1
Low maternal education	41.8	46.2	47.5	41.2	41.3	46.3	25.0	33.3	1	1
Seasons at birth										
Spring	15.6	14.4	22.8	28.2	45.6	36.6	16.7	20.0	0.008	0.136
Summer	20.5	24.1	32.7	25.2	17.4	21.9	25.0	40.0	0.564	1
Autumn	40.9	39.7	26.7	27.2	17.4	19.5	25.0	20.0	0.12	0.284
Winter	22.9	21.7	17.8	19.4	19.6	21.9	33.3	20.0	1	1

Values are percent or means (SD). Symptom dynamic phenotypes (Dyn.) are shown on white background and reference phenotypes (Ref.) are shown on grey shading background. Symptom dynamic phenotypes were defined by average entropy of transition states, reference phenotypes by weeks with any respiratory symptom. Differences in the distribution of characteristics across phenotypes were assessed using Chi-squared tests for categorical variables, and Kruskal–Wallis for continuous variables. P-values are Bonferroni corrected, accounting for multiple testing.

Figures

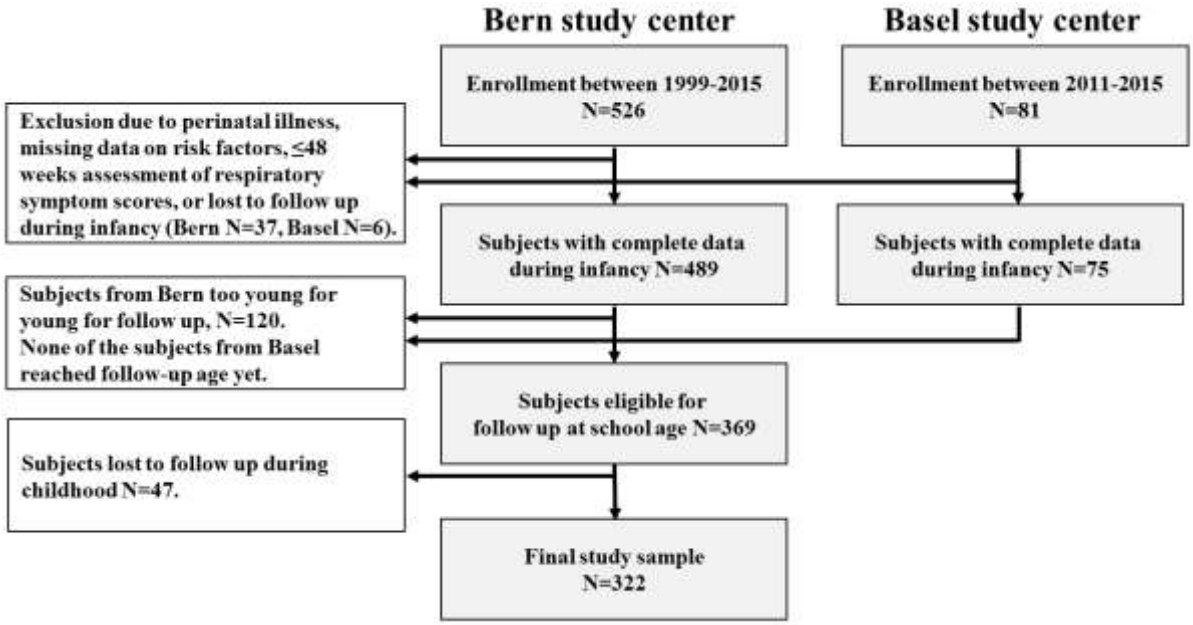

Figure S1 Flow Chart of the study population.

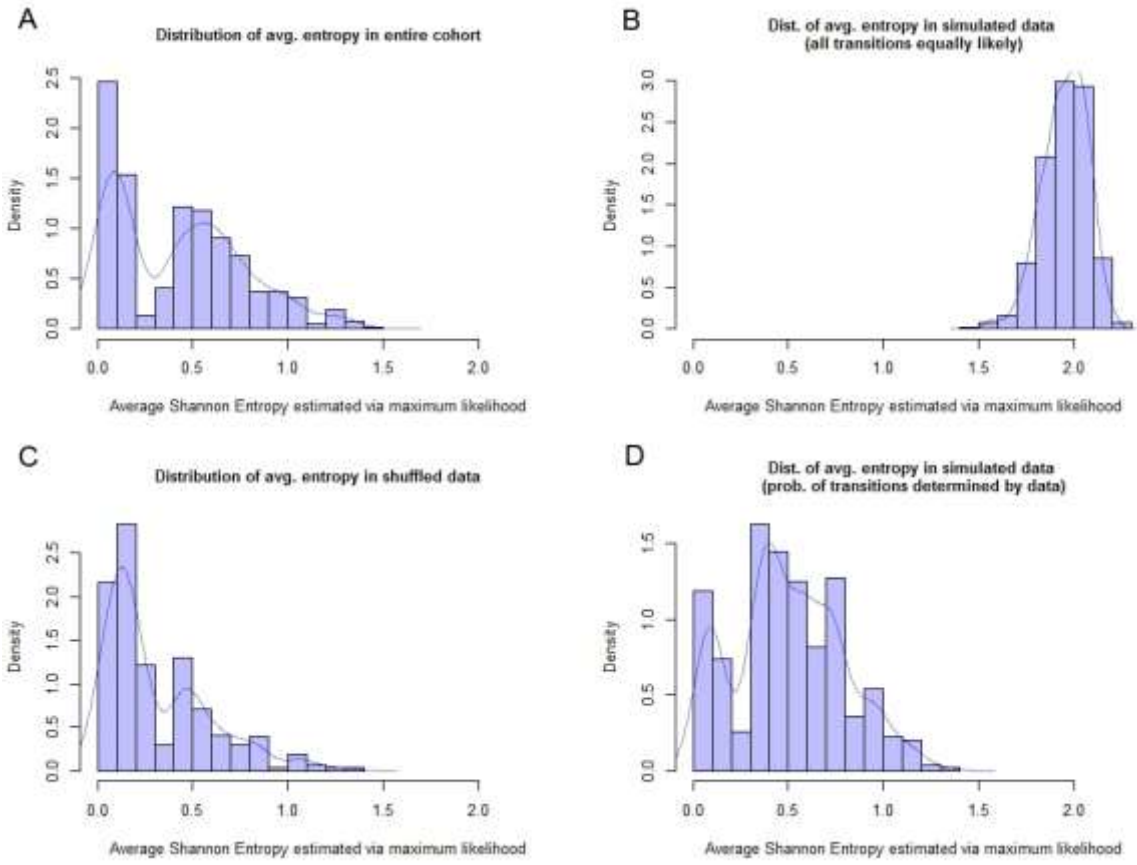

Figure S2 Histograms of estimated entropy in the cohort and in simulated and perturbed data sets. A) Histogram of average estimated entropy of the rows of the Markov matrix of each participant in the cohort. B) Histogram of average estimated entropy of the rows of the Markov matrix in a simulated data set in which all symptom transitions are assumed to be equally probable. C) Histogram of average estimated entropy of the rows of the Markov matrix of each participant after each participant's chronological order of reported symptoms was randomly permuted. D) Histogram of average estimated entropy of the rows of the Markov matrix in a simulated data set in which the probabilities of symptom transitions were determined by the pooled cohort data. The entropy in all four panels was estimated using the maximum likelihood estimator of the entropy.

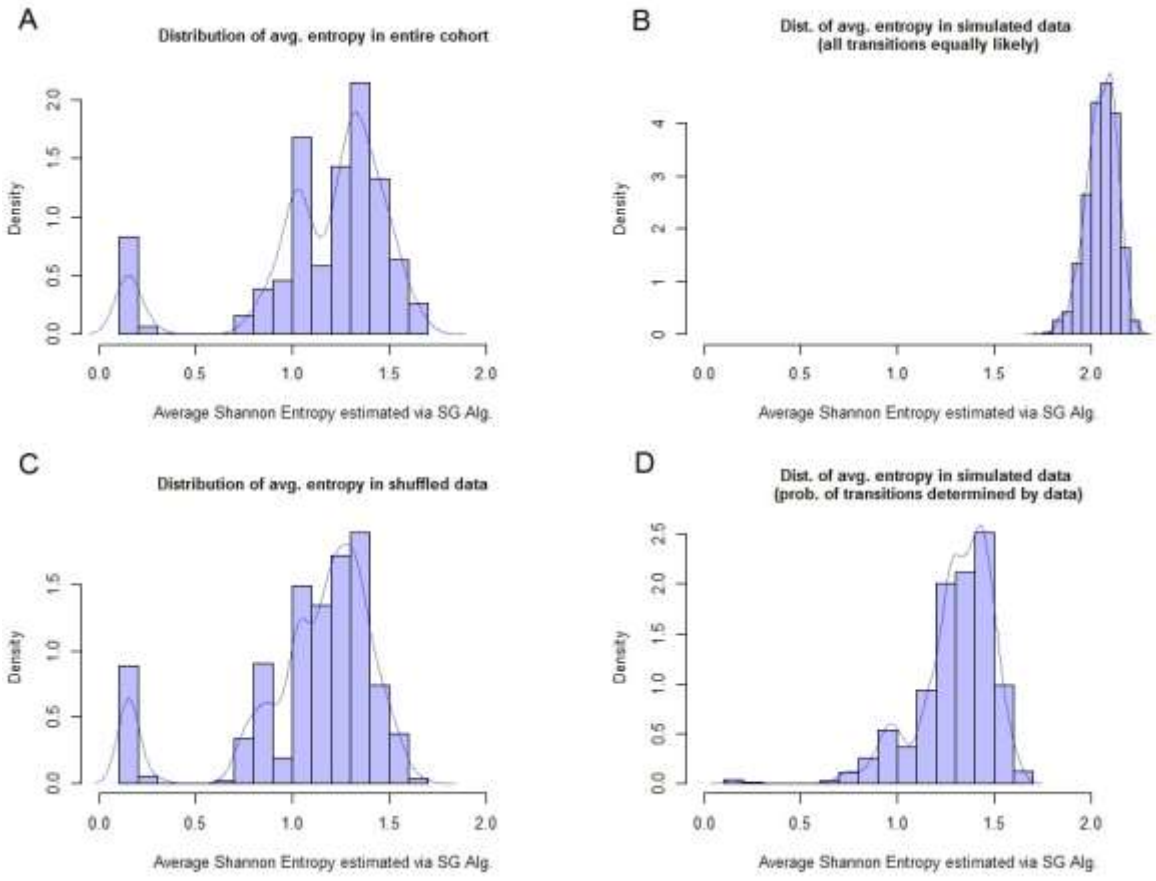

Figure S3 Histograms of estimated entropy in the cohort and in simulated and perturbed data sets when controlling for unobserved events. A) Histogram of average estimated entropy of the rows of the Markov matrix of each participant in the cohort. B) Histogram of average estimated entropy of the rows of the Markov matrix in a simulated data set in which all symptom transitions are assumed to be equally probable. C) Histogram of average estimated entropy of the rows of the Markov matrix of each participant after each participant's chronological order of reported symptoms was randomly permuted. D) Histogram of average estimated entropy of the rows of the Markov matrix in a simulated data set in which the probabilities of symptom transitions were determined by the pooled cohort data. The entropy in all figures was estimated using an estimator that corrects for unobserved events (Schuerman and Grassberger algorithm (SG Alg.) [11]). Compared to Figure S2A, a shift towards higher entropy values can be observed.

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3.4.3 Analytical approach: *in vitro* air pollution exposure

Effects of gasoline and ethanol-gasoline exhaust exposure on human bronchial epithelial and natural killer cells in vitro

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[*] Equal contribution.

Under revision Toxicol. in vitro

Effects of gasoline and ethanol-gasoline exhaust exposure on human bronchial epithelial and natural killer cells *in vitro*

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Highlights (in a separate file, 3-5 bullet points, <80 characters or 20 words each)

- The use of ethanol-gasoline blends may reduce dependency on fossil fuels
- Toxicity of exhaust from modern gasoline cars has not yet been thoroughly investigated
- Toxic effects of ethanol-gasoline blend exhaust are unknown
- We exposed human bronchial epithelial cells for 6hrs to fresh car exhaust
- Exposure to exhaust from pure gasoline or ethanol-gasoline blend had only minor toxic effects

Abstract

Air pollution exposure, including passenger car emissions, may cause substantial respiratory health effects and cancer death. In western countries, the majority of passenger cars are driven by gasoline fuel. Recently, new motor technologies and ethanol fuels have been introduced to the market, but potential health effects have not been thoroughly investigated.

We developed a coculture model composed of bronchial epithelial cells (ECs) and natural killer cells (NKs) mimicking the human airways to compare toxic effects between pure gasoline (E0) and ethanol-gasoline-blend (E85, 85% ethanol, 15% gasoline) exhaust emitted from a flexfuel gasoline car. We drove a steady state cycle, exposed ECs for 6hrs and added NKs. We assessed exhaust effects in ECs alone and in cocultures by RT-PCR, flow cytometry, and oxidative stress assay.

We found no toxic effects after exposure to E0 or E85 compared to air controls. Comparison between E0 and E85 exposure showed a weak association for less oxidative DNA damage after E85 exposure compared to E0. Our results indicate that short-term exposure to gasoline exhaust may have no major toxic effects in ECs and NKs and that ethanol as part of fuel for gasoline cars may be favorable.

Keywords

Airways; Gasoline exhaust; ethanol-gasoline blend; diesel exhaust particles; Cocultures; DNA damage; flexfuel

1. Introduction

Air pollution is an important cause of cardiopulmonary and cancer mortality (Kurt et al. 2016). In Europe, about half a million premature deaths per year are attributed to exposure to ambient air pollution (WHO 2014), and road traffic is a major source of particulate air pollution. Most cars in western countries are driven by gasoline fuel, and the World Health Organization recognizes potential adverse health effects by classifying gasoline exhaust as "possibly carcinogenic to humans" (IARC 2014). There are currently ongoing initiatives to promote the use of ethanol-gasoline blends to potentially reduce emissions of harmful air pollutants, and lower dependency on fossil fuels (Guarieiro 2013).

Few studies have investigated the adverse effects of gasoline exhaust (Lund et al. 2007, Mauderly et al. 2014, Reed et al. 2008), summarized in (Claxton 2015). However, these studies mostly used older gasoline technologies, and toxicological studies on emissions from modern gasoline engine technologies (e.g. gasoline direct injection (GDI)) and gasoline-ethanol blends are rare (McDonald et al. 2007). A recent study reported that exposure of murine lung slices to GDI engine exhaust upregulated genes related to the metabolism of polycyclic aromatic hydrocarbons and oxidative stress (Maikawa et al. 2016). Slightly increased cytotoxicity was reported in primary airway epithelial cells (ECs) upon exposure to aged gasoline particles (Kunzi et al. 2015), whereas acute exhaust exposure of a GDI flex-fuel vehicle using pure gasoline and ethanol-gasoline blends to an airway coculture model (consisting of ECs, macrophages and dendritic cells) showed no toxic effects (Bisig et al. 2016). Importantly, the latter study did not include primary natural killer cells (NKs) in their coculture model. However, NKs have central immune regulatory functions. They are involved in the control of lung cancers and the defense against viral pathogens in the lung (Vivier et al. 2008). Furthermore, cell-cell interactions between ECs and NKs are important to regulate the activation status of NKs (Vivier et al. 2008,

Whitsett and Alenghat 2015). Therefore, it may be important to study toxic effects of gasoline exhaust on both ECs and NKs.

ECs are the predominant cell type in the respiratory tract (Ochs and Weibel 2008) and are vulnerable to inflammation, viral infections and malignant degeneration. They serve as a physical barrier for pathogens, are important regulators of airway immune responses (Müller and Jaspers 2012, Swamy et al. 2010), and communicate with other cells via soluble factors and cell-cell interactions (Whitsett and Alenghat 2015). ECs recruit and activate immune cells, including NKs, via cytokine release (Vivier et al. 2008), and regulate NKs' activation status via expression of surface receptors, including UL16-binding proteins (ULBPs) and MHC class I polypeptide-related sequence (MIC) A/B (Obeidy and Sharland 2009). Expression of EC stress receptors, including ULBPs and MICA/B, can be upregulated by exposure to oxidative stressors, e.g. hydrogen peroxide (Borchers et al. 2006), or air pollutants (Müller et al. 2013).

NKs are classified as innate lymphoid cells (Spits et al. 2013) and their activity is regulated via expression of inhibitory (e.g. CD158b and CD159a), and activating (e.g. CD314 and CD335), receptors on their surface. HLA or MHC class I molecules binding of inhibitory receptors reduces NKs' activation, ensuring the recognition of "self", and avoiding the killing of healthy, autologous cells. ULBPs or MICA/B from ECs can bind to CD314 (also called NKG2D), and viral hemagglutinins can bind to CD335 (also called NKp46), both resulting in increased cytokine release and killing potential of NKs. NKs' killing potential and cytokine release (e.g. interleukin (IL) 4 and interferon (IFN) γ) is crucial for early pathogen defense, especially against viruses, and the immuno-surveillance of tumor development (Campbell and Hasegawa 2013). A potential reduction of the cytotoxic activity and/or a change of the cytokine release due to car exhaust exposure may hence increase the susceptibility to viral infections and tumor development (Guillerey and Smyth 2016).

A central feature of the human airway's complexity are cell-cell interactions, and coculture models are suggested as more favorable models than monocultures to study the effects of exhaust emission (Alfaro-Moreno et al. 2008, Müller et al. 2010, Rothen-Rutishauser et al. 2008). Therefore, we developed a novel coculture model with a human bronchial EC line and primary human NKs to study toxic effects of gasoline and ethanol-gasoline blend exhaust. We used freshly generated exhaust from a modern car engine (containing both particles and gaseous compounds) to best mimic exhaust exposure to human lungs.

2. Material and Methods

2.1 Study design

This experimental *in vitro* study describes the effects of pure conventional lead free gasoline (E0) and conventional lead free gasoline with 85% ethanol (E85) exhaust compared to air control exposures in two cell culture models (monocultures of ECs, cocultures of ECs and NKs) (Figure 1).

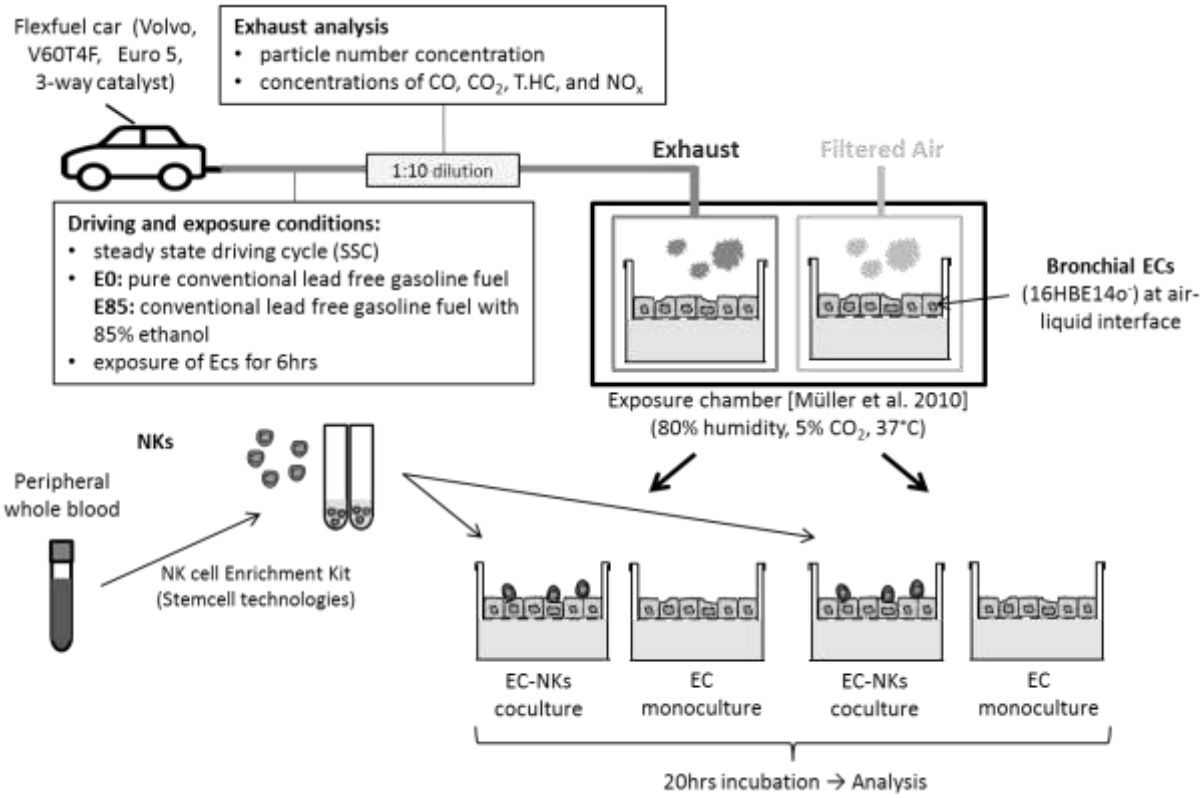

Figure 1. Schematic overview of the study design. *CO = carbon monoxide, CO_2 = carbon dioxide, T.HC = total hydrocarbons, NO_x = nitrogen oxides, EC = epithelial cell, NKs = natural killer cells.*

2.2 Cell cultures

2.2.1 Bronchial epithelial cells

We maintained the human bronchial EC line 16HBE14o$^-$ (kindly provided by Dr. Gruenert, University of California, San Francisco) as previously described (Bauer et al. 2015). Briefly, we kept the cells at 37°C and 5% carbon dioxide (CO_2) in collagen and fibronectin (BD Bioscience,

Allschwil, Switzerland) coated tissue culture flasks in minimum essential medium (MEM) with 1% L-glutamine, 1% penicillin/streptomycin (both Sigma-Aldrich, Buchs, Switzerland), and 10% heat inactivated fetal bovine serum (FBS; BioConcept, Allschwil, Switzerland), and split them three times per week. For experimental cultures, we seeded $5x10^4$ cells in 500µl medium in the apical, and 1ml medium in the basolateral chamber into transwells (Corning 12-well transwell polyester membrane Insert, pore size 0.4µm; Sigma-Aldrich), grew them for 6 days until the monolayer was confluent and exposed them for 20hrs at the air-liquid interface (ALI) before using them for experiments.

2.2.2 Natural killer cells (NKs)

We enriched primary NKs from the peripheral blood of ten healthy non-smoking volunteers [age mean (standard deviation) 36.6 (12.4) years, BMI 22.9 (4.2), female/male ratio 7:3]. The study was approved by the Ethics Committee Nordwest- und Zentralschweiz, Switzerland. Written informed consent of the donors was obtained. NKs from six donors were used to establish the coculture model and the validation experiments. NKs from four donors were used for the two exposure series (E0 and E85, with air controls each, Figure 1). We performed the isolation of peripheral blood mononuclear cells using a density gradient of Lymphoprep in SepMate tubes (both StemCell Technologies, Grenoble, France), and enriched NKs using the EasySep Human NK cell Enrichment Kit (StemCell Technologies), both according to manufacturer's instructions. NKs were re-suspended in RPMI 1640 media with L-glutamine completed with 10% FBS and 1% penicillin/streptomycin.

2.2.3 Coculture model of bronchial ECs and NKs

We added $2.5x10^5$ NKs in 100µl media to the confluent bronchial EC monolayer at the ALI for incubation of 20hrs at 37°C, 5% CO_2 and 80% humidity. We compared endpoints of ECs and NKs in cocultures to ECs and NKs in monocultures to assess potential coculturing effects. In order to

study the effects of car exhaust emissions, we exposed the ECs for 6hrs to the exhaust in the Laboratory for Exhaust Emission Control, transported them to the cell culture lab of the University Children's Hospital Basel, and added the NKs approximately 2hrs after the end of the 6hr exposure period.

2.3 Microscopic characterization of the coculture model

We fixed the cells in 4% paraformaldehyde (PFA; Electron Microscopy Sciences, Lucerna-Chem, Lucerne, Switzerland) and stained them with hematoxylin-eosin for histological analysis.

For analysis with laser scanning microscopy (LSM), NKs were stained with DiO (Vybrant Cell-Labeling Solution; Molecular Probes, Thermo Fisher Scientific, Reinach, Switzerland), according to manufacturer's instructions, and added to the confluent ECs at ALI. After 20hrs, cocultures were fixed with 4% PFA and washed with PBS (with Ca^{2+}, Mg^{2+}; Sigma-Aldrich). Cocultures were stained with rhodamine phalloidin (Molecular Probes, Thermo Fisher Scientific) for the f-actin cytoskeleton, according to manufacturer's instructions, and mounted in a Vectashield mounting medium (Vector Laboratories, Reactolab, Servion, Switzerland). Samples were analyzed with a Zeiss LSM 510 Meta and images were processed with the IMARIS Software from Bitplane.

2.4 Validation of the coculture model with diesel particulate matter exposure and unspecific stimulation

We weighed 300µg of diesel particulate matter (DPM) (1650b, National Institute of Standards and Technology, USA) in a 0.5ml low retention Eppendorf tube and transferred the DPM into the loading chamber of a Dry Powder Insufflator™ (PennCentury, USA). One puff (200µl, corresponding to about 150µg DPM) was sprayed on one insert with confluent ECs. 4hrs later NKs or media (as a control) were added to the cells. 20hrs later the cells were fixed using Trizol (Lubioscience, Lucerne, Switzerland) and stored at -80°C until analysis of gene expression (RT-PCR) was performed.

For validation of the coculture model, cells were stimulated with the unspecific stimuli phorbol 12-myristate 13-acetate (PMA; Acros Organics, Thermo Fisher Scientific; 50ng/ml) and ionomycin (MP Biomedicals, Lucerna-Chem; 1µg/ml) for 4hrs and subsequently analyzed with flow cytometry.

2.5 Exposure to gasoline and ethanol-gasoline exhaust emissions

2.5.1 Exposure system and protocol

We utilized a previously established and described exhaust exposure system (Müller et al. 2010) and exposure protocol (Bisig et al. 2016). Briefly, a GDI flex-fuel gasoline passenger car (Volvo V60 T4F, with 3-way catalyst, Euro 5 standard) was driven at a steady state cycle with stepwise decreasing velocity from 95km/h, 61km/h, 45km/h to 26km/h, and idling for 20min each. Freshly produced exhaust was diluted 10x with filtered ambient air using a Minidiluter (MD19-2E, Matter Engineering) and transferred to the exposure chamber with a constant flow of 2l/min. ECs as monocultures at ALI were exposed to freshly produced car exhaust (E0 or E85) or filtered ambient air (as control) under controlled conditions (37°C, 5% CO_2, ~80% humidity). The exposures were performed for 2hrs and 6hrs, for a better overview only the data points after 6hrs are shown, the 2hr exposures showed very similar trends as the 6hr exposures. Feasibility of the exposure conditions has been previously shown (Müller et al. 2012, Steiner et al. 2013a, Steiner et al. 2013b). Thereafter, half of the ECs were cultured as monocultures and NKs were added to the other half of the ECs to obtain cocultures. No washing was performed to remove potentially deposited exhaust particles. 20hrs later the samples were harvested for analysis. We repeated exposure to E0 and E85 each on four different days, resulting in a repetition number (n) of 4.

2.5.2 Fuel and exhaust characterization

We used pure conventional lead free gasoline (E0) and conventional lead free gasoline with 85% ethanol (E85). The Laboratory for IC-Engines and Exhaust Emission Control of the Bern University of Applied Sciences analyzed the particle number (PN) concentration; using a condensation particle counter (TSI 3790), and the concentrations of carbon monoxide (CO), CO_2, nitrogen oxides (NO_x), and total hydrocarbon (T.HC) using an automotive emission analyzer (Horiba MEXA-9400H). Exhaust characterization was performed as previously described (Bisig et al. 2016).

2.6 Endpoints

2.6.1 Quantitative real-time RT-PCR

mRNA was isolated using the RNA Clean&Concentrator (Zymo Research). cDNA was prepared using the GoScript Reverse Transcription System (Promega). Quantitative real-time RT-PCR was done with GoTaq qPCR Master Mix (Promega) in 15µl reaction volume. *β-actin* was used as housekeeping gene. We used the following primer: *β-actin* - forward (FW) CCTCGCCTTTGCCGATCC and reverse (RV) CTCGTCGCCCACATAGGAAT, *ULBP2* – FW GGCAACAAGACAGTCACACCT and RV GTACTGGGTTCTGTGCTTTCCA, *MICA* – FW AAGACAACAGCACCAGGAGC and RV TCCATTCCTCAGTCTCCAGGT, *IL-8* – FW CTGATTTCTGCAGCTCTGTG and RV ATTTCTGTGTTGGCGCAGTG, and *IP-10* – FW CTAGAACTGTACGCTGTACC and RV CTTGATGGCCTTCGATTCTG. Thermal cycles for all genes were carried out using the CFX Connect Real-time System from Bio-Rad at 95°C for 1min, followed by 45 cycles of 95°C for 5sec, and 60°C for 30sec, followed by the melt curve (increase from 65°C to 95°C with 1°C each 5sec).

2.6.2 Flow cytometry analysis

Brefeldin A (eBioscience, Vienna, Austria) was added for the last 4hrs of the post-exposure incubation to block the Golgi apparatus and accumulate cytokines in the cytosol. We lifted the cells of the transwells by accutase treatment (Sigma-Aldrich; 30min at 37°C), divided the samples into various tubes for different antibody (AB) panels (Table 1), and stained the cells with viability dye (Fixable Viability Dye eFluor 450, eBioscience) following the supplier's instructions. For the staining of surface markers, cells were re-suspended in flow staining buffer (PBS without Ca^{2+}, Mg^{2+} (Sigma-Aldrich), 1% FBS, 0.9% sodium azide (Sigma-Aldrich)) and incubated with ABs for 20min at room temperature, and subsequently fixed with 2% PFA for 10min. After surface marker staining, intracellular staining of NKs was done with the Intracellular Fixation & Permeabilization Buffer Set (eBioscience) according to the manufacturer's protocol (30min incubation at room temperature with ABs). All samples were re-suspended in flow staining buffer for acquisition with a BD LRS Fortessa flow cytometer (BD Bioscience). Data was analyzed using FlowJo software version 10.

Table 1. Flow cytometry antibody cocktails used for different endpoints.

	AF488	**AF700**	**APC**	**APC-Cy7**	**BV605**	**FITC**	**PE**	**PE-Cy7**	**PE-TR**
Isotype control EC SM	IgG2a[1]		IgG2[3]	CD45[6]	IgG1[1]		IgG2[3]		
EC SM	MICA/B[1]		ULBP2/5/6[3]	CD45[6]	CD183[1] (CXCR3)		ULBP3[3]		
Isotype control NK cell SM		IgG2a[3]	IgG1[1]	CD45[6]	IgG1[1]	IgG1[1]	IgG2[4]	IgG1[1]	IgG2[5]
NK cell SM		CD159a[3] (NKG2A)	CD314[1] (NKG2D)	CD45[6]	CD183[1] (CXCR3)	CD335[1] (NKp46)	CD158b[4]	CD16[1]	CD56[5]
NK cell ICS			IFN-γ[2]	CD45[6]		grzB[4]	IL-4[2]		

CD45 was used for differentiation between ECs and NKs. Abbreviations: SM, surface marker; ICS, intracellular staining; AF, AlexaFluor; APC, allophycocyanin; APC-Cy7, allophycocyanin-cyanine 7; BV605, brilliant violet 605; FITC, fluorescein isothiocyanate; PE, phytoerythrin; PE-Cy7,

phytoerythrin-cyanine 7; PE-TR, phytoerythrin-texas red. The antibodies were purchased from the following companies: [1]BioLegend (Lucerna-Chem), [2]eBioscience, [3]R&D Systems (Bio-Techne, Zug, Switzerland); [4]BD Bioscience; [5]Molecular Probes; [6]Tonbo (LuBio Science, Lucerne, Switzerland)

2.6.3 NKs' killing potential

We assessed NKs' killing potential with the 7-AAD/CFSE Cell-mediated Cytotoxicity Assay Kit (Cayman Chemical, Biozol, Eiching, Germany) according to the manufacturer's instructions. Briefly, we added $5x10^4$ pre-stained human chronic myelogenous leukemia cells (K562 cell line, Sigma-Aldrich), serving as target cells, to the $2.5x10^5$ NKs in the coculture transwells, ending up with a target:NKs ratio of 1:5, and incubated them for the last 4hrs of the post-exposure incubation. Samples were measured by flow cytometry. Data are presented as a percentage of dead target cells minus background cytotoxicity (percentage of dead target cells incubated with assay kit dyes only). For positive controls, NKs only and NKs in cocultures were stimulated with human recombinant IL-12 (BioLegend, 10ng/ml) for 20hrs. Stimulated NKs alone showed a 30% increase, and cocultured NKs an 8% increase in killing potential compared to unstimulated cells (data not shown).

2.6.4 Oxidative DNA damage

DNA was analyzed using the OxyDNA Assay Kit (Calbiochem, MERCK Millipore, Schaffhausen, Switzerland). Cells were detached from the transwell membranes using accutase (15min, 37°C), fixed with 2% PFA, and permeabilized with 70% ethanol (BioUltra 99.8%, Sigma-Aldrich). After re-suspension in PBS (without Ca^{2+}, Mg^{2+}), cells were washed with wash solution and 5µl FITC conjugate was added. After 1h incubation, cells were washed twice and DNA damage indicated by the bound FITC-labelled dye was measured using flow cytometry (BD LSR Fortessa). As positive controls, cells were incubated with methylene-blue (Sigma-Aldrich; 3mM) for 1h at

37°C, and after removal of the methylene-blue, exposed for 30min to light at room temperature, resulting in a 44% increase of oxidative DNA damage (data not shown).

2.6.5 Cellular oxidative stress

We assessed glutathione (GSH) using the Glutathione Assay Kit (Cayman Chemical, Biozol) following the supplier's protocol. The basolateral medium was removed to avoid measurement interferences with FBS, and the cells were collected using the lysis buffer of the kit. Deproteination of samples with metaphosphoric acid (Sigma-Aldrich) was done before performing the assay. We normalized the measured levels of GSH to the levels of total protein in the samples, which were assessed using the Pierce BCA Protein Assay Kit (Thermo Scientific, Thermo Fisher Scientific), according to manufacturer`s instructions for the assay kit's microplate procedure. Microplates were analyzed with the Synergy H4 Hybrid Microplate Reader (BioTek, Lucerne, Switzerland). Cells exposed to tert-butylhydroperoxid (Sigma-Aldrich; 10mM) for 2.5hrs served as positive controls, resulting in lower GSH levels (11.5 % on average, data not shown) and thus increased oxidative stress.

2.6.6 Data analysis and statistics

For statistical analysis, we used Prism GraphPad (Version 6.05, La Jolla, USA). Data are shown as median [range]. Comparisons of monocultures with cocultures were analyzed with Wilcoxon signed-rank test. For comparison between E0 and E85 exhaust, samples were normalized to corresponding air controls, and compared using Mann-Whitney test (for EC monocultures), or Wilcoxon signed-rank test (for cocultures). $P<0.05$ was considered statistically significant. Results shown are representative of 4 independent experiments, unless otherwise stated.

3. Results

3.1 Characterization of the novel EC-NKs coculture model

To study interactions of bronchial ECs and NKs, we developed a novel coculture model by adding primary peripheral blood NKs to the apical side of a confluent monolayer of the human bronchial 16HBE14o$^-$ EC line cultured at the ALI. Histological analysis using light microscopy and LSM images showed the presence of NKs on the apical side of ECs (white arrow heads, Figure 2), indicating direct cell-cell contact between cocultured ECs and NKs. To ensure viability of ECs and NKs in cocultures, we analyzed the percentage of dead ECs and NKs via flow cytometry. No effect on the viability of ECs or NKs after coculturing was found (Table 2, Table 3).

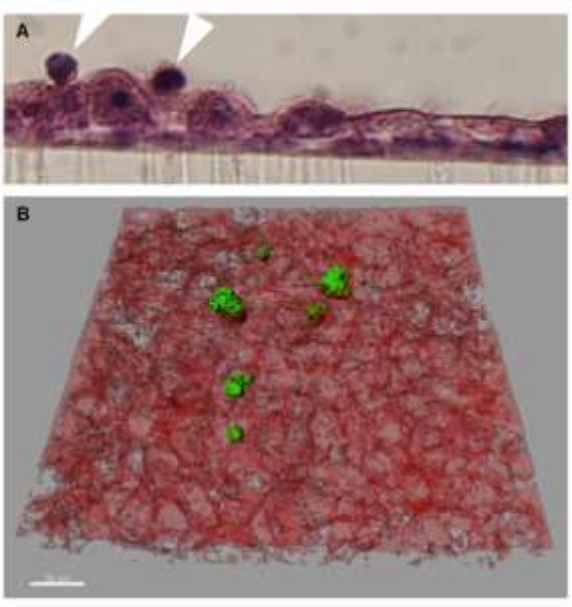

Figure 2. (A) Histological image of the haemotoxylin-eosin stained coculture model *consisting of 16HBE14o$^-$ bronchial ECs and primary NKs (white arrow heads).* ***(B) Laser scanning microscope (LSM) image of a coculture.*** *3D projection showing live stained NKs (green) and the f-actin cytoskeleton (red, transparent rendering).*

EC surface receptor expression of ULBP2/5/6, MICA/B and CD183 were unchanged in cocultures compared to ECs in monocultures. However, the expression of the EC stress receptor ULBP3

significantly decreased after coculturing (Table 2). Cocultured NKs showed significantly higher expression of CD16, CD56, CD158b, CD159a and CD183; whereas CD314 and CD335 expression was lower compared to NKs only (Table ***3***). Intracellular IFN-γ production was not affected by coculturing, while granzyme B and IL-4 production were significantly decreased (Table ***3***). NKs' killing potential was significantly reduced in cocultures compared to NKs alone (Table ***3***), while ECs alone did not affect the viability of the target cells (data not shown).

Table 2. Comparison of ECs in monoculture and ECs in coculture with NKs.

		EC monoculture	**EC coculture**	**p-value**
Cytotoxicity (% of dead ECs)		60.3 [41.1-82.6]	58.8 [32.3-75.0]	0.106
EC surface markers (MFI)	MICA/B	81.2 [16.8-275]	70.7 [4.80-268]	0.963
	ULBP1	14.9 [0.19-55.1]	14.5 [-0.6-38.9]	0.946
	ULBP2/5/6	405 [119-1011]	336 [107-804]	0.130
	ULBP3	305 [82.8-852]	268 [60.7-736]	***0.024**
	CD183	2645 [996-7011]	2756 [1647-7493]	0.995
DNA damage (MFI)		7885 [70-15492]	6466 [32-14333]	0.426

*Values are presented as median [range] of percentage of dead cells or MFI. Data were analyzed by Wilcoxon signed-rank test, n=13-18, *p<0.05. Abbreviations: MFI, mean fluorescence intensity.*

Table 3. Comparison of NKs only and NKs in coculture with ECs.

		NKs only	NKs in coculture	p-value
Cytotoxicity (% of dead NKs)		6.60 [3.12-13.2]	3.80 [1.7-20.5]	0.051
NK cell surface markers (MFI)	CD16	36992 [-328-59782]	48941 [21086-71514]	***<0.001**
	CD56	421 [-6.15-894]	769 [611-1594]	***<0.001**
	CD158b	942 [399-4320]	1388 [633-4590]	***0.003**
	CD159a	218 [-311-268]	274 [-343-423]	***0.029**
	CD183	681 [-198-1432]	1112 [-314-3949]	***0.011**
	CD314	437 [-21.5-701]	393 [-52.4-639]	***0.034**
	CD335	596 [-2.9-769]	529 [-299-625]	***<0.001**
NK cell intracellular markers (MFI)	grzB	431 [204-900]	394 [180-851]	***0.016**
	IFN-γ	143 [-121-322]	128 [-68.6-249]	0.520
	IL-4	367 [251-1217]	283 [98.2-477]	***0.003**
NKs' killing potential (% of dead target cells)		70.2 [28.7-8.5]	25.6 [4.8-73.1]	***<0.001**
DNA damage (MFI)		3828 [8.8-6688]	1852 [6.8-6405]	***0.003**

*Values are presented as median [range] of percentage of dead cells or MFI. Data were analyzed by Wilcoxon signed-rank test, n=13-18, *p<0.05. Abbreviations: MFI, mean fluorescence intensity.*

3.2 Validation of the coculture model

In order to validate that our novel coculture model is feasible to assess exhaust related cell damage, we studied the response to DPM exposure, known to have toxic effects on ECs and NKs (Boland et al. 1999, Muller et al. 2013, Takizawa et al. 1999). DPM exposure was weakly associated with increased mRNA expression of stress receptors *ULBP2* and *MICA,* and of the pro- inflammatory cytokine *IL-8* in mono- as well as in cocultures (p=0.125 and p=0.06, respectively). There was weak evidence of reduced *IP-10* expression following DPM exposure (p=0.06) (Figure 3).

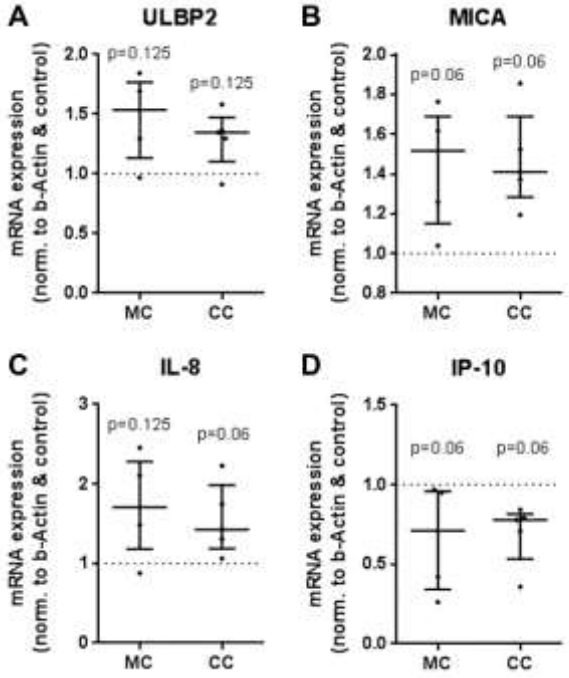

Figure 3. Effect of DPM on ECs. *Data are normalized to the housekeeping gene β-actin and to the negative control, shown as median with interquartile range and single values as diamonds. Tested with Mann-Whitney Test for monocultures, and Wilcoxon singed-rank test for cocultures. Abbreviations: CC, coculture; MC, monoculture.*

As an additional validation, we exposed cocultures to PMA/ionomycin, which was weakly associated with decreased surface expression of MICA/B, ULBP2/5/6, and ULBP3 and increased expression of CD183 in cocultured ECs compared to unstimulated controls (p=0.125, Figure 4).

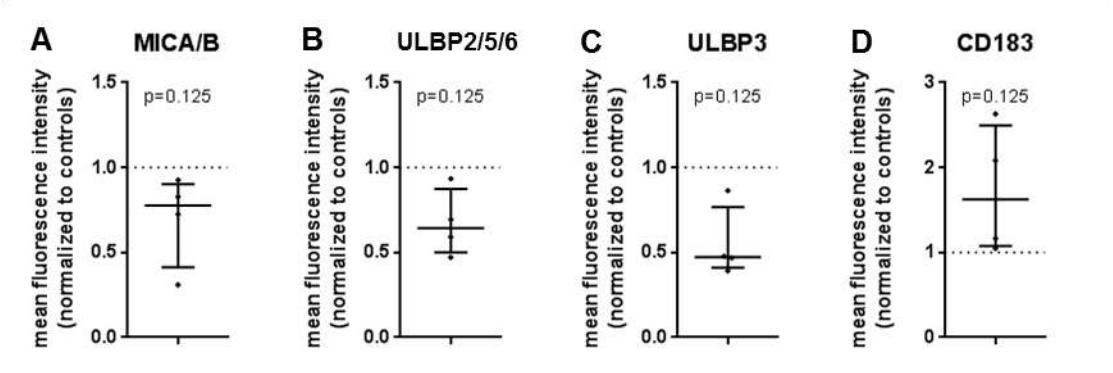

Figure 4. Effect of PMA/ionomycin stimulation on EC surface receptor expression of cocultures. *Data are normalized to the negative controls and are shown as median with interquartile range and single values as diamonds, tested with Wilcoxon singed-rank test.*

PMA/ionomycin resulted in a weak evidence for decreased expression of CD16, CD56, CD158a, CD183, CD335 in NKs in cocultures and NKs only, and increased the CD159a expression in cocultures (Figure 5A-G). Increased intracellular production of IFN-γ in cocultures, as well as in monocultures, and decreased granzyme B production in cocultures was weakly associated with PMA/ionomycin stimulation, while IL-4 production was not changed (Figure 5H-J).

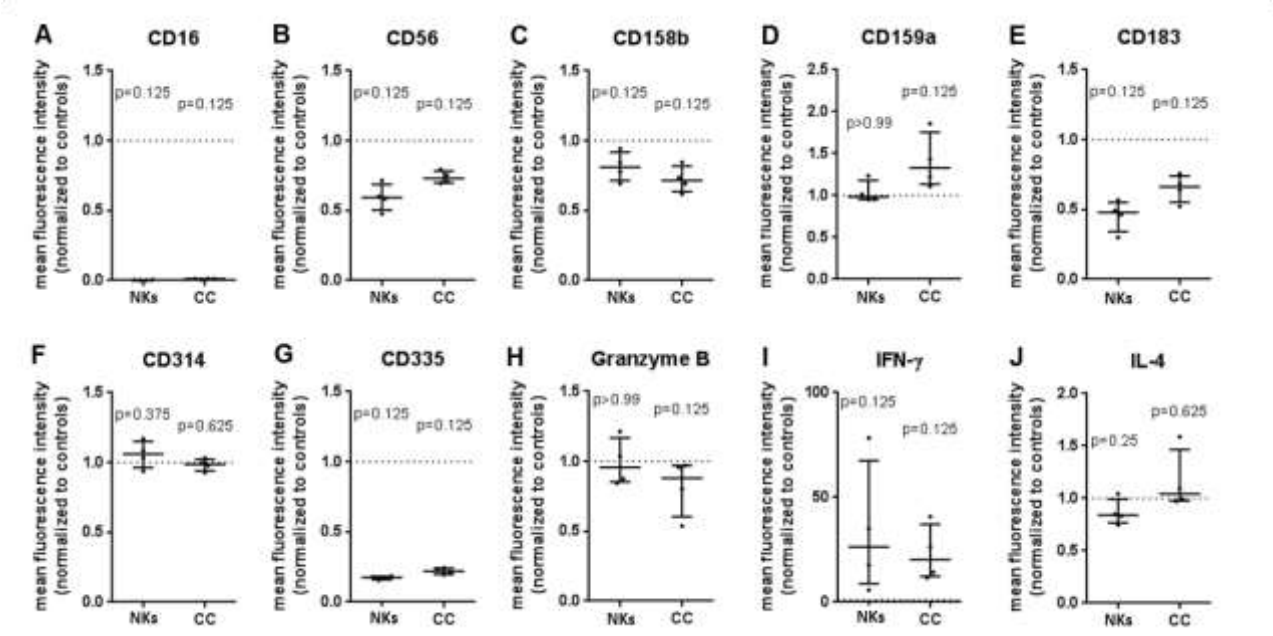

Figure 5. Effect of PMA/ionomycin stimulation on immunophenotype and intracellular markers of NKs. *Data are normalized to the unstimulated controls and shown as median with interquartile range and single values as diamonds, tested with Wilcoxon singed-rank test. Abbreviations: CC, coculture; NKs, natural killer cells.*

3.3 Exhaust characterization

We measured volatile exhaust components (CO, T.HC, and NOx) and the PN concentration (Figure 6). No differences between E0 and E85 exhaust emissions were found for CO, NO_x, and PN concentration, while T.HC was significantly lower in E85 compared to E0 exhaust.

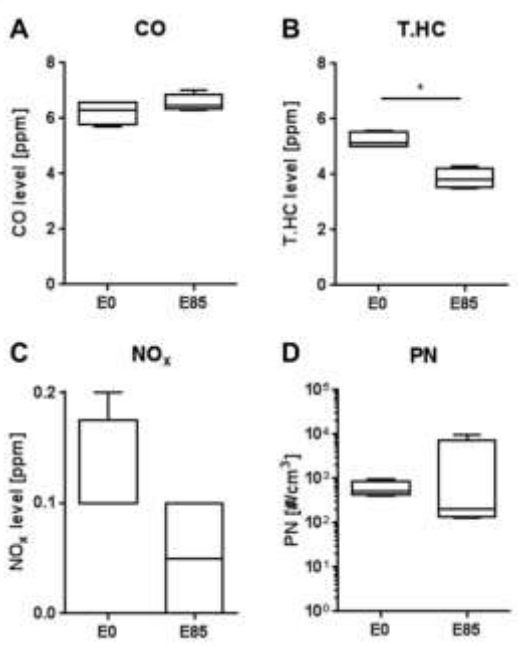

Figure 6. Exhaust characterization. (A-C) *Volatile Fraction and* ***(D)*** *Particle Number Concentration. Data are shown as Box- and whisker-plots. *p<0.05 tested with Mann-Whitney Test. Abbreviations: CO, carbon monoxide; T.HC, total hydrocarbons; NO_x, nitrogen oxides; PN, particle number.*

3.4 Comparison of E0 and E85 effects on ECs and NKs in monocultures and cocultures

We normalized all values to the corresponding air controls to determine whether E0 and E85 exhaust induced different effects in our cell cultures (Table 4 for EC endpoints,

Table 5 for NKs endpoints).

Table 4. Comparison of effects of exposure to E0 or E85 exhaust in ECs of monocultures or cocultures.

			E0	E85	p-value
Flow cytometry measurement					
Cytotoxicity (% dead EC)		**MC**	**0.87 [0.69- 0.99]**	**1.03 [0.93- 1.32]**	**0.06**
		CC	0.84 [0.74- 0.94]	0.98 [0.83- 1.30]	0.25
EC surface markers (MFI)	**MICA/B**	**MC**	1.75 [1.07- 2.44]	1.05 [0.83- 1.20]	0.11
		CC	1.53 [1.01- 3.02]	1.07 [0.30- 1.35]	0.25
	ULBP2/5/6	**MC**	1.38 [1.17- 1.73]	1.23 [0.90- 1.48]	0.49
		CC	1.30 [1.15- 1.92]	1.13 [0.28- 1.61]	0.38
	CD183	**MC**	1.12 [0.77- 1.87]	0.86 [0.64- 1.60]	0.49
		CC	1.09 [0.93- 1.27]	1.00 [0.85- 1.07]	0.38
DNA damage (MFI)		**MC**	**1.62 [0.89- 10.7]**	**0.82 [0.41- 1.07]**	**0.06**
		CC	1.25 [0.94-2.43]	0.97 [0.79- 0.99]	0.20
Quantitative real-time RT-PCR					
ULBP2		**MC**	0.88 [0.44-2.77]	0.70 [0.06-0.79]	0.57
		CC	1.05 [0.49-4.68]	1.23 [0.28-3.95]	>0.99
MICA		**MC**	0.83 [0.59-3.44]	1.22 [0.05-33.69]	0.83
		CC	1.10 [0.94-4.89]	1.55 [0.46-7.11]	>0.99
IL-8		**MC**	0.49 [0.28-0.91]	0.71 [0.06-20.48]	0.57
		CC	0.80 [0.60-4.11]	1.33 [0.22-14.88]	0.25
IP-10		**MC**	0.71 [0.16-0.81]	18.78 [0.69-33.06]	0.23
		CC	1.30 [0.96-1.72]	2.02 [0.05-4.7]	>0.99
Colorimetric assay					
Oxidative Stress (GSH/total protein)		**MC**	1.06 [1.02- 1.08]	1.19 [0.92- 1.20]	0.34
		[#]CC	0.99 [0.78- 1.31]	0.99 [0.96- 1.00]	>0.99

Data are normalized to corresponding air controls (resulting in 1 = no effect). Values are presented as median [range] of percentage of dead cells, MFI, relative gene expression or ratio of GSH relative to total protein. [#]includes ECs and NKs, since the cells cannot be separated for this assay. Data of EC monocultures were analyzed by Mann-Whitney test and those of cocultures by Wilcoxon signed-rank test. Abbreviations: CC, coculture; GSH, glutathione; MC, monoculture, MFI, mean fluorescence intensity.

Table 5. Comparison of effects of exposure to E0 and E85 in NKs cocultured with ECs.

		E0	E85	p-value
Cytotoxicity (% dead NKs)		0.76 [0.52-1.67]	1.05[1.01-1.39]	0.38
NK cell surface markers (MFI)	**CD16**	1.02 [0.98-1.04]	0.97 [0.96-1.02]	0.25
	CD56	1.01 [0.91-1.08]	0.99 [0.92-1.09]	>0.99
	CD158b	1.01 [0.95-1.15]	1.02 [0.90-1.13]	0.63
	CD159a	0.99 [0.81-1.07]	1.15 [1.03-1.92]	0.13
	CD183	1.39 [0.86-1.48]	1.27 [0.97-1.29]	0.50
	CD314	1.97 [0.10-6.70]	1.20 [0.90-1.34]	0.50
	CD335	1.22 [0.92-1.48]	1.28 [1.10-1.41]	0.63
NK cell intracellular markers (MFI)	**grzB**	1.00 [0.92-1.13]	1.05 [0.88-1.12]	>0.99
	IFN-γ	0.96 [0.88-1.00]	1.08 [0.85-1.79]	0.38
	IL-4	1.00 [0.88-1.60]	0.92 [0.86-1.03]	0.38
Killing potential (% dead target cells)		0.92 [0.77-1.14]	0.92 [0.86-1.09]	0.88
DNA damage (MFI)		1.13 [0.87-1.84]	0.71 [0.59-1.04]	0.13

Data are normalized to corresponding air controls (resulting in 1 = no effect, negative values were adjusted to positive values by adding the same fixed value to all data points of the same endpoints). Values are presented as median [range] of percentage of dead cells, or MFI. Data were analyzed by Wilcoxon signed-rank test. Abbreviations: grzB, granzyme B; MFI, mean fluorescence intensity.

The exposure upon E85 exhaust was weakly associated with slightly more killed cells in monocultures compared to the exposure to E0 exhaust (p=0.06, Table 4). There was no difference in cytotoxicity between E85 and E0 exhaust upon cocultures or NKs (Table 4, Table 5). The expression of EC surface receptors did not differ between E0 and E85 exhaust exposed ECs in mono- or cocultures (Table 4). Expression of NK cell surface receptors and the production of intracellular mediators did not differ between cells exposed to E0 or E85 exhaust (Table 5). NKs' killing potential was not different between both exposure conditions (Table 5). We observed a weak association of increased oxidative DNA damage (p=0.06) after exposure to E0 exhaust compared to E85 in ECs of monocultures, but not in ECs of cocultures or NKs (Table 4,

Table 5). Gene expression of ULBP2, MICA, IL-8 or IP-10 was not different between E0 and E85 exposure (Table 4). The level of oxidative stress was not different between exposure to E0 or E85 exhaust (Table 4).

4. Discussion

We developed a novel coculture model of bronchial ECs and NKs mimicking the human airways to study effects of exposure to E0 and E85 exhaust *in vitro.* Coculturing reduced the ULBP3 expression of ECs, altered the receptor expression of NKs, and reduced their killing potential. We validated the novel coculture model showing that DPM exposure changed gene expression of ECs, and unspecific stimulation with PMA/ionomycin altered surface receptor expression of ECs and NKs. The exposure to E0 and E85 exhaust had only minor toxic effects on ECs and NK cells compared to air. Looking at the results more closely, we found a weak association for higher cytotoxicity, but lower oxidative DNA damage in ECs after exposure to E85 exhaust compared to E0 (Table 4). The increase for cytotoxicity was only about 18% in ECs exposed to E85 compared to E0, and therefore the biological relevance may be questionable. However, the level of oxidative damage in ECs exposed to E0 reached twice the level of ECs exposed to E85. While the death of ECs leads to the replacement of the cells, oxidative DNA damage may be a starting event for cancer development (Clift and Rothen-Rutishauser 2013), and thus may be more relevant for long term effects.

One strength of the study is the exposure model (Müller et al. 2010), using whole fresh gasoline exhaust which enabled us to expose cells to complete gasoline exhaust mixtures, including gaseous and particulate components. This exposure setup represents more closely real-life exposure conditions. Given that (pro-) inflammatory effects have also been attributed to non-particulate matter components of exhaust emissions (Holder et al. 2007, Lund et al. 2007, Reed et al. 2008), the use of whole exhaust exposure to assess the toxicity of exhaust emissions is

crucial. Our exposure system was controlled for temperature, humidity, and CO_2 concentration. Furthermore, the setup includes exposure of cells to filtered ambient air, which serve as a corresponding control and to which effects of exhaust exposure can be normalized. This allowed for a comprehensive evaluation of the toxic effects of exhaust emissions in a biologically relevant *in vitro* airway exposure model.

In this study, we used human cells to avoid potential translation difficulties from animal data. We used the human bronchial EC line 16HBE14o$^-$, which has already been used in several *in vitro* air pollution studies (Bauer et al. 2015, Baulig et al. 2003, Müller et al. 2012, Steiner et al. 2013b), and represents a good model of the bronchial part of the human airways. As for NKs, we used primary cells from healthy human donors, representing an important cell type in the respiratory system. Combining ECs and NKs in cocultures allowed us to take into account that the human airways consist of numerous different cell types, which interact with each other (Ochs and Weibel 2008). The characterization of our coculture model showed changes in almost all studied markers of NKs upon coculturing with ECs. This illustrates the ability of NKs to adapt to their microenvironment and thus to adjust their function to varying conditions. The coordinated responses in cocultures represent a more realistic model of the physiological setting in the body than single cell types (Alfaro-Moreno et al. 2008, Bauer et al. 2015, Müller et al. 2010), and therefore, our results mimic more closely *in vivo* conditions. We used a cell culture model specifically developed to study respiratory physiology. This model is exposed to air (by air-liquid interface) and mimics the human airways more closely than submerged conditions (Ghio et al. 2013), and therefore is a favorable setting to study air pollutant exposure.

Our study is limited by the *in vitro* study design, focusing on two cell types only. Therefore, with our model, we only represent part of the complexity of the airways and cannot conclude on

exhaust effects on other cells types of the lung. We could only repeat the experiments four times. This is due to restriction of financial and human resources and the limited time the Laboratory for Exhaust Emission Control was available for our project.

Another study used the exact same exposure system and set-up to investigate the effects of gasoline exhaust emissions in a triple cell coculture model of 16HBE14o$^-$ ECs, human monocyte-derived macrophages and human monocyte-derived dendritic cells (Bisig et al. 2016). They found no effects of gasoline exhaust emissions in the cell model and no differences between the exposure to pure gasoline and ethanol-gasoline blend (E85 or gasoline with 10% ethanol) exhaust emissions. Their findings, along with our results, therefore, suggest only minor toxic effects of gasoline exhaust. However, these findings stand in contrast to older studies investigating toxic effects of gasoline exhaust. These older studies found lung cytotoxicity, suppression of macrophages and oxidant stress in rodents (Mauderly et al. 2014), upregulated factors of vascular remodeling and increase in vascular oxidative stress in mice (Lund et al. 2007) and mild lung irritation and decrease oxidant production by alveolar macrophages in a sub-chronic setting with rodents (Reed et al. 2008). However, it has to be considered that all of those studies used older engine technologies, and not the newer GDI technology that was used in our study. Thus, the differences in findings may reflect technological progress. Two other studies investigated the effects of methanol, another gasoline fuel blend, and found less adverse effects in several cell lines for exposure to organic extracts of methanol exhaust compared to organic extracts of gasoline exhaust (Che et al. 2010, Zhang et al. 2007). These findings, and our results of reduced oxidative DNA damage after E85 exposure compared to E0, are further supported by Muñoz et al., reporting reduced genotoxic potential of PAHs (Munoz et al. 2016) due to blending of gasoline with ethanol.

Other studies have associated the exposure to diesel exhaust particles or fine particulate matter to stress or inflammatory signals in ECs (Auger et al. 2006, Holder et al. 2007). ULBPs and MICA/B are known to be highly expressed on stressed ECs after infections, mutations and oxidative stress (Borchers et al. 2006), and are ligands for the activating receptor CD314 on the surface of NKs (Obeidy and Sharland 2009). We found, however, no increased expression of ULBPs, MICA/B or CD314 and no signs for a changed function of NKs cocultured with ECs exposed to E0 exhaust. We found that neither exposure to E0 nor to E85 exhaust - both conditions with low particle numbers - affected NKs. However, when directly comparing the oxidative DNA damage induced by E0 and E85, exposure to E0 exhaust resulted in increased DNA damage compared to E85. We speculate that the higher hydrocarbon concentration in the E0 exhaust, known to cause DNA damage (Toraason et al. 2001), may explain this finding.

With regards to the increasing number of passenger cars worldwide, the predominance of gasoline-driven cars in western countries, and the increasing popularity of ethanol-gasoline blends, it is of high importance to gain more insight into the potential health effects of those fuel blends. We found no major toxic effects of pure gasoline or ethanol-gasoline exhaust emissions from a modern flex-fuel car in EC monocultures or cocultures of ECs and NKs. Therefore, based on our findings, we speculate, that the augmented use of ethanol as part of fuel for gasoline cars does not seem to be harmful. As our study investigated only acute effects in two cell types, additional *in vitro* studies with other cells types, and animal and human *in vivo* studies examining chronic exposure scenarios, are needed to further support our findings.

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6. Declaration of interest statement

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Andreas Mayer is the owner and general manager of ‘‘TTM Andreas Mayer’’, Switzerland, an emission consulting company. As with all of the authors, however, he declares to have no conflicts of interest.

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4 General discussion

In summary, this PhD thesis adds further information on early lung development from infancy until school age. Following our aim to improve our understanding of infant lung function, we studied the methodological challenges of two different infant lung function tests. First, we studied MBW measurements, which provide information on lung volumes and ventilation homogeneity. We investigated the magnitude of MBW measurement errors from the lung function of healthy infants, and were able to outline recommendations that greatly improve the quality of the outcomes. Second, we studied Rint measurements that provide information on airway obstruction. We demonstrated that Rint measurements in unsedated infants shortly after birth are feasible despite their time-consuming nature. This technique allows for the determination of airway obstruction during the vulnerable phase of lung development.

We studied airway inflammation shortly after birth using FeNO levels, in order to further understand early disease pathologies in asthma and CF. In regards to asthma development, we found that FeNO measured in the naïve airways of newborns was not associated with asthma at school age. Thus, FeNO measurements in infancy were not helpful to predict the risk for asthma development. We also compared FeNO levels between healthy newborns and infants with CF. Our finding of reduced FeNO levels in infants with CF compared to healthy controls contributed to our understanding of early airway pathology in CF disease.

In order to address the complexity of airway disease, we applied three different approaches to studying the pathophysiology: i) we assessed different risk factors (genetic, immunological) and used epidemiological models to study their association with asthma development. We found that a key immune regulator YKL-40, and polymorphisms from the gene encoding this protein, may be associated with asthma at school age; ii) We applied a time-series approach and used prospectively assessed respiratory symptom scores during infancy to develop a novel method to characterize these symptoms. Utilizing this method, we were able to observer-independently assess dynamic patterns of symptoms. This method could be useful in prospective cohort studies,

and could be applied to predict asthma development; iii) we took an analytical approach, to study the *in vitro* effect of air pollution within a newly developed cell-culture model. We exposed different human respiratory cells to gasoline and ethanol-gasoline exhaust. Comparison between gasoline and ethanol-gasoline exposure showed a weak association for less oxidative DNA damage after ethanol-gasoline exposure.

The relevant conclusions drawn from each project, including future directions, are described below.

4.1 Recommendations to improve infant lung function tests

In this study of healthy infants, we quantified the magnitude of MBW measurement errors. We demonstrated that non-systematic, inter-center differences in MBW outcomes in infants can be greatly reduced by including correctly recorded environmental conditions in the software analysis algorithms and by making minimal alterations in these algorithms. We provided several simple recommendations for the user. This included, e.g. to examine the recorded temperature during measurements in the software, and make any necessary adjustments. Along with the increasing need to include infant lung function tests in multicenter studies for patients with CF [95, 96], arises the need to obtain higher quality measurements, and this should be the focus of both clinical scientists and equipment manufacturers. We hope the recommendations outlined here will help improve the quality and reliability of MBW measurements in specialized and non-specialized settings.

The quality of Rint measurements has never been systematically studied in infants. We demonstrated the feasibility of Rint measurements in unsedated infants shortly after birth. We further provided first evidence of higher airway resistance in preterm compared to term-born infants. Variability of measurements was high in both groups, but higher in term compared to preterm infants. Variability of measurements could be considered noise, but could also be regarded as a biological signal *per se*. The less variability of Rint in preterm infants may also

suggest an association between immaturity and breath control. The clinical relevance of our findings needs to be evaluated. Future studies will need to assess whether Rint could be used as a prognostic marker for subsequent respiratory morbidity, taking into consideration the high variability of measurements *per se*, requiring further interpretation.

4.2 New insights on nitric oxide metabolism

We measured postnatal airway FeNO in unselected healthy newborns during natural sleep and found no association with asthma at school age. This finding contradicted studies measuring FeNO at later time points during childhood, describing an association with subsequent respiratory disease [36, 37]. This caused us to speculate that nitric oxide metabolism may play a role in the pathophysiology of childhood asthma only *after* exposure to environmental factors.

Using the same methodology, we compared postnatal FeNO between healthy infants and infants with CF disease. We demonstrated that FeNO is lower in CF patients compared to controls. Further, we found that this effect is even more pronounced in infants homozygous for class I and/or class II mutations without residual function in the Cystic Fibrosis Transmembrane Regulator (CFTR). This finding indicates that FeNO is reduced in CF *a priori*, and may be considered a proxy of CFTR function. It was previously shown that FeNO is associated with reduced neutrophil sequestration, bacteriostatic properties, and mucociliary transport [97], and therefore may be an important player in the progression of CF lung disease.

4.3 Effects of polymorphisms in *CHI3L1* and YKL-40 levels with asthma development

We studied the association of a key immune regulator YKL-40, and genetic polymorphism from the gene encoding this protein, with asthma at school age. We found no robust association between genetic variation in *CHI3L1* and asthma development, but found some indication that the SNP rs10399805 might be related to asthma diagnosis at 6 years of age. In order to test the robustness of our findings, large replication studies within a prospective study design in an

unselected population are warranted. In previous studies, the biomarker YKL-40 was shown to be elevated in children and adults with severe asthma [59, 61, 98]. In our healthy study population, we found a trend of association between elevated cord blood YKL-40 levels and asthma. This finding requires further validation before YKL-40 may be considered an early biomarker for asthma development in unselected infants.

4.4 A novel method to characterize respiratory symptoms

We developed a novel method to characterize the transition probabilities between respiratory symptoms. We found that different symptom dynamic patterns were associated with wheezing and atopy at school age. Furthermore, these symptom dynamics enabled us to identify four distinct phenotypes, of which one consisted of predominantly male subjects of asthmatic mothers, exposed to environmental tobacco smoke (ETS) exposure. These findings indicate that symptom dynamics contain relevant information on risk factors for asthma development. The developed method could potentially be applied in various research settings, since it is based only on respiratory symptom scores, which can be assessed without laboratory equipment or specially trained experts.

4.5 A novel cell model to study air pollution exposure

We developed a novel coculture model consisting of human bronchial epithelial and natural killer cells, to study toxic effects of air pollution. In our *in vitro* study, we demonstrated that pure gasoline or ethanol-gasoline exhaust exposure emissions of a modern car had no major toxic effects on these cells. Comparison between gasoline and ethanol-gasoline exposure showed a weak association for less oxidative DNA damage after ethanol-gasoline exposure. In our study, only acute effects in two cell types were investigated, and additional *in vitro* studies with other cells types could help elucidate the effects of gasoline in other cell types of the lung.

4.6 Physiological mechanisms

The pathophysiological mechanisms of airway disease during childhood are complex, and many factors contribute to disease severity. Findings from this study may further contribute to our understanding of the mechanisms relevant in early life.

We provided further insights into FeNO metabolism during early childhood, and speculated on the basis of our findings that environmental factors modify FeNO levels. While FeNO levels may be modified by the environment, there was also indication that the genetic background partly predetermines FeNO levels. FeNO levels in newborns were decreased in infants with CF, and this effect was even more pronounced in those infants with no residual CFTR function. This indicates that FeNO levels are reduced *a priori* due to the genetic profile. Studies support the idea that the genetic background influences FeNO levels. Our study group has previously reported several genetic determinants located in the 6q12 gene locus which modify FeNO levels in healthy newborns [99]. Another study in adult asthmatics reported an association between polymorphisms in the inducible nitric oxide synthase (iNOS) and FeNO levels [100].

Applying our novel method, which captures symptom dynamic patterns, we were able to identify a group of infants at high risk for the development of wheezing symptoms during childhood. Interestingly, a large proportion of infants in this group were exposed to ETS, which underlies the impact of this exposure on airway disease development. While ETS exposure is clearly a relevant risk factor for respiratory symptoms, there are several other factors that determine a child's response to respiratory infections. Studies suggest that the severity of respiratory infections in children depends not only on the virus type [101], but also its modification by microbial composition [102] and environmental factors [8, 87]. Interplay between viral stimuli, the microbial composition and the genetic predisposition are suggested to affect airway epithelial function [103, 104]. We speculate that infants with more fluctuations during symptom deterioration and recovery are those infants with an epithelial dysfunction, and are thus at an increased risk for developing persistent wheezing. Markers associated with asthma exacerbations

(e.g. cytokine expression, impaired interferon response) were identified previously [103]. In order to test our hypothesis of an epithelial dysfunction during infancy relevant for future disease, prospective studies assessing these markers during respiratory infections are needed.

4.7 Relevance

Results from this PhD thesis provide new insights on early lung development, and may contribute to a better assessment of airway pathologies. Especially in infants with CF, maintenance of normal lung function is crucial, since reduced lung function is associated with increased morbidity and mortality [105]. Infant lung function can assess lung pathologies at an early stage, which can then lead to the application of therapies before airway damage is permanent. This can ultimately improve long term outcome. We addressed relevant methodological issues in infant lung function testing and provided recommendations on how to improve the quality of outcomes. These results could lead to an improved assessment of early airway damage, and may improve the clinical management of CF patients.

It is advantageous to assess airway function using objective lung function tests. In young children, these tests are time-consuming and expensive, and therefore only applied in specialized centers. Simpler, but still objective methods to assess airway function are therefore needed. We were able to develop a method that can observer-independently characterize respiratory symptoms, and provides relevant information on airway mechanics. This method can broadly be applied, since no technical equipment is needed, only the simple assessment of respiratory symptoms.

4.8 Conclusions and outlook

For the different projects in this PhD, different scientific recommendations for next steps could be described. In order to understand the relevance of our recommendations to improve outcomes from infant lung function measurements, application in larger study populations would be necessary. Applying our recommendations in multicenter trials could also improve comparability and reduce inter-center differences.

We provided new insights on FeNO metabolism and speculated that environmental exposures modify nitric oxide synthase (NOS). In order to confirm that environmental exposures indeed modify NOS expression during infancy, frequent longitudinal assessment of FeNO levels and NOS expression would be necessary. Our findings should encourage further research on factors impacting NO metabolism during infancy. In particular, modifying environmental factors, e.g. air pollution and aeroallergens, should be in focus, since exposure to these can be actively reduced. These risk factors may therefore serve as targets for new preventive strategies in childhood asthma development. In regards to FeNO assessed in patients with CF, a further scientific step would be to continue follow-up of the patients during childhood in order to see if FeNO levels in infancy may be of prognostic value for later disease severity.

Our novel method to characterize respiratory symptoms was useful to predict later asthma development in an unselected population. We would suggest that further research seek to validate the developed method in high-risk populations, as well, in which clinical application may be of even greater interest. It is advantageous that our method is based only on respiratory symptom scores, which can be assessed without laboratory equipment or specially trained experts. This opens up the possibility for application in telemonitoring-settings, in which the assessment of symptoms is crucial [106].

We also highlighted the toxic effects of car exhaust exposure within an *in vitro* setting. From a public health perspective, it is imperative that we apply methods which further decrease the toxic effects of air pollution. For example, our cell culture model could be used to investigate whether

newly available particle filters for gasoline cars are useful to reduce the toxic effects of car exhaust.

Some of these steps are currently being undertaken in ongoing projects within our group, with which I am also involved. Some specific questions can, however only be answered in different specific populations, e.g. in high-risk populations for asthma development. Results from these studies will hopefully increase our knowledge of potentially harmful exposures and may improve clinical management.

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6 Appendix of additional manuscripts

During my PhD studies, I was also involved in several projects which did not directly relate to the hypotheses of my thesis. These projects extended my knowledge and broadend my scientific experience.

1) A long-standing and close collaboration between the BILD cohort study and the Swiss Tropical and Public Health Institute Basel (Swiss TPH) enabled me to be involved in a collaboration work between the institutions. Working together with Dr. Danielle Vienneau (Swiss Tropical and Public Health Institute, Basel, Switzerland) and Mrs. Manuella Lech Cantuaria (The Mærsk Mc-Kinney Møller Institute, Faculty of Engineering, University of Southern Denmark, Odense, Denmark) we investigated the association between residential exposure to road traffic noise during pregnancy and postnatal stress, as assessed by the concentration of glucocorticoid metabolites at 5 weeks of age.

2) I also had the opportunity to take part in a project which investigated the relevance of viral and bacterial pathogens for subsequent respiratory diseases during infancy. Together with Dr. Roland Neumann (University Children's hospital Basel, Switzerland) and PD Dr. Markus Hilty (Institute for Infectious Diseases, University of Bern, Bern), we investigated the hypothesis that a distinct microbial composition during the first symptomatic viral infection is associated with subsequent respiratory symptoms.

3) I was involved in a study designed to explore factors influencing aerobic exercise capacity, expressed as peak oxygen consumption (VO_{2peak}) with a specific focus on transmembrane conductance regulator (CFTR) genotype in children and adults with CF. This project was done under under the lead of Dr. Thomas Radtke and Prof. Susi Kriemler (both Epidemiology, Biostatistics and Prevention Institute, University of Zurich, Switzerland).

6.1 Glucocorticoid metabolites in newborns: a marker for traffic noise related stress?

Lech Cantuaria M[*], **Usemann J**[*], Proietti E, Blanes-Vidal V, Dick B, Frey MB, Flück C, Rüedi S, Héritier H, Wunderli J.M., Latzin P, Frey U, Röosli M, Vienneau D on behalf of the BILD study group

[*] Equal contribution.

Manuscript ready for submission

Glucocorticoid metabolites in newborns: a marker for traffic noise related stress?

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Appendix

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ABSTRACT

Background: Traffic noise has been associated with an increased risk for several non-auditory health effects, which may be explained by a noise-induced release of stress hormones (e.g. glucocorticoids). Although several studies in children and adults have shown an increased secretion of glucocorticoids after exposure to noise, information regarding newborns is scarce.

Objectives: To investigate the association between residential exposure to road traffic noise and postnatal stress, as assessed by the concentration of glucocorticoid metabolites at five weeks of age.

Methods: Residential noise exposure was estimated for each infant based on spatially detailed modeled data for all buildings. Adjusted multivariable linear regression models were used to estimate the association between noise exposure and the concentration of nine glucocorticoid metabolites measured in urine of 165 infants from a prospective birth cohort in Bern, Switzerland. Noise exposure was assessed by three different metrics (i.e. Lden, LeqD or LeqN) and categorized into tertiles: low (reference), medium and high categories.

Results: Borderline positive associations were found between high road traffic noise and cortisone (% change: 22.57% [95% confidence interval: -1.8, 53.0%]) and β-cortolone (51.5% [-0.9, 131.5%]), in comparison to infants exposed to low noise levels. On the contrary, newborns exposed to higher noise levels showed a borderline significant reduction of tetrahydrocortisol (-23.7% [-42.8, 1.9%]) and α-cortolone (-18.3% [-33.6, 0.6%]) concentrations.

Conclusions: Our findings suggest a potential effect of noise on glucocorticoid metabolism in early postnatal life. A possible physiological relevance and association with short- and long-term adverse health effects needs to be further investigated.

Keywords: Road traffic; Noise; Glucocorticoids; Cortisol; Postnatal stress; Environmental stressors

Appendix

1. Introduction

In the past few decades, there has been a growing concern about health impacts triggered by environmental noise from different sources, such as industry, neighbors and transportation. As one of the most widespread sources of environmental stress, noise from transportation is considered a major threat for public health. Transportation noise has been identified as a major contributor to the environmental burden of disease in Europe (Hänninen et al. 2014; WHO 2011), and a recent evaluation in Switzerland found that the burden of transportation noise was equal to that of air pollution in terms of total external costs (Vienneau et al. 2015b).

Studies have demonstrated an association between exposure to transportation noise and an increased risk for several non-auditory health effects in adults, including reduced quality of life (Dratva et al. 2010; Héritier et al. 2014), hypertension (Haralabidis et al. 2008; Paunović et al. 2014; van Kempen and Babisch 2012), ischemic heart disease (Babisch 2014; Héritier et al. 2017; Seidler et al. 2016; Sørensen et al. 2012; Vienneau et al. 2015a), respiratory health (Recio et al. 2016b), and diabetes (Eze et al. 2017; Sørensen et al. 2013). In relation to studies in children, most are focused on blood pressure (Babisch et al. 2009; Belojevic et al. 2008), cognitive function (Haines et al. 2001; Stansfeld et al. 2017) and respiratory outcomes (Ising et al. 2003, 2004a, 2004b).

Noise induced health effects are postulated to occur through the activation of either a direct or indirect pathway (Münzel et al. 2016). In the direct pathway, noise directly triggers an instantaneous activation of the central nervous system; in the indirect pathway stress markers are activated through annoyance (Babisch 2002). Regardless of the pathway, noise exposure results in stress responses, characterized by activation of the neuroendocrine system (i.e. hypothalamus-pituitary-adrenal (HPA) axis and sympathetic-adrenal-medulla axis), and a subsequent release of stress hormones, e.g. glucocorticoids.

The stimulation of glucocorticoid production results in several regulatory effects on physiological functions in the human body. The secretion of glucocorticoids induces immune

responses, and might act on both the production of cytokines and lymphocyte proliferation (Dobbs et al. 1996). Overproduction of glucocorticoids also affects blood glucose levels by inhibiting insulin secretion and increases the concentration of lipids and lipoproteins (e.g. cholesterol and triglycerides) (Aich et al. 2009; Qureshi et al. 2009; Recio et al. 2016a).

A number of studies have empirically demonstrated that exposure to transportation noise indeed increase stress-induced secretion of glucocorticoids, mostly cortisol. For instance, Ising et al. (2004b) and Wagner et al. (2010) showed an association between elevated salivary cortisol levels and traffic noise in children and adults, respectively. Furthermore, a substantial increase in morning saliva cortisol levels in women exposed to aircraft noise higher than 60 dB was reported by Selander et al. (2009). While the association is well reported in children and adults, this has never been investigated in newborn infants who are particularly vulnerable to environmental exposures and likely to experience long term effects.

We aimed to investigate the association between residential exposure to road traffic noise (during and shortly after pregnancy) and postnatal stress, as assessed by the concentration of glucocorticoid metabolites measured in urine from infants at five weeks of age.

2. Materials and Methods

2.1 Study design and subjects

This study included a subgroup of 205 healthy infants from the prospective Basel-Bern Infant Lung Development (BILD) birth cohort, recruited antenatally between 2005 and 2011 in the region of Bern, Switzerland. We determined the residential history during pregnancy and after birth, and assessed pre- and early postnatal risk factors (e.g. tobacco smoke exposure, socio-economic status, delivery mode) (Bradley and Corwyn 2002; Floyd et al. 1993; Latimer et al. 2012; Rice et al. 2007) via standardized questionnaires (Fuchs et al. 2012). Addresses were geocoded using a reference file from the Swiss Federal Statistical Office (Neuchâtel). Exclusion criteria for the study were delivery <35 weeks gestational age and problems during the extraction

of urine samples and/or analysis. The study was approved by the Ethics Committee of Bern, Switzerland. Written informed consent was obtained from parents before enrollment.

2.2 Exposure assessment

Noise exposure assessment was based on detailed noise modeling for the year of 2011 from the SiRENE (Short and Long Term Effects of Traffic Noise Exposure) study (Karipidis et al. 2014). The SiRENE database included road traffic noise estimates (dB) for façade points for each dwelling in every building in Switzerland. Data (VECTOR25) from the Swiss Federal Office of Topography (Swisstopo 2007) was used to characterize the buildings and dwelling units from the period of 1998 to 2006, with height and number of floors for each building estimated by a digital surface model combined with a digital terrain model for Switzerland. Road traffic noise was calculated at a maximum of 3 façade points per building façade and floor, with a minimum spacing distance of 5 meters.

The noise exposure metrics used in our study included: Lden, average sound level over all 24h periods of a year, with an additional 5dB for the evening (19:00 – 23:00) and an additional 10dB for the night (23:00 – 07:00) hours; and LeqD and LeqN, which respectively represent the average sound level over all day (07:00 – 23:00) and night (23:00 – 07:00) periods of a year. Since no residential floor information was available for the BILD study participants, we used noise estimates from the middle floor of the relevant residential building. Noise levels from the façade point with the maximum Lden for each participant were assigned, i.e. selected the most exposed façade point for the residential unit based on the Lden. Exposure variables were censored at 35 dB (Lden, LeqD) or 25 dB (LeqN).

Since traffic-related air pollution was shown to affect glucocorticoids metabolism (Rüedi et al. 2013), we also assessed residential nitrogen dioxide (NO_2) exposure averaged for the in-utero period of each infant. We estimated NO_2 exposure using a validated time-space hybrid model for our study area (Proietti et al. 2016).

2.3 Outcome assessment

To assess urinary glucocorticoid levels, parents provided diapers with clean urine from infants at five weeks of age (hereby referred as "newborns" throughout the paper). Within 24 hours, the urine was centrifuged out from the diapers at 4378 rpm for 8 minutes at room temperature (Multifuge 3SR+, Thermo Fisher Scientific) and immediately stored at −20 °C. After sample processing (i.e. pre-extraction, enzymatic hydrolysis, extraction from the hydrolysis mixture, derivatization, gel filtration), gas chromatography-mass spectrometry was carried out to quantitate the different stress hormones as previously described (Dhayat et al. 2015; Garde et al. 2004; Shackleton 1986). This work was conducted at the Department of Nephrology, Hypertension and Clinical Pharmacology, University Hospital of Bern, Switzerland, using a gas chromatograph 7890A from Agilent Technologies (La Jolla, California, USA) coupled to a mass selective detector (Hewlett-Packard 5975C). Measured steroids were then standardized to urinary creatinine concentration (QuantiChrom Creatinine Assay, DICT-500; BioAssay Systems, Hayward, CA, USA) and expressed in μg/mmol creatinine (Garde et al. 2004; Dhayat et al. 2015).

2.4 Statistical analysis

Multivariable linear regression models were used to evaluate the association between road traffic noise (i.e. as determined separately by Lden, LeqD or LeqN) and the concentration of each glucocorticoid metabolite. The noise exposure variables were categorized into tertiles (subsequently referred to as "low", "medium" and "high" categories).

We restricted our analysis to the nine glucocorticoid metabolites: eight of which were detectable in the urine of all the study participants (cortisol, 5α-tetrahydrocortisol (α-THF), tetrahydrocortisol (THF), α-cortol, cortisone, tetrahydrocortisone (THE), α-cortolone, β-cortolone) and the ninth had negligible missing data (β-cortol, for which only two of 165 observations were missing). Since the distribution of concentrations for many of the

glucocorticoid metabolites was strongly right-skewed, data were normalized by natural logarithmic transformation.

All models were calculated unadjusted (i.e. crude), adjusted for anthropogenic factors including sex, gestational age and weight at the time of urine sample (i.e. basic) and further adjusted for potential confounders (i.e. full) identified in previous studies as relevant risk factors (Dhayat et al. 2015; Rüedi et al. 2013): delivery mode; pathological cardiotocogram (CTG); maternal smoking during pregnancy; educational status of the mother; season at birth; vaginal infection; maternal atopy; and existence of older siblings. We used backwards step selection, retaining all statistically significant variables ($p < 0.1$) or variables that, when removed, changed the risk estimate by at least 10% of the previous value.

All statistical analyses were performed in R software (version 3.1.2).

3. Results

3. 1 Summary statistics

From the 205 enrolled newborns, we excluded those with low gestational age (n=2), jellified urine samples (n=35) and unreadable chromatogram (n=3), giving a total sample of 165 (80.5%) newborns. Of those, 88 (53%) were male, the mean (SD) gestational age was 39.5 (1.3) weeks and weight at the time of the urine sample was 4.3 (0.6) kg. 21% of the newborns were delivered by Cesarean section and 5% of the mothers smoked during pregnancy (Table 1). The medians for long-term exposure to road traffic noise were 52.4 dB(A) for LeqD, 45.6 dB(A) for LeqN and 54.4 dB for Lden (Table 2). The cortisol and cortisone concentration (in μg/mmol creatinine) measured from urine were 109.5 (72.4) and 352.8 (205.8), respectively. Details on the 9 glucocorticoid metabolites are provided in Table 3.

All noise exposure metrics were highly correlated with each other (Pearson's correlation (r) ranged between 0.99 and 1.00). Noise and NO_2 exposure during pregnancy showed low correlation coefficients ($r = 0.33$ for all exposure metrics).

3.2 Association between noise and glucocorticoid metabolite concentration

As depicted in Figure 1, traffic noise exposure was found to be borderline associated with change in concentrations of four glucocorticoid metabolites in the urine of newborns, with indications of a dose-response (DR): cortisone (+ DR), β-cortolone (+ DR), THF (- DR) and α-cortolone (- DR).

Borderline positive associations (defined as p-value<0.1) were found between road traffic noise and cortisone for newborns in our high exposure category (%change = 22.6%; 95%CI: -1.8, 53.0%, p-value = 0.074) (Figure 1) compared to newborns in our low exposure category. We saw an increase in β-cortolone concentration for newborns in the high category (%change = 51.5%; 95%CI: -0.9, 131.5%, p-value = 0.057), whereas the borderline significant association for α-cortolone concentration in the high exposed category was in the opposite direction (%change = -18.3%; 95%CI: -33.6, 0.6%, p-value = 0.059). Newborns exposed to higher noise also showed a borderline significant reduction in THF concentration (%change = -23.7%; 95%CI: -42.8, 1.9%, p-value = 0.069).

Similar results were obtained for the crude and basic models for Lden (Table S1), and likewise results for models based on the alternative noise metrics LeqD and LeqN and different exposure classifications were comparable (Figures 2 and S3, and Tables S2 and S3).

4. Discussion

In this study of unselected newborns, we found borderline significant associations between high traffic noise exposure and the concentration of several steroid metabolites measured in urine (Figure 1).

Glucocorticoids metabolites, a subset of steroid hormones, can be further divided into branch E, with glucocorticoid progenitor cortisone, and branch F, with glucocorticoid progenitor cortisol. The enzyme 11β-hydroxysteroid dehydrogenase (HSD) type 1 converts cortisone to cortisol, whereas 11β-HSD type 2 does the opposite. Both enzymes regulate the amount of available progenitor glucocorticoids in each branch. Glucocorticoid metabolites downstream from the

progenitor are then generated by different enzymes. We found exposure to higher noise levels increased the concentration of cortisol and decreased the concentration of downstream metabolites (α-THF, THF, β-cortol) in the F branch. In the E branch, cortisone was increased while the downstream metabolite α-cortolone was decreased and β-cortolone was increased.

There are several influencing factors through which noise exposure could modify the metabolite concentrations. This includes the availability of co-factors (i.e. associated enzymes that catalyze the metabolite synthesis), negative feedback mechanisms which reduce the metabolite synthesis, or the activity of enzymes involved in the glucocorticoid pathway (Miller and Auchus 2011). It is possible that noise exposure modifies the amount of glucocorticoid metabolites by changing the activity of the enzymes 11β-HSD type I and 11β-HSD type 2. However, since we measured the glucocorticoid metabolome rather than enzyme activity directly, our hypothesis needs confirmation by mechanistic studies. Interestingly, previous studies in rats suggested that the enzyme activity of 11β-HSD type 2 is modified by prenatal (Peña et al. 2012) and social stress (Vodička et al. 2014).

The most plausible mechanism to explain noise-induced health effects is related to changes in the HPA axis activity that, in turn, increases levels of secreted glucocorticoids. Several studies have indicated the activation of the HPA axis due to exposure to noise from transportation (Ising et al. 2004b; Selander et al. 2009). With respect to noise exposure during pregnancy, a study performed in pregnant monkeys revealed higher levels of cortisol in the offspring of mothers exposed to noise during gestation than mothers unexposed (Clarke et al. 1994).

It has been previously suggested that both fetal and early postnatal life environment are important determinants of the risk of metabolic and cardiovascular diseases in later life (Barker 1995; Barker et al. 1989). It has also been suggested that stress after birth is associated with reduced neurodevelopment (reviewed in Flacking et al. 2012). A number of studies, when seeking to explain the association between low birth weight and a range of health diseases in

adulthood, have pointed to excess of glucocorticoids as a key factor for long-term effects in the offspring (Edwards et al. 1993; Edwards et al. 1996; Gohlke et al. 2015).

Although exposure to higher levels of glucocorticoid may result in beneficial short-term metabolic consequences, excess is related to increased blood pressure and alterations of the glucose metabolism in the long term (Edwards et al. 1996). These effects can persist and increase the risk of cardiovascular, metabolic, neuroendocrine and behavioral disorders later in life (Seckl 2001). Studies in rats have suggested that increased fetal exposure to glucocorticoids may explain higher risks of hypertension (Benediktsson et al. 1993), diabetes (Nyirenda et al. 1998) and endocrine system alterations (Barbazanges et al. 1996) in adulthood. Furthermore, the glucocorticoid receptor has several interactions with the epigenome, which may explain the aforementioned long term effects (Hunter et al. 2015).

The precise exposure window related to the changes in glucocorticoids concentration observed in our study is still unclear, i.e. whether the change in concentrations was due to maternal stress during pregnancy or stress in early infancy. Regardless of the uncertainty about the relevant exposure time, we have demonstrated that individuals exposed to higher levels of noise had higher changes in glucocorticoid concentrations, indicating traffic noise exposure as a potential stressor shortly after birth. Whether these changes lead to adverse health effects, and whether those effects persist later in life, still needs to be determined.

Strengths and limitations

One of the main strengths of our study is the standardized assessment of detailed prenatal risk factors, enabling us to control for potential confounders. Glucocorticoid measurements were all performed at the same time point (5 weeks of age), eliminating a known age-dependent impact on measurements (Dhayat et al. 2015). Since the samples were collected at different time points over the day, however, possible effects due to the diurnal rhythm of metabolites could not be ruled out.

Appendix

Our models were adjusted for air pollution which, while not highly correlated in our study, can be correlated with traffic noise due to the common source. We also used spatially detailed noise and air pollution models in assigning individual level exposures at the home address and investigated the effects of day and night noise separately. Due to the high correlation among the noise metrics (Pearson's correlation > 0.99 for all combinations), we obviously observed very similar associations regardless of the metric and, therefore, cannot assess if day or night exposure is more influential on change in glucocorticoid concentrations. The noise metrics further represent the longer-term average rather than noise specifically for pregnancy and 5-weeks after birth. This is considered adequate given that daily fluctuations in noise levels are similar day-to-day, and noise is less influenced by meteorology than air pollution. Further, it was not feasible to employ spot noise measurements for this study.

Detailed information on the floor where the residence was located was not available, reducing precise assessment of the noise exposure. However only 13.3% participants resided in buildings taller than three floors, thus our assumption of the middle building floor is reasonable for most participants. As in most studies, noise exposure was only assessed at home and we further did not have information on window opening behavior, placement of the infant's sleeping room, or amount of time spent outside the home. This last point is less relevant for the newborns included in this study since healthy babies in Switzerland are typically cared for by mothers at home after a brief hospital stay. Mothers and infants may of course enjoy some daytime hours outdoors, but we also adjusted for season of birth to account for potential differences in exposure due to outdoor activity and window opening behavior.

It may also be viewed as a limitation that glucocorticoid metabolites were assessed at one time point, and are therefore just snap-shot. This should be considered in the interpretation of our results, given the possible short-term physiologic alterations during the first year of life (Dhayat et al. 2015). Only by longitudinal assessment of noise exposure based on measurements, in line with repeated measurements of cortisol metabolites, conclusions on a sustained and

physiologically relevant effect of noise exposure upon cortisol metabolites is possible. Finally, we recognize our small sample size to the large confidence intervals and borderline associations. Thus, future research on traffic noise exposure and the release of stress hormones in a larger sample is recommended to confirm our findings.

5. Conclusions

To the extent of our knowledge, this is the first study to suggest a potential effect of traffic noise exposure on glucocorticoid metabolism in early postnatal life. Further prospective studies are needed to better understand the clinical relevance of our findings, especially in subjects exposed to high noise levels where potential adverse effects may be more relevant.

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Table 1: Demographic data for the 165 newborns.

	Summary statistics
Anthropometric data	
Gestational age at birth (weeks) [a]	39.5 ± 1.3
Weight at the time of urine collection (kg) [a]	4.3 ± 0.6
Length at the time of urine collection (cm) [a]	54.2 ± 2.0
Sex (male) [b]	88 (53)
Family history	
Maternal education (low) [b]	30 (18)
Maternal education (medium) [b]	56 (34)
Maternal education (high) [b]	79 (48)
Older siblings [b]	97 (59)
Pregnancy history	-
Caesarean section [b]	34 (21)
Vaginal infection during pregnancy [b]	42 (25)
Maternal smoking during pregnancy [b]	8 (5)
Pathological cardiotocogram [b]	18 (11)
NO_2 exposure during pregnancy ($\mu g/m^3$) [a]	17.1 ± 5.3

Notes:
[a] Result is given in mean ± standard deviation
[b] Result is given in number (%)

Table 2: Noise exposure data for the 165 newborns.

	Median	**IQR**	**Range for tertiles**		
			T1	**T2**	**T3**
LeqD (day) road noise exposure (dB(A))	52.4	45.4 – 57.9	35.0 – 46.8	46.8 – 56.2	56.2 – 69.5
LeqN (night) road noise exposure (dB(A))	45.6	38.2 – 50.7	25.0 – 39.5	39.5 – 49.0	49.0 – 62.4
Lden road noise exposure (dB)	54.4	47.0 – 59.6	35.0 – 48.4	48.4 – 57.9	57.9 – 71.2

Appendix

Table 3: Glucocorticoid metabolite concentrations (in µg/mmol creatinine) in newborns.

Metabolite [a]	**Mean ± SD**	**IQR**
Cortisol	109.5 ± 72.4	63.2 – 122.6
5α-Tetrahydrocortisol (α-THF)	55.8 ± 53.6	23.0 – 75.2
Tetrahydrocortisol (THF)	31.1 ± 63.1	12.7 – 28.2
α-Cortol	181.3 ± 178.8	64.6 – 233.6
β-Cortol	72.3 ± 109.3	33.4 – 70.4
Cortisone	352.8 ± 205.8	196.4 – 461.0
Tetrahydrocortisone (THE)	1632.0 ± 844.4	986 - 2053
α-Cortolone	227.8 ± 125.0	139.2 – 298.6
β-Cortolone	691.6 ± 395.1	413.8 – 917.4

Notes:

a. Glucocorticoid metabolite concentrations were measured for all newborns (n=165), except for β-cortol (n=163)

Figures

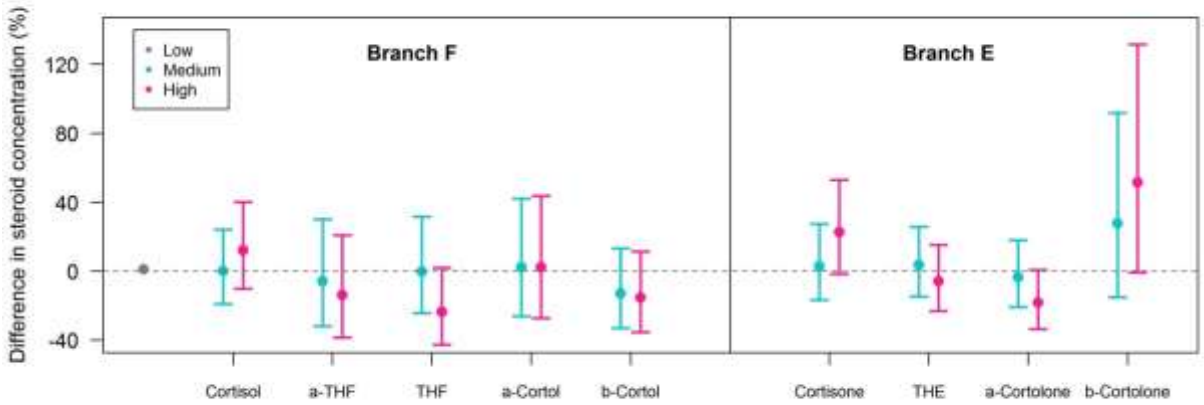

Figure 1. Association between road traffic noise (determined by Lden) and the concentration of glucocorticoid metabolites in newborn's urine. Points represent the percent change in concentration derived from full adjusted models in comparison to newborns in the low exposure (i.e. reference) category; error bars show 95% CI. Glucocorticoids metabolites can be divided into branch E, with glucocorticoid progenitor cortisone, and branch F, with glucocorticoid progenitor cortisol.

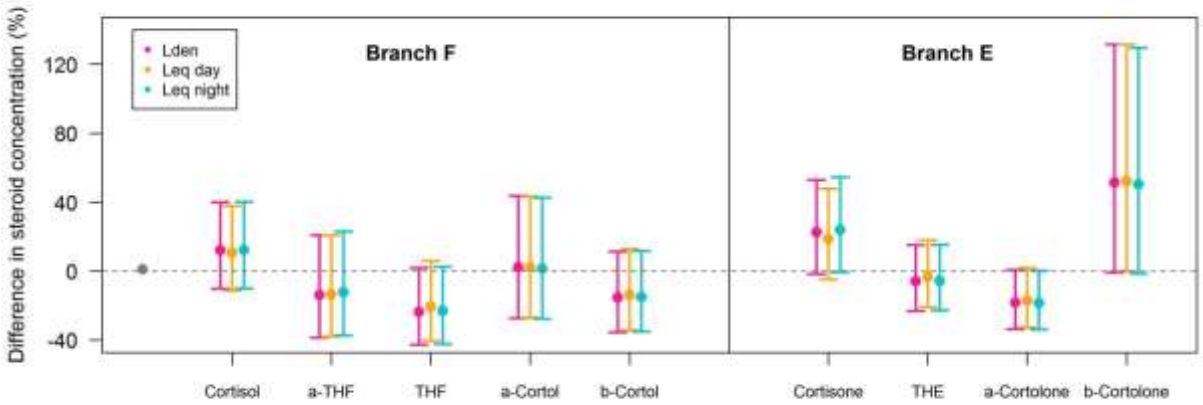

Figure 2. Comparison of the associations for high exposure categories based on different traffic noise exposure metrics (i.e. Lden, LeqD and LeqN) and the concentration of glucocorticoid metabolites in newborn's urine. Points represent the percent change in concentration derived from full adjusted models in comparison to newborns in the low exposure (i.e. reference) category; error bars show 95% CI. Glucocorticoids metabolites can be divided into branch E, with glucocorticoid progenitor cortisone, and branch F, with glucocorticoid progenitor cortisol.

Supplemental Material

Glucocorticoid metabolites in newborns: a marker for traffic noise related stress?

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Appendix

Table S1. Association between road traffic noise (determined by Lden) and concentreation of glucocorticoid metabolites [a]

Cortisol metabolite		**Crude model** Noise exposure (Lden) level [d]		**Basic model** [b] Noise exposure (Lden) level [d]		**Adjusted model** [c] Noise exposure (Lden) level [d]	
		Medium	**High**	**Medium**	**High**	**Medium**	**High**
Cortisol	%change	2.8 (-16.2, 26.1)	15.9 (-5.7, 42.5)	3.8 (-15.1, 26.9)	15.2 (-6.5, 41.9)	0.1 (-19.2, 24.0)	12.1 (-10.3, 40.1)
	p-value	0.795	0.162	0.717	0.185	0.991	0.316
5α-Tetrahydrocortisol (α-THF)	%change	-6.47 (-31.4, 27.5)	-2.7 (-28.8, 33.0)	-6.7 (-31.8, 27.5)	-4.4 (-30.8, 32.2)	-5.9 (-32.0, 30.1)	-13.8 (-38.5, 20.8)
	p-value	0.673	0.864	0.662	0.786	0.712	0.39
Tetrahydrocortisol (THF)	%change	-8.23 (-30.3, 20.8)	-16.9 (-37.1, 9.7)	-7.2 (-29.2, 21.8)	-19.8 (-39.5, 6.2)	-0.2 (-24.4, 31.7)	**-23.7 (-42.8, 1.9)**
	p-value	0.542	0.192	0.592	0.125	0.989	**0.069**
α-Cortol	%change	4.7 (-25.0, 46.1)	9.6 (-21.7, 53.3)	7.3 (-21.2, 46.1)	7.3 (-22.1, 47.7)	2.3 (-26.3, 42.0)	2.2 (-27.3, 43.7)
	p-value	0.789	0.596	0.655	0.667	0.892	0.901
β-Cortol	%change	-13.7 (-32.9, 10.9)	-5.1 (-26.3, 22.2)	-12.9 (-32.2, 11.9)	-8.0 (-29.0, 19.0)	-13.0 (-33.2, 13.2)	-15.3 (-35.5, 11.3)
	p-value	0.251	0.685	0.282	0.525	0.301	0.236
Cortisone	%change	4.6 (-14.7, 28.1)	**22.4 (-0.3, 50.2)**	5.5 (-13.8, 29.1)	**22.8 (-0.4, 51.3)**	2.8 (-16.9, 27.2)	**22.6 (-1.8, 53.0)**
	p-value	0.666	**0.055**	0.604	**0.056**	0.797	**0.074**
Tetrahydrocortisone (THE)	%change	4.1 (-13.9, 25.8)	1.9 (-15.9, 23.4)	4.8 (-13.4, 26.6)	3.0 (-15.4, 25.3)	3.5 (-14.8, 25.6)	-5.9 (-23.1, 15.2)
	p-value	0.681	0.848	0.632	0.772	0.732	0.557
α-Cortolone	%change	-1.0 (-18.4, 20.1)	-7.1 (-23.6, 13.0)	-0.9 (-18.3, 20.3)	-10.5 (-26.7, 9.4)	-3.5 (-21.0, 17.9)	**-18.3 (-33.6, 0.6)**
	p-value	0.918	0.462	0.931	0.281	0.729	**0.059**
β-Cortolone	%change	25.8 (-13.4, 82.7)	**51.5 (3.9, 120.8)**	26.3 (-13.4, 8)	**53.4 (3.9, 126.6)**	27.6 (-15.1, 91.8)	**51.5 (-0.9, 131.5)**
	p-value	0.231	**0.032**	0.227	**0.033**	0.243	**0.057**

Notes:

a. Results are shown in percent change in concentration (95%CI) in comparison with newborns in the low exposure category (i.e. reference category); p-values < 0.1 are indicated in bold print.

b. The Basic model was adjusted for sex, gestational age at birth and weight at the time of urine collection.

c. The Adjusted model was further adjusted for maternal smoking during pregnancy, delivery mode, occurrence of vaginal infection, maternal education, existence of older siblings, season at birth and NO_2 exposure during pregnancy.

d. Categorized into tertiles (i.e. <48.4: low; 48.4-57.9: medium; >57.9: high).

Table S2. Association between road traffic noise (determined by Leq during the day) and concentrations of glucocorticoid metabolites [a]

Cortisol metabolites		Crude model Noise exposure (LeqD) level [d]		Basic model [b] Noise exposure (LeqD) level [d]		Adjusted model [c] Noise exposure (LeqD) level [d]	
		Medium	**High**	**Medium**	**High**	**Medium**	**High**
Cortisol	%change	2.1 (-16.9, 25.3)	16.4 (-5.2, 42.9)	4.2 (-14.9, 27.6)	14.3 (-7.0, 40.5)	1.1 (-18.4, 25.3)	10.6 (-11.3, 37.9)
	p-value	0.844	0.148	0.692	0.206	0.92	0.374
5α-Tetrahydrocortisol (α-THF)	%change	-4.6 (-30.1, 30.3)	-4.7 (-30.2, 30.1)	-5.3 (-30.9, 29.8)	-6.1 (-31.8, 29.4)	-6.1 (-32.1, 30.0)	-13.5 (-38.1, 20.8)
	p-value	0.768	0.763	0.736	0.703	0.707	0.396
Tetrahydrocortisol (THF)	%change	-9.3 (-31.2, 19.7)	-15.9 (-36.2, 11.0)	-7.3 (-29.5, 21.9)	-19.3 (-38.9, 6.6)	-3.1 (-26.7, 28.2)	-20.6 (-40.4, 5.9)
	p-value	0.493	0.223	0.587	0.133	0.827	0.118
α-Cortol	%change	1.2 (-27.6, 41.3)	13.3 (-18.9, 58.3)	7.1 (-21.6, 46.1)	7.6 (-21.6, 47.6)	2.3 (-26.3, 42.0)	2.2 (-27.0, 43.3)
	p-value	0.948	0.465	0.668	0.652	0.894	0.898
β-Cortol	%change	-14.4 (-33.5, 10.2)	-4.6 (-25.8, 22.7)	-13.7 (-33.0, 11.0)	-7.3 (-28.2, 19.7)	-14.2 (-34.1, 11.7)	-13.9 (-34.3, 12.8)
	p-value	0.229	0.716	0.253	0.561	0.257	0.28
Cortisone	%change	5.7 (-13.9, 29.7)	**20.7 (-1.6, 48.1)**	7.5 (-12.3, 31.8)	**19.6 (-2.8, 47.2)**	5.5 (-14.8, 30.7)	18.6 (-4.9, 47.8)
	p-value	0.597	**0.073**	0.487	**0.092**	0.624	0.132
Tetrahydrocortisone (THE)	%change	1.8 (-15.9, 23.2)	4.2 (-13.9, 26.1)	3.2 (-14.7, 25.0)	4.6 (-13.9, 27.1)	1.3 (-16.6, 23.0)	-3.4 (-20.9, 18.1)
	p-value	0.854	0.672	0.744	0.648	0.898	0.738
α-Cortolone	%change	-1.4 (-18.8, 19.8)	-6.6 (-23.1, 13.4)	-1.2 (-18.7, 20.0)	-9.8 (-26.0, 10.0)	-4.3 (-21.7, 16.9)	**-17.1 (-32.6, 1.9)**
	p-value	0.89	0.49	0.901	0.311	0.666	**0.076**
β-Cortolone	%change	23.9 (-14.8, 80.3)	**53.2 (5.3, 122.8)**	25.0 (-14.5, 82.6)	**54.1 (4.8, 126.6)**	26.7 (-15.8, 90.5)	**52.2 (0.0, 131.5)**
	p-value	0.263	**0.027**	0.251	**0.029**	0.258	**0.052**

Notes:

a. Results are shown in percent change in concentration (95%CI) in comparison with newborns in the low exposure category (i.e. reference category); p-values < 0.1 are indicated in bold print.

b. The Basic model was adjusted for sex, gestational age at birth and weight at the time of urine collection.

c. The Adjusted model was further adjusted for maternal smoking during pregnancy, delivery mode, occurrence of vaginal infection, maternal education, existence of older siblings, season at birth and NO_2 exposure during pregnancy.

d. Categorized into tertiles (i.e. <46.8: low; 46.8-56.2: medium; >56.2: high).

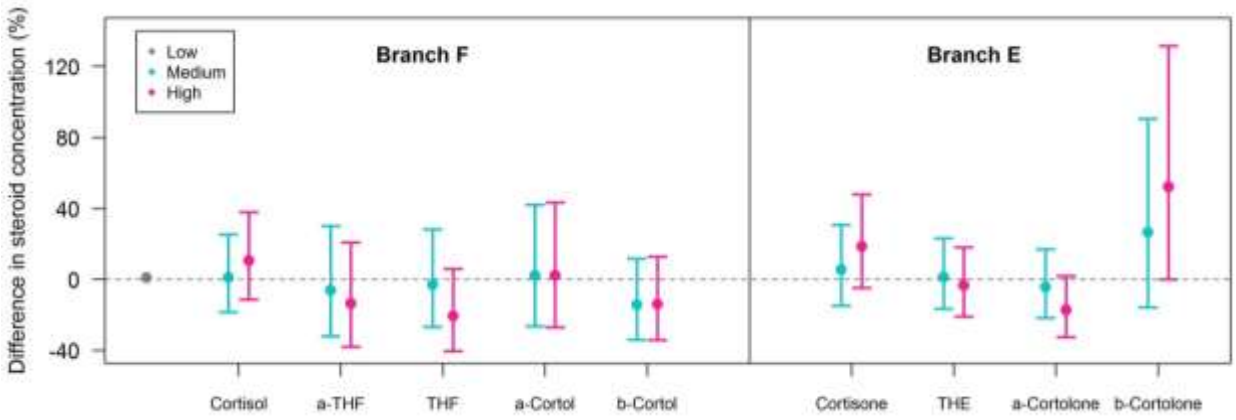

Figure S1. Association between daytime road traffic noise and the concentration of glucocorticoid metabolites in newborn's urine. Points represent the percent change in concentration derived from full adjusted models in comparison to newborns in the low exposure (i.e. reference) category; error bars show 95% CI. Glucocorticoids metabolites can be divided into branch E, with glucocorticoid progenitor cortisone, and branch F, with glucocorticoid progenitor cortisol.

Table S3. Association between road traffic noise (determined by Leq during the night) and concentrations of glucocorticoid metabolites [a]

Cortisol metabolites		**Crude model** Noise exposure (LeqN) level [d]		**Basic model [b]** Noise exposure (LeqN) level [d]		**Adjusted model [c]** Noise exposure (LeqN) level [d]	
		Medium	**High**	**Medium**	**High**	**Medium**	**High**
Cortisol	%change	3.6 (-15.5, 27.1)	16.4 (-5.2, 42.9)	4.6 (-14.4, 27.9)	15.6 (-6.0, 42.2)	0.3 (-19.1, 24.4)	12.2 (-10.1, 40.1)
	p-value	0.734	0.15	0.66	0.172	0.977	0.31
5α-Tetrahydrocortisol (α-THF)	%change	-2.0 (-28.1, 33.7)	-0.3 (-27.0, 36.1)	-2.2 (-28.5, 33.7)	-2.0 (-29.0, 35.2)	-2.8 (-29.8, 34.7)	-12.2 (-37.3, 23.0)
	p-value	0.901	0.983	0.889	0.901	0.866	0.45
Tetrahydrocortisol (THF)	%change	-6.4 (-28.9, 23.3)	-16.1 (-36.3, 10.7)	-5.2 (-27.7, 24.5)	-18.9 (-38.7, 7.3)	1.1 (-23.5, 33.6)	**-23.2 (-42.4, 2.5)**
	p-value	0.638	0.217	0.703	0.144	0.941	**0.075**
α-Cortol	%change	5.7 (-24.2, 47.5)	10.1 (-21.2, 53.8)	7.9 (-20.7 – 47.0)	7.5 (-21.8, 47.8)	1.1 (-27.3, 40.5)	1.5 (-27.8, 42.6)
	p-value	0.743	0.575	0.628	0.656	0.949	0.931
β-Cortol	%change	-12.5 (-32.0, 12.5)	-4.3 (-25.6, 23.0)	-11.4 (-31.0, 14.0)	-7.1 (-28.2, 20.1)	-12.5 (-32.9, 14.1)	-14.9 (-35.2, 11.7)
	p-value	0.298	0.732	0.349	0.575	0.325	0.248
Cortisone	%change	6.8 (-12.8, 30.9)	**23.6 (0.8, 51.6)**	7.8 (-11.9, 31.8)	**24.0 (0.7, 52.6)**	4.9 (-15.3, 29.8)	**23.8 (-0.7, 54.4)**
	p-value	0.525	**0.043**	0.468	**0.044**	0.665	**0.06**
Tetrahydrocortisone (THE)	%change	5.7 (-12.6, 27.8)	2.7 (-15.2, 24.2)	6.4 (-12.0, 28.7)	3.7 (-14.7, 26.1)	4.0 (-14.4, 26.4)	-5.7 (-22.9, 15.4)
	p-value	0.567	0.788	0.521	0.715	0.695	0.572
α-Cortolone	%change	-0.6 (-18.1, 20.6)	-6.9 (-23.3, 13.1)	-0.4 (-17.9, 20.9)	-10.2 (-26.4, 9.6)	-4.2 (-21.6, 17.2)	**-18.6 (-33.8, 0.2)**
	p-value	0.953	0.474	0.971	0.291	0.68	**0.054**
β-Cortolone	%change	25.6 (-13.5, 82.4)	**51.1 (3.8, 119.8)**	26.0 (-13.5, 83.7)	**52.9 (3.7, 125.5)**	26.2 (-16.3, 90.1)	**50.3 (-1.6, 129.5)**
	p-value	0.233	**0.033**	0.231	**0.033**	0.268	**0.061**

Notes:

a. Results are shown in percent change in concentration (95%CI) in comparison with newborns in the low exposure category (i.e. reference category); p-values < 0.1 are indicated in bold print.

b. The Basic model was adjusted for sex, gestational age at birth and weight at the time of urine collection.

c. The Adjusted model was further adjusted for maternal smoking during pregnancy, delivery mode, occurrence of vaginal infection, maternal education, existence of older siblings, season at birth and NO_2 exposure during pregnancy.

d. Categorized into tertiles (i.e. <39.5: low; 39.5-49.0: medium; >49.0: high).

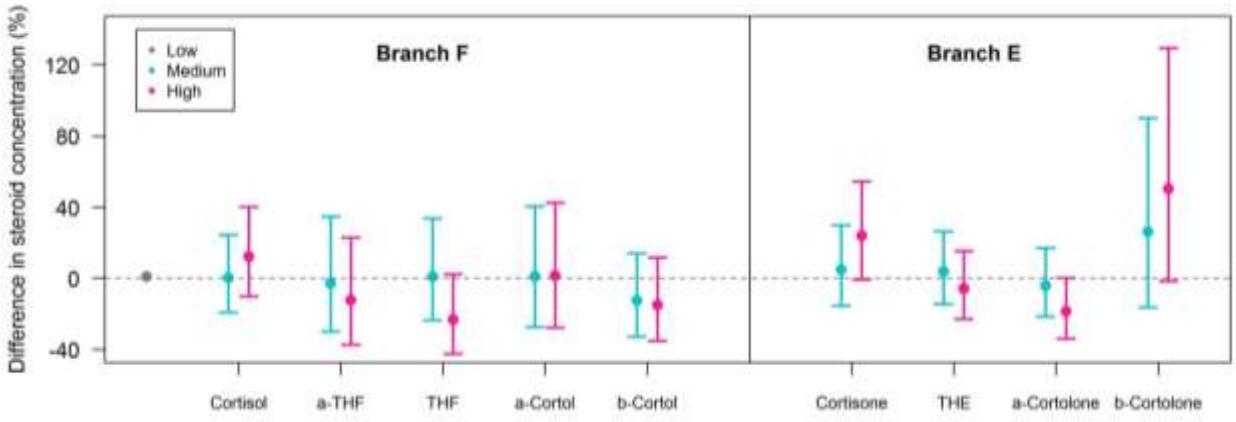

Figure S2. Association between night time road traffic noise and the concentration of glucocorticoid metabolites in newborn's urine. Error bars show 95% CI. Points represent the percent change in concentration derived from full adjusted models in comparison to newborns in the low exposure (i.e. reference) category; error bars show 95% CI. Glucocorticoids metabolites can be divided into branch E, with glucocorticoid progenitor cortisone, and branch F, with glucocorticoid progenitor cortisol.

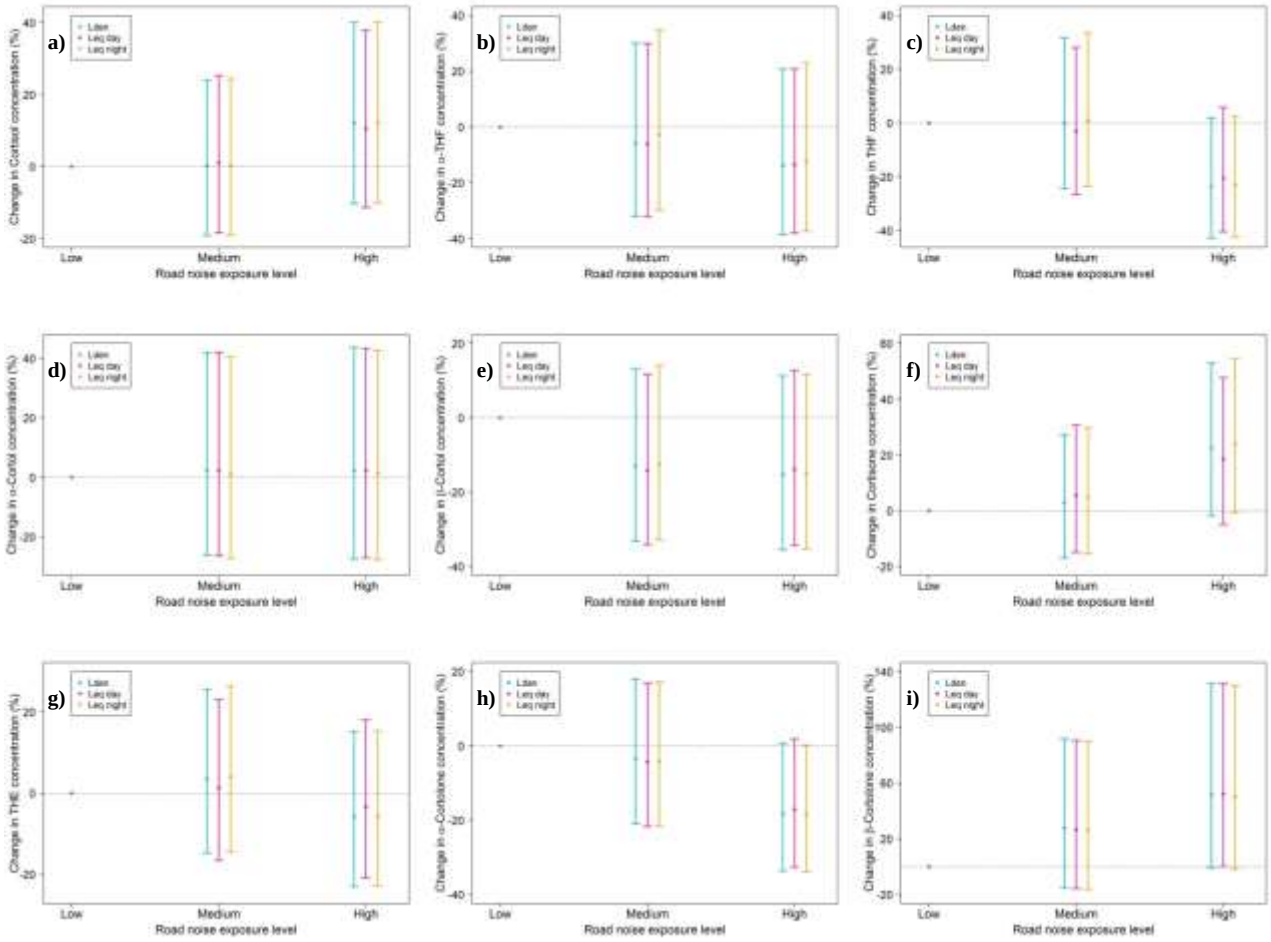

Figure S3. Comparison of the associations for the different traffic noise exposure metrics (i.e. Lden, LeqD and LeqN) and the concentration of each glucocorticoid metabolite: a) cortisol; b) 5α-tetrahydrocortisol (α-THF); c) tetrahydrocortisol (THF); d) α-cortol; e) β-cortol, f) cortisone, g) tetrahydrocortisone (THE); h) α-cortolone; i) β-cortolone. Points represent the percent change in concentration; error bars show 95% CI. Reference category: low noise exposure. Full adjusted models.

6.2 Upper airway microbiota is associated with symptom persistence after the first acute symptomatic respiratory tract infection in infants

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Manuscript ready for submission

Title: Upper airway microbiota is associated with symptom persistence after the first acute symptomatic respiratory tract infection in infants

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Author contributions: *RPN and MH contributed equally to this study. RPN, MH and UF designed the experiments. RPN, MH and UF wrote the study protocols. RPN, UF, PL, JU and IK were responsible for patient recruitment and clinical data collection. MH and MM were responsible for sample preparation and 16S-rRNA sequencing and for post-processing of data. LM was responsible for viral analyses. RN, BX and UF were responsible for statistical analyses. RPN, MH, BX and UF were involved in data interpretation and drafting of the manuscript. Critical review of the paper, all authors.

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Running title: Nasal microbiota in infants at first ARI

Character count running title: 40/50 characters.

Descriptor number: 10.11 Pediatrics: Respiratory Infections.

Word count main text: 3550/3500 words.

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject: Acute respiratory tract infections (ARI) with viral and bacterial pathogens in the first year of life have been implicated in the development of chronic respiratory disease. The impact of viral infections on the upper airway microbiota remains largely unknown.

What This Study Adds to the Field: Five infant clusters were identified based on their distinct nasal microbiota at the onset of first ARI in infancy. Three weeks after the onset of the first ARI, these clusters reduced from five to three; whereby distinct microbial composition was associated with symptom persistence. The type of respiratory virus was not predictive of symptom persistence.

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

ABSTRACT

Rationale: Infections with viral and bacterial pathogens in the first year of life have been implicated in the development of chronic respiratory disease, but the interplay between viruses, bacteria and host is complex, and underlying mechanisms remain largely unknown.

Objectives: To prospectively determine whether the nasal microbiota changes between the onset of the first symptomatic acute respiratory tract infection (ARI) in the first year of life and three weeks later, and to explore possible associations of the nasal microbiota with host, environmental and viral factors, as well as with duration of respiratory symptoms.

Methods: Nasal microbiota profiles of 167 infants in their first year of life were determined at the onset of the first ARI, and three weeks later, by 16S ribosomal ribonucleic acid PCR amplification and subsequent pyrosequencing. Infants were clustered based on their nasal microbiota using hierarchical clustering methods at both time points.

Measurements and Main Results: We identified five dominant infant clusters with distinct microbiota at the onset of ARI in symptomatic formerly infection-naïve infants; these five clusters reduced to only three clusters after three weeks. In these three clusters, symptom persistence was overrepresented in the Streptococcaceae-dominated cluster and underrepresented in the cluster dominated by “Others” ($P < 0.001$). Duration of symptoms was not associated with type of respiratory virus.

Conclusions: Infants with prolonged respiratory symptoms after their first ARI tend to exhibit distinct microbial compositions three weeks after the ARI onset, indicating close microbiota-host interactions which seem to be important for symptom persistence and recovery.

Word count abstract: 247/250 words.

Keywords: microbiota; infant; infection, respiratory tract; symptoms, respiratory.

INTRODUCTION

The increasing prevalence of wheezing disorders in infants and preschool children has become a major health issue and its complex causes remain largely unknown (1). Viral infections are well known triggers for episodic wheeze, as well as for multiple trigger wheeze, and represent a risk factor for the subsequent development of asthma (2-4). In addition to viral infections, bacterial colonization of the airways in neonates with certain species of bacteria, particularly *Moraxella catarrhalis*, *Haemophilus influenzae* and *Streptococcus pneumoniae,* has been shown to be associated with the development of childhood asthma (5, 6). It has been hypothesized that viral infections may sustainably alter epithelial function, increasing the risk for chronic airway disease (7), and that changes in microbial diversity may play a role; particularly in symptomatic infants (8-10). It has also been recognized that airway microbiota in patients with asthma exhibit distinct differences in airway microbiota compared to healthy subjects (11-13). Bacterial airway colonization seems to influence and modulate respiratory morbidity in various conditions such as asthma, cystic fibrosis and chronic obstructive pulmonary disease (14-16).

Composition of airway microbiota is highly dynamic and various environmental factors have been shown to influence colonization patterns. Antimicrobial exposure and vaccinations, as well as tobacco smoke exposure, can lead to changes of the airway microbiota (17, 18). In a prospective cohort study, Mika et al. (19) investigated the nasal microbiota longitudinally in infants during the first year of life, in the absence of acute respiratory tract infections. They showed that the composition of nasal microbiota in infants is highly dynamic and seems to be predominantly influenced by age and season. However, little is known about how and to what degree acute respiratory tract infections (ARI), which are often caused by viruses, influence the dynamically changing microbiota. In particular, this influence on the prevalence of certain commensal bacteria, shown to be associated with asthma development (5). Interactions

between viruses and bacteria are well known (20), but their relation to respiratory symptoms in the first year of life has not been studied. Recently, Korten et al. (8) demonstrated for human rhinovirus (HRV) that the microbiota primarily changes in cases of symptomatic ARI, but not in cases of asymptomatic HRV colonization. Thus, we hypothesize that the first *symptomatic* viral infection in infection-naïve infants may induce distinct changes of microbiota following all commonly observed respiratory viruses. We also hypothesize that distinct clusters of infants with specific microbial response patterns can be identified, which could be associated with severity of clinical symptom response and host risk factors, such as atopy, as suggested by Teo et al (6).

In a prospective birth cohort of infection-naïve healthy infants, we aimed 1) To determine whether clusters based on the composition and diversity of the upper respiratory tract microbiota changed between the onset of the first symptomatic ARI, caused by common respiratory viruses, and three weeks later; and 2) To investigate the association of host factors, environmental factors and respiratory viruses with infant clusters based on bacterial composition; 3) To explore the duration of respiratory symptoms in relation to microbiota or virus type, after the onset of symptoms.

METHODS

Study design

This study was performed as part of the Basel Bern Infant Lung Development (BILD) cohort study (21). The study was approved by the local ethics committees (Ethics Committee of the Canton of Bern; Ethics Committee of Basel (EKBB)). Informed written parental consent was obtained prior to enrolment in the study. Infants from this prospective birth cohort were followed by weekly standardized telephone interviews, as previously described (22). Parents

were advised to call the study team as soon as their child presented with symptoms of an acute respiratory tract infection (ARI) which was defined as more than 2 consecutive days of cough and/or wheeze accompanied by fever (>38°C), rhinitis, otitis media or pharyngitis (21). Nasal swabs were taken at the onset of ARI (swab A), and three weeks later (swab B), as described in the Supplementary methods E1. Nasal swabs were analyzed by real-time polymerase chain reaction (PCR) assays targeting five different respiratory viruses (human rhinovirus/enterovirus (HRV/EV), respiratory syncytial virus (RSV), human metapneumovirus (HMPV), human coronavirus (HCoV), human parainfluenzavirus (HPIV)), which have been shown to be the most common viruses in our population (3). Details on the methods of viral analyses are provided in an online data supplement (Supplementary methods E1). The results of viral studies of a subset of infants, also reported in this study, have been reported previously (3). Extensive data on several host factors were collected using questionnaires (14). **Microbiota analysis**

Nasal swabs were prospectively taken at the onset of the first ARI and three weeks later. Nasal swabs were further analyzed by amplification of bacterial 16S ribosomal ribonucleic acid (rRNA) by PCR and 454 Titanium FLX sequencing, as described previously (17). Details on the microbiota analysis can be found in an online supplement (Supplementary methods E1).

Estimation of relative bacterial abundances and microbiota clustering

Sequencing products were analyzed using Pyrotagger, with the definition of operational taxonomic units (OTUs) based on 97% similarity, estimation of chimeras, and taxonomic assignments (23). Bacterial abundances were calculated using Pyrotagger, as described elsewhere (17, 23). Resulting OTUs were assigned to the five most abundant families with the remaining families grouped together as “Others”. These families were used as input variables for hierarchical clustering at the onset of ARI (swab A) and three weeks later (swab B). Details on the clustering method are provided in an online supplement (Supplementary methods E1).

Calculation of α and β diversity was performed, as described previously (19); details can be found in an online data supplement (Supplementary methods E1).

Statistical analyses

Descriptive statistics were used for exploration and analysis of the association between host and environmental factors and viruses with nasal microbiota. Assuming non-normal distribution, Kruskal-Wallis and Wilcoxon signed rank sum tests were used. Fisher's exact tests, Chi-square tests and enrichment analyses (24) were employed where appropriate. A *P* value of < 0.05 was considered statistically significant. Season and age dependency (19) of the clusters was evaluated in a sensitivity analysis using logistic regression analyses. Statistical analyses were performed using Stata 11 software (StataCorp. 2009. Stata Statistical Software: Release 11. College Station, Texas, United States) and Matlab 2015b (Mathworks, Natick, Massachusetts, United States).

RESULTS

Study population

A total of 252 infants met the criteria for an ARI in the study period. Nasal swabs were available from 200 infants. After exclusion of low-quality samples, nasal swabs of 197 infants were analyzed, including 183 complete nasal swab pairs, i.e. available data from the onset of ARI (swab A) and three weeks later (swab B). Prior to final analysis, an additional 16 study participants were excluded due to antibiotic exposure within 4 weeks prior to either swabbing, resulting in 167 nasal swab pairs (Figure 1). Table 1 summarises sociodemographic and clinical characteristics of the study population.

Results of viral analysis

Real-time PCR detected viruses in swab A and swab B in 108 study participants (64.7 %). The most abundant viruses in swab A were RSV (17.8 %), followed by HRV/EV (13.8 %), HPIV (11.4 %), HMPV (6.0 %) and HCoV (5.4 %). Three weeks after the onset of ARI (swab B) the number of detected viruses was lower (except for HCoV), the most frequently detected viruses were RSV (11.4 %), followed by HRV/EV (10.8 %), HCoV (6.0 %), HPIV (3.0 %), and HMPV (3.0 %). In 59 subjects (35.3 %) none of the targeted viruses could be detected. Positive virus detection at swab B, different to the result at the onset of infection (swab A), could be found in 42 subjects (25.1 %).

Description of bacterial composition and diversity

Composition of the microbial communities consisted of five most abundant families (Moraxellaceae, Streptococcaceae, Corynebacteriaceae, Pasteurellaceae, and Staphylococcaceae); remaining families were grouped together as “Others” (Table 2). Among “Others”, the most prevalent families included Neisseriaceae, Prevotellaceae, Acidaminococcaceae and Flavobacteriaceae. Median (IQR) bacterial density was not significantly different between swabs at both time points (swab A: 41.5 (37.8) vs swab B: 32.4 (43.1) ng/µL). Moraxellaceae and Streptococcaceae represented the two most abundant bacterial families, together accounting for more than 50 % of bacterial abundance in either swab. Bacterial abundances of Corynebacteriaceae and “Others” at the onset of ARI were significantly lower than three weeks later (median (IQR) swab A: 0.1 % (0.7) vs swab B: 0.2 % (1.1), P = 0.002 and median (IQR) swab A: 6.6 % (14.7) vs swab B: 9.4 % (27.3), P = 0.004 respectively). Abundances of the remaining bacterial families (Moraxellaceae, Streptococcaceae, Pasteurellaceae and Staphylococcaceae) were not significantly different between onset of ARI and three weeks later (Table 2). Shannon Diversity Index (SDI) and

bacterial richness of all samples were lower at the onset of ARI (SDI median (IQR) swab A: 1.0 (0.8), bacterial richness median (IQR) 33 (35)) compared to three weeks later (SDI median (IQR) swab B: 1.1 (1.2), bacterial richness median (IQR) 38 (36); *P* = 0.013 and 0.052 respectively) (Table 2).

Infant cluster analysis based on bacterial families

Five main clusters could be identified in swab A (n = 165), whereas only three main clusters could be identified in swab B (n = 155) (Figure 2). The five most prevalent clusters at swab A were cluster A1 (Moraxellaceae-dominated, 33.9 %), cluster A2 (Moraxellaceae & Streptococcaeae-dominated, 30.9 %), cluster A3 (Streptococcaceae-dominated, 18.8 %), cluster A4 ("Others"-dominated, 8.5 %) and cluster A5 (Pasteurellaceae-dominated, 7.9 %). The three clusters based on bacterial abundances in swab B were cluster B1 (Moraxellaceae-dominated, 61.9 %, cluster B2 ("Others"-dominated, 21.9 %) and cluster B3 (Streptococcaceae-dominated, 16.1 %) (Figure 2). The most abundant cluster in both swabs was dominated by Moraxellaceae, which showed the lowest bacterial diversity (SDI median (IQR) A1: 0.67 (0.63); B1: 0.88 (0.76)). The number of infants in this cluster increased in size between the onset of ARI and three weeks later from 33.9 % to 61.9 % (Figure 3). Cluster A4 ("Others"-dominated) at the onset of ARI resembles in its composition cluster B2 three weeks later and more than doubles in size (from 8.5 % to 21.9 %) (Figures 2 and 3). In infants of these two clusters, the bacterial diversity was highest (SDI median (IQR) A4: 2.54 (1.52); B2: (2.40 (0.56)). Differences in the SDI and Jaccard dissimilarity indices between infant clusters were significant (P < 0.001) (Supplementary table E1).

Influence of host and environmental factors on infant clusters

Infant clusters were tested for differences based on the following factors: age, season at first ARI, sex, cohabitating siblings, cesarean section, atopy, childcare attendance, breastfeeding

status and environmental tobacco smoke exposure. Statistically significant differences between clusters at the onset of infection could be seen in infants with atopy risk, with higher proportions in cluster A2 and A5 (76.5 % and 84.6 % respectively) compared to cluster A3 (38.7 %) (*P* = 0.008) (Supplementary table E2). In swab B, childcare attendance was significantly different between clusters with an overrepresentation of childcare attendance in the Moraxellaceae-dominated cluster B1 (*P* = 0.030) (Supplementary table E3). For all the other aforementioned host and environmental factors no significant differences could be observed (Supplementary tables E2 and E3).

Respiratory viruses and infant clusters

At the onset of infection (swab A), HMPV occurrence was significantly different between clusters, with the highest detection rate in the Streptococcaceae-dominated cluster A3 (16.1 %) (*P* = 0.030). For all other tested respiratory viruses (HRV/EV, RSV, HCoV, HPIV) no significant difference in occurrence between clusters could be found (Supplementary table E4). Three weeks after the onset of infection (swab B), presence of HRV/EV, as well as of HCoV, was significantly different among clusters (*P* = 0.030 and *P* = 0.007 respectively). HRV/EV was overrepresented in the Moraxellaceae-dominated cluster B1 (14.6 %) vs B2 (2.9 %) and B3 (0.0 %). HCoV was highest in cluster B3 (20.0 %) vs B1 (4.2 %) and B2 (0.0 %). HRV and HCoV were both underrepresented in the “Others”-dominated cluster B2 (*P* = 0.020 and *P* < 0.001 respectively) (Supplementary table E5).

Occurrence of any new virus in swab B compared to viral detection in swab A was significantly different among clusters (*P* = 0.042), with an overrepresentation in cluster B1 (29.2 %) and an underrepresentation in cluster B2 (8.8 %). New detection of HRV/EV and HCoV in swab B, compared to the onset of infection, was also significantly different among clusters (*P* = 0.017 and *P* = 0.021 respectively) (Supplementary table E5).

Duration of respiratory symptoms and infant clusters

Weekly recorded symptom persistence after the onset of infection showed a mean (SD, range) duration of 2.3 (1.5, 1-11) weeks. Presence of symptoms two weeks after the onset of ARI showed a borderline significant difference between clusters determined at swab A ($P = 0.061$): symptomatic infants were more common in cluster A2 (37.3 %, $P = 0.025$) and underrepresented in clusters A3 (12.9 %, $P < 0.01$) and A4 (7.1 %, $P < 0.01$) (Table 3).

Presence of symptoms three weeks after the onset of ARI was different between clusters in swab B ($P < 0.001$). There were less symptomatic infants in cluster B2 (5.9 %, odds ratio (95 % CI) 0.2 (0.0-0.7)) and more symptomatic infants in clusters B1 (29.2 %) and B3 (56.0 %, odds ratio (95 % CI) 3.3 (1.3-8.0)) (Figure 3, Table 4, Supplementary table E6). Adjusting for age and season did not change these associations (Supplementary table E7). Interestingly, SDI in swab B was much higher in cluster B2 (SDI median (IQR): 2.40 (0.56)) compared to B1 (0.88 (0.76)) and B3 (1.06 (0.69)) ($P < 0.001$) (Supplementary table E1).

Overall, mainly in clusters with lower diversity and overrepresentation of Moraxellaceae or Streptococcaceae (A1, B1, B3), the proportion of persistently symptomatic patients was higher, whereas infants in clusters characterized by high diversity and overrepresentation of “Others” (A4, B2), showed more rapid symptom recovery following ARI. (Figure 3, Table 4).

Duration of respiratory symptoms and respiratory viruses

Enrichment analysis revealed an overrepresentation of HCoV in cluster B3, in which the presence of symptoms at swab B, as well as being continuously symptomatic for ≥ three weeks, is overrepresented (Supplementary table E5). Despite this finding, type of virus at swab A and swab B was not associated with duration of respiratory symptoms (Supplementary tables E8 - E10).

DISCUSSION

Using an unsupervised approach in a representative prospective central European white healthy birth cohort (21), we could show that the nasal microbiota of infection-naïve infants exhibited five distinct major microbial patterns at the onset of the first acute respiratory tract infection, largely independent of the type of common respiratory virus. Three weeks after the onset of the ARI, these five distinct clusters became three dominant clusters. The duration of respiratory symptoms was associated with clusters based on microbiota three weeks after the onset of infection, but not with commonly observed respiratory viruses, indicating close microbiota-host interactions.

Bacterial composition of nasal swabs in our study consisted primarily of five families (Moraxellaceae, Streptococcaceae, Corynebacteriaceae, Pasteurellaceae, and Staphylococcaceae) which is comparable to previous studies on microbiota in infants and young children (6, 8, 17, 19, 25).

With the exception of childcare and atopy, we did not observe any other host or environmental effects on microbiota cluster composition. Childcare attendance was higher in infants of the Moraxellaceae-dominated cluster B1. The association of increased abundance of *Moraxella* in infants attending childcare has been similarly observed by Teo et al. (6).

At the onset of infection (swab A), higher rates of HMPV in the Streptococcaceae-dominated cluster A3 could be found. A similar observation has been described previously by Verkaik et al. Frequent nasopharyngeal carriage of *Streptococcus pneumoniae* in children seemed to increase susceptibility to HMPV (26). Three weeks after the onset of infection there was a higher proportion of HCoV detection in the Streptococcaceae-dominated cluster B3, compared to the other clusters. However, the rate of HCoV detection was too low to allow meaningful statistical analyses. In the literature, no specific effect of HCoV on Streptococcaceae could be found.

HRV/EV detection was higher in the Moraxellaceae-dominated cluster B1. This is consistent with published observations. Korten et al. reported an increase of Moraxellaceae in infants with symptomatic HRV/EV infections (8). Kloepfer et al. found similarly increased detection rates of *Moraxella catarrhalis* and *Streptococcus pneumoniae* associated with HRV/EV (27).

We observed less symptomatic infants in the ”Others”-dominated cluster B2 three weeks after the onset of infection. Bacterial abundances of Staphylococcaceae and Corynebacteriaceae were significantly higher in cluster B2 than in clusters B1 or B3 (Figure 2). Similarly, Teo et al. recognized *Staphylococcus* and *Corynebacterium* as commensals, which seem to be associated with respiratory health (19). Three weeks after the onset of ARI, we found most symptomatic infants in the Streptococcaceae-dominated cluster B3. Similarly, Kloepfer et al. identified detection of *Streptococcus pneumoniae* as a relevant co-factor associated with increased respiratory symptoms with concurrent HRV infection (27).

In our study, duration of symptoms was not associated with type of virus which is consistent with observations by Carlsson et al (28). He described the duration of wheezy episodes in high-risk infants and young children as independent of the microbial trigger. However, numbers of detected viruses in our study might have been too low to detect associations between virus-type and symptom duration.

Strength of the study is the relatively high number of studied infants from a prospective birth cohort with comprehensive information on antenatal and postnatal history, including weekly telephone interviews (21). However, due to the high variability of the microbiota, it is possible that significant differences in the bacterial composition associated with certain viruses and host factors could only become obvious with an even higher number of infants. A limitation of our study is that the age at onset of ARI naturally varied, since the focus of this prospective study was to catch the very first symptomatic ARI in infants. Our study design precludes information

on the nasal microbiota in an asymptomatic period immediately prior to the first ARI. Additional information on this could help recognize changes in the bacterial composition which might have preceded symptoms of the ARI, and could have contributed to an understanding of the cause-effect relationships between microbiota, viruses and respiratory symptoms. We analyzed only five different viruses in our samples and, therefore, might have missed the presence of other viruses. However, we deliberately focused on the five targeted viruses that have previously been shown to be the most common viruses in our population (3).

Respiratory tract microbiota seems to affect susceptibility to viral infections, which has been shown in animal, in-vitro and clinical studies (as reviewed by (20, 29)). Viral-bacterial interactions are complex and certain combinations of viruses and bacteria can exert either synergistic or inhibitory effects. It seems that certain bacteria can lead to an enhanced or reduced inflammatory response, and thereby modulate the burden of respiratory symptoms (10). The clusters three weeks after the onset of infection that were associated with increased symptoms were dominated by Moraxellaceae and Streptococcaceae, two potentially pathogenic bacterial families, which might result in a sustained ARI with symptoms. In a recent study, young children showed an exaggerated inflammatory response to RSV in the presence of nasopharyngeal microbiota clusters enriched with *Haemophilus influenzae* and *Streptococcus pneumoniae* (10). In contrast, cluster B2, which exhibited fewer symptoms, contained higher abundances of Corynebacteriaceae and Staphylococcaceae. These organisms have similarly been recognized by Teo et al. to be associated with respiratory health (6). Nasopharyngeal *Staphylococcus aureus* was found to be associated with lower disease severity in RSV infections (10). Biesbroek et al. reported less ARI in infants with nasopharyngeal microbiota dominated by *Corynebacterium* and *Dolosigranulum* (25). Whether these organisms are simply biomarkers indicating respiratory health, or whether they have immediate protective effects on the

respiratory tract, remains unclear. Cluster B2 identified three weeks after the onset of ARI was also characterized by higher bacterial diversity compared to the other clusters. Lower diversity in infants with longer persistence of symptoms could reflect an outgrowth of certain potentially pathogenic bacterial families e.g. Moraxellaceae, or potentially a lack of beneficial microbiota. It has been shown that the production of pro-inflammatory cytokines by dendritic cells is higher after stimulation by *Moraxella catarrhalis* and *Haemophilus influenzae* compared to *Prevotella* strains, common commensals of the respiratory tract microbiota. Co-stimulation with *Prevotella* reduces the *Haemophilus influenzae*-induced production of pro-inflammatory cytokines (30). This could indicate that commensals, such as *Prevotella* and others, are not only filling a niche, but are also functionally important for the maintenance of "healthy" equilibrium.

Overall, our data show that high diversity of upper respiratory tract microbiota at the very first ARI and three weeks later seems to be beneficial for health. Treatment with antibiotics seems attractive, but data on antibiotic effects in the manipulation of airway microbiota in infants and children is scarce. A prerequisite would be to know the "ideal" microbial composition associated with respiratory health and the highest resilience in order to optimize antimicrobial treatment. Immunomodulatory substances, such as OM-85, a bacterial lysate, could be another potential modifier of the airway microbiota (31).

In conclusion, nasal microbiota at the onset of the first ARI in infants is different compared to three weeks later. The fact that infants with prolonged respiratory symptoms after ARI, compared to infants with quicker recovery, tend to exhibit distinct microbial compositions indicates the presence of close microbiota-host interactions. Future studies will need to reveal how the microbial composition influences the dynamic equilibrium of viruses and bacteria, and how this is related to inflammation, mucosal function, immune development and subsequent chronic lung disease.

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Appendix

Table 1. Baseline characteristics of study population

Variable		
Total participants, n (% male)		167 (57)
Gestational age, weeks; mean (SD*)		39.3 (2.0)
Birth weight, kg; mean (SD)		3.3 (0.55)
Season at birth	Spring	62 (37)
	Summer	44 (26)
	Autumn	32 (19)
	Winter	29 (17)
Age at ARI†, weeks; mean (SD; range)		29.9 (12.5; 2-52)
Season at ARI	Spring	40 (24)
	Summer	14 (8)
	Autumn	38 (23)
	Winter	75 (45)
Atopy		109 (65)
Childcare attendance		32 (19)
Breastfeeding at time of ARI		99 (60)
Environmental tobacco smoke exposure		24 (14)
Other siblings (living in the same household)		106 (63)
One sibling		75 (45)
≥ two siblings		31 (19)

Data are presented as n (%) unless otherwise noted. *SD, standard deviation; †ARI, acute respiratory tract infection. ‡Swab A taken at the onset of ARI, swab B taken three weeks later.

Table 2. Abundances of bacterial families and Shannon Diversity Indices (SDI)

Bacterial family	**At onset of ARI*** **(swab A)**	**Three weeks after onset of ARI*** **(swab B)**
Moraxellaceae	52.4 (66.5)	49.5 (66.2)
Streptococcaceae	19.2 (41.03)	16.31 (33.9)
Corynebacteriaceae	0.1 (0.7)	0.2 (1.1)[†]
Pasteurellaceae	0.4 (1.9)	0.3 (1.5)
Staphylococcaceae	0.1 (0.6)	0.1 (0.6)
"Others"	6.6 (14.7)	9.4 (27.3)[†]
SDI, median (IQR)	1.0 (0.8)	1.1 (1.2)[‡]
Richness, median (IQR)	33 (35)	38 (36)[‡]

*ARI, acute respiratory tract infection. [†]Abundance of Corynebacteriaceae and "Others" significantly different between swab A and swab B (P = 0.002 and 0.004 respectively, Wilcoxon signed rank sum test); [‡]Shannon Diversity Indices (SDI) and bacterial richness are lower at time point A compared to time point B (P = 0.013 and 0.052 respectively, Wilcoxon signed rank sum test). Data are presented as median % (IQR) for bacterial families and as median (IQR) for SDI and bacterial richness.

Table 3. Infant clusters at the onset of ARI* (swab A) and presence of symptoms

Cluster	**A1**	**A2**	**A3**	**A4**	**A5**	***P* value**
(n= 165)	**(n= 56)**	**(n = 51)**	**(n= 31)**	**(n = 14)**	**(n = 13)**	
Symptomatic after 1 week	38 (67.8)	**43 (84.3)** ↑	19 (61.3)	8 (53.3)	9 (69.2)	0.121[†]
Symptomatic after 2 weeks	17 (30.4)	**19 (37.3)** ↑	***4 (12.9)*** ↓	***1 (7.1)*** ↓	4 (30.8)	0.061[‡]
Symptomatic after 3 weeks	19 (33.9)	12 (23.5)	8 (25.8)	4 (28.6)	5 (38.5)	0.754[‡]
Continuously symptomatic for ≥ 3weeks	13 (23.3)	9 (17.6)	***2 (6.5)*** ↓	1 (7.1)	3 (23.1)	0.329[‡]

*ARI, acute respiratory tract infection. Data are presented as n (%). Statistical analyses were performed using a Chi-square[†] or Fisher's exact test[‡]. Significant overrepresentation (bold letters, ↑) and significant underrepresentation (bold/italic letters, ↓) of presence of symptoms in the clusters was assessed by enrichment analysis.

Table 4. Infant clusters three weeks after onset of ARI* (swab B) and presence of symptoms

Cluster	**B1**	**B2**	**B3**	***P* value**
(n = 155)	**(n = 96)**	**(n = 34)**	**(n = 25)**	
Symptomatic after 1 week	70 (72.9)	22 (64.7)	16 (64.0)	0.135†
Symptomatic after 2 weeks	28 (30.2)	***5 (14.7)*** ↓	9 (36.0)	0.454†
Symptomatic after 3 weeks	27 (29.2)	***2 (5.9)*** ↓	**14 (56.0)** ↑	< 0.001‡
Continuously symptomatic for ≥ 3weeks	16 (16.7)	***1 (2.9)*** ↓	**9 (36.0)** ↑	0.010‡

*ARI, acute respiratory tract infection. Data are presented as n (%). Statistical analyses were performed using a Chi-square† or Fisher's exact test‡. Significant overrepresentation (bold letters, ↑) and significant underrepresentation (bold/italic letters, ↓) of presence of symptoms in the clusters was assessed by enrichment analysis.

Appendix

FIGURES

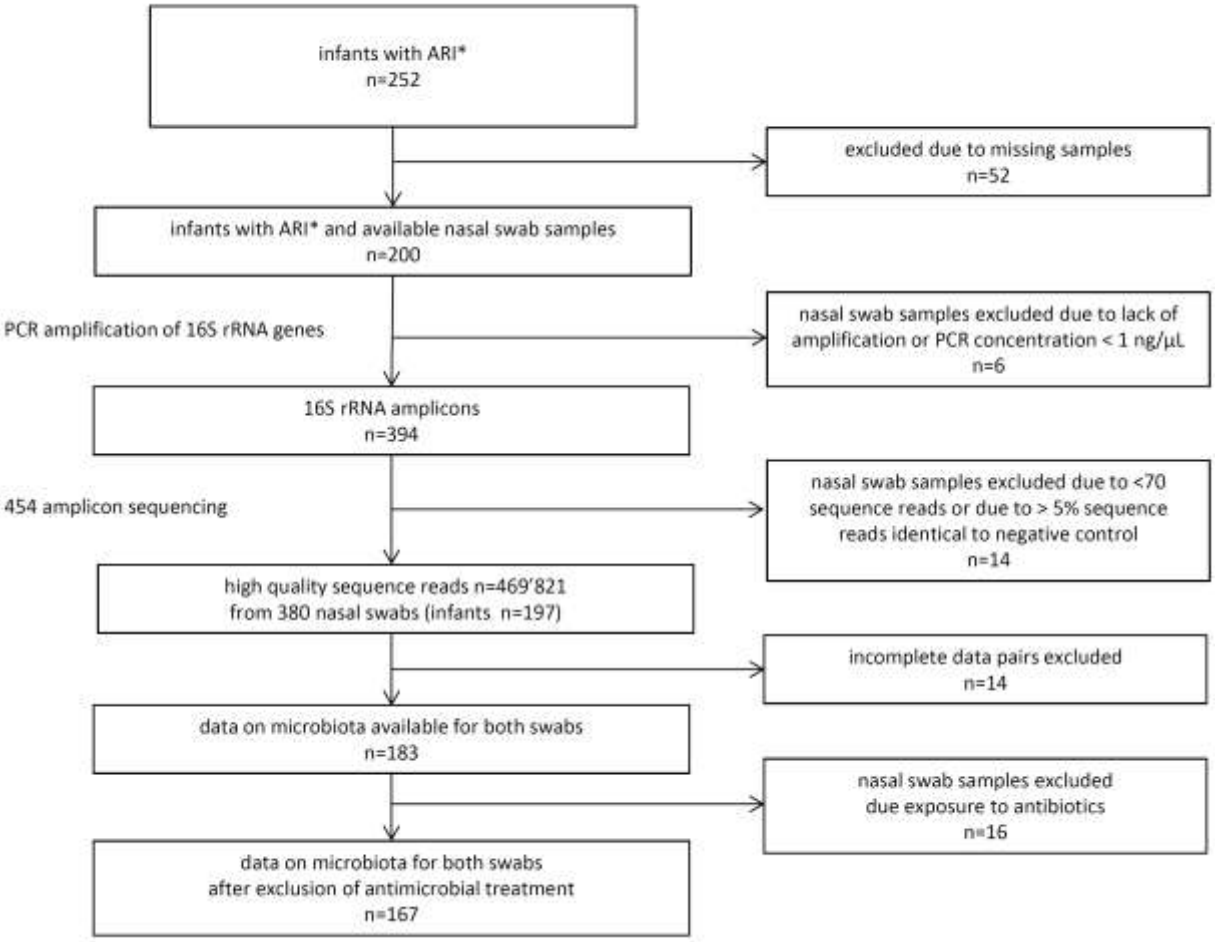

Figure 1. Flow chart of the study population

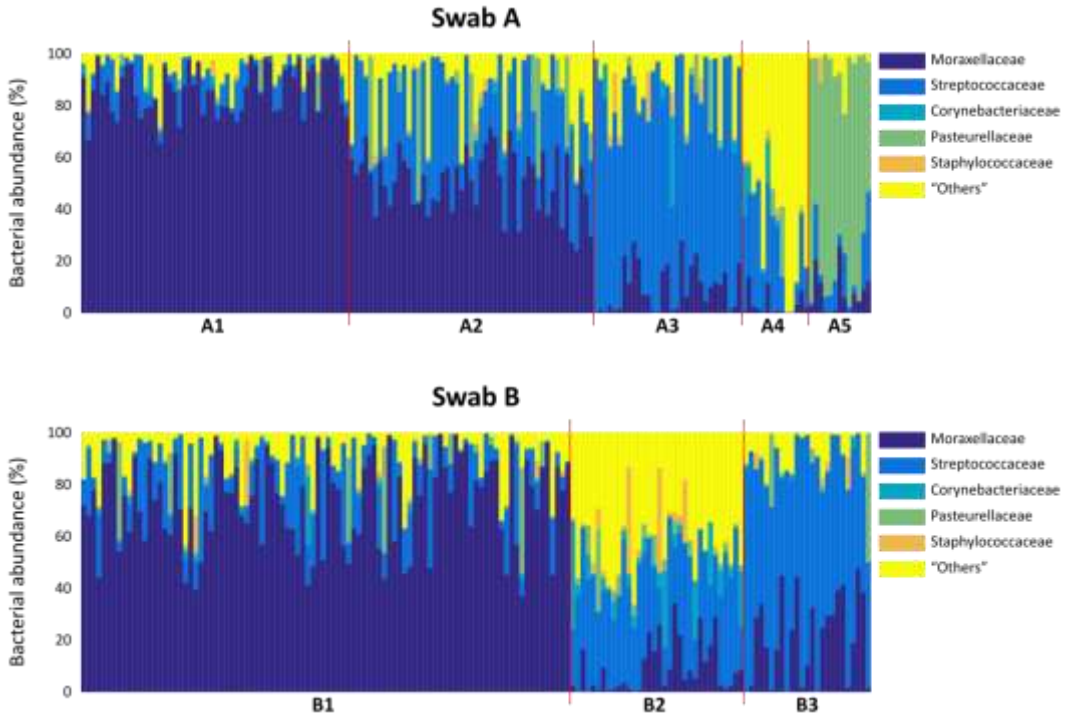

Figure 2. Composition of the most common clusters at the onset of the first ARI (swab A) and three weeks later (swab B) illustrated as bacterial abundances of the 5 most common bacterial families with the remaining grouped as “others”. Clusters with sizes of < 5 % are not depicted.

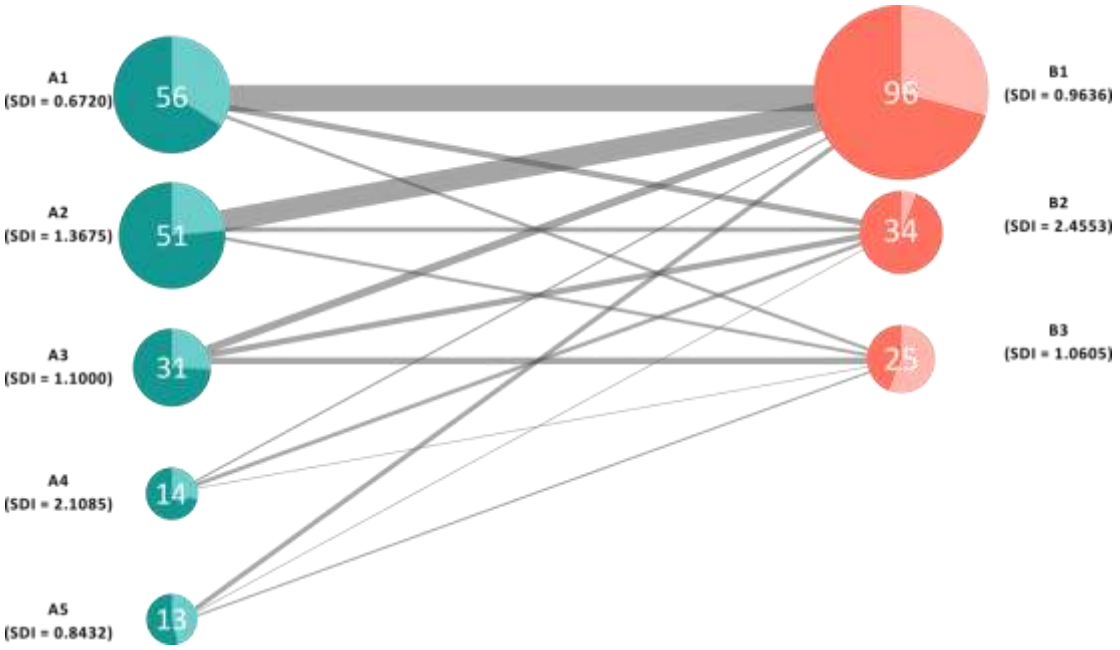

Figure 3. Results of hierarchical clustering based on the microbial composition at the onset of the first ARI (swab A) and three weeks later (swab B). The size of the circles and the numbers inside indicate the number of individuals assigned to each cluster. The connecting bars indicate the transition from one particular cluster to another between swabs A and B; the size of the bars corresponds to the number of transitioning individuals.

Areas in the circles of darker green on the left side of the panel indicate the proportion of infants at swab A in each cluster who were asymptomatic at swab B compared to the proportion of symptomatic infants at swab B (lighter green).

Areas in the circles of darker orange on the right side of the panel indicate the proportion of asymptomatic infants at swab B compared to the proportion of symptomatic infants (lighter orange). SDI, Shannon Diversity Index.

Supplementary methods E1

Viral analysis

Nasal swabs were analyzed by real-time polymerase chain reaction (PCR) assays targeting five different respiratory viruses (human rhinovirus/enterovirus (HRV/EV), respiratory syncytial virus (RSV), human metapneumovirus (HMPV), human coronavirus (HCoV), human parainfluenzavirus (HPIV)), which have been shown previously to be the most common viruses in our population (1). One part of the samples has been analysed previously as described by Regamey et al. (1). The other part was studied using commercially available real-time PCR kits (HCoV/HPIV r-gene®, Rhino/EV/Cc r-gene®, RSV/hMPV r-gene®, Argene, bioMérieux, Marcy-l'Etoile, France); the analysis was performed according to the manufacturer's recommendation.

Definition of acute respiratory tract infection and symptom score

An acute respiratory tract infection (ARI) was defined as more than two consecutive days of cough and/or wheeze accompanied with fever (>38°C), rhinitis, otitis media or pharyngitis (1). Wheeze was defined as a squeaky noise or whistling from the chest audible to parents. Respiratory symptoms were assessed using a standardized scoring system (2). Throughout the first year of life i.e. also during the time of the ARI any use of antibiotics was recorded (3).

Nasal swab collection

Nasal swabs were collected at the onset of the first ARI (swab A) and three weeks later (swab B) at the homes of the infants by a research nurse using Neonatal Flocked Swabs (UTM, Copan, Italy) and transport tubes (UTM, Copan, Italy). The samples were immediately snap-frozen and then stored at minus 80°C in phosphate-buffered saline containing 40 U/mL RNase-inhibitor (Roche, Basel, Switzerland).

Definition of host factors

A detailed pre- and postnatal questionnaire allowed collecting extensive data on several host factors which were of interest in our study. Atopy was defined as positive if the infants' mother, father or siblings were suffering from eczema, allergic rhinitis or asthma. Siblings were counted when living in the same household as the study participant. Environmental tobacco smoke (ETS) exposure was defined as positive if there was at least one parent smoking tobacco in the same household. Data on nutrition including breastfeeding were collected throughout the first year of life in weekly telephone interviews.

Microbiota analysis

Bacterial 16S ribosomal ribonucleic acid PCR amplification and 454 Titanium FLX sequencing

Amplification of bacterial 16S ribosomal ribonucleic acid (rRNA) by PCR and 454 Titanium FLX sequencing has been described previously (4, 5). In summary, after DNA extraction the variable regions V3 to V5 of bacterial 16S rRNA were amplified by using the multiplex identifier tagged primer pair 341F/907R. After elution of the PCR reactions by 40 µL of double-distilled water, the concentration was measured with the Agilent 2100 Bioanalyzer (Agilent Technologies, Basel, Switzerland). Amplification products with a concentration of less than 1.0 ng/ µL which corresponds to less than 1 pg/ µL bacterial DNA were excluded from this study according to recent recommendations (6). Samples were pooled using 40 ng/ µL of purified PCR product,

resulting in 8 amplicon pools labelled with unique multiplex identifiers. The reads from 454 Titanium amplicon sequencing were submitted to the National Center for Biotechnology Information Sequence Read Archive. Bacterial density was described as the concentration of PCR product within a sample.

Calculation of α and β diversity

Alpha diversity analysis was performed using the "diversity" function of the "vegan" software package of R, version 3.02 (http://www.R-project.org) calculating the Shannon Diversity Index (SDI) to describe within-community diversity. For community comparisons between swab A and swab B the β diversity was calculated using the "vegdist" function of the "vegan" software package of R. We chose the Manhattan-type Jaccard dissimilarity index as weighted (abundance-based) β diversity.

Clustering of microbiota

The hierarchical clustering was performed using Matlab 2015b (Mathworks, Natick Massachusetts, United States). The five most frequent bacterial families with the remaining families grouped together as "Others" were used as input variables for clustering of both swabs. The optimal number of clusters was determined by Silhouette index (7) and KL index (8) for both swabs. Both indices suggested 6 clusters for swab A. Since the sixth cluster was too small for phenotyping purposes (n = 2 infants), it was thus excluded from the study, resulting in five clusters, including 165 infants. For swab B, KL index suggested five clusters and Silhouette index suggested seven clusters. Also taking into account the clusters' sizes, the optimal cluster number for swab B was six. However, as the last three clusters were too small for phenotyping purposes (n ≤ 6 infants), they were not considered for the current study, resulting in three clusters, including 155 infants. The clustering was also validated with the K-means clustering method, which resulted in similar cluster numbers and classes for both swabs.

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Supplementary table E1. Shannon Diversity Indices (SDI) and Jaccard dissimilarity indices in infant clusters

Swab A. At the onset of ARI*

	Cluster A1	Cluster A2	Cluster A3	Cluster A4	Cluster A5	*P* value[§]
Index						
SDI[†]	0.67 (0.63)	1.18 (0.68)	1.02 (0.94)	2.54 (1.52)	0.87 (0.76)	< 0.001
Jaccard[‡]	0.35 (0.43)	0.56 (0.35)	0.73 (0.37)	0.83 (0.28)	0.81 (0.26)	< 0.001

Swab B. Three weeks after the onset of ARI*

	Cluster B1	Cluster B2	Cluster B3	*P* value[§]
Index				
SDI[†]	0.88 (0.76)	2.40 (0.56)	1.06 (0.69)	< 0.001
Jaccard[‡]	0.46 (0.34)	0.74 (0.21)	0.52 (0.49)	< 0.001

*ARI, acute respiratory infection. [†]SDI, Shannon diversity index. [‡]Jaccard dissimilarity index. [§]Kruskal-Wallis test. Data are presented as median, (IQR).

Supplementary table E2. Host and environmental characteristics in infant clusters present at the onset of ARI* (swab A)

Cluster	**A1**	**A2**	**A3**	**A4**	**A5**	***P* value**
(n = 165)	**(n = 56)**	**(n = 51)**	**(n = 31)**	**(n = 14)**	**(n = 13)**	
Age, months	7.2 [3.8-8.8]	7.4 [5.3-8.8]	7.3 [3.7-9.0]	7.2 [4.8-9.4]	5.7 [4.3-8.2]	0.920
Seasons						
Spring	13 (23.2)	12 (23.5)	7 (22.6)	3 (21.4)	5 (38.5)	0.807†
Summer	4 (7.1)	**2 (3.9)** ↓	**5 (16.1)** ↑	1 (7.1)	1 (7.7)	0.364†
Autumn	12 (21.4)	11 (21.6)	8 (25.8)	2 (14.3)	**5 (38.5)** ↑	0.650†
Winter	27 (48.2)	26 (51.0)	11 (35.5)	8 (57.1)	**2 (15.4)** ↓	0.105†
Sex, male	33 (58.9)	26 (51.0)	18 (58.1)	**11 (78.6)** ↑	7 (53.9)	0.609‡
Siblings	35 (62.5)	31 (60.8)	20 (64.5)	8 (57.1)	10 (76.9)	0.761‡
Cesarean section	6 (10.7)	8 (15.7)	6 (19.4)	3 (21.4)	2 (15.4)	0.835†
Atopy	36 (64.3)	**39 (76.5)** ↑	***12 (38.7)*** ↓	9 (64.3)	**11 (84.6)** ↑	0.008‡
Childcare	13 (23.2)	9 (17.7)	7 (22.6)	3 (21.4)	***0 (0.0)*** ↓	0.479†
Breastfeeding	31 (55.4)	33 (64.7)	17 (54.8)	8 (57.1)	10 (76.9)	0.768‡
ETS§	6 (10.7)	5 (9.8)	6 (19.4)	**4 (28.6)** ↑	3 (23.1)	0.355†

*ARI, acute respiratory tract infection. Data are presented as median [interquartile range] or n (%). Statistical analyses were performed using a Kruskal Wallis test for age and Fisher's exact test† or Chi-square test‡ for categorical variables. Significant overrepresentation (bold letters, ↑) and significant underrepresentation (bold/italic letters, ↓) of presence of symptoms in the clusters was assessed by enrichment analysis. §ETS, environmental tobacco smoke exposure.

Supplementary table E3. Host and environmental characteristics in infant clusters three weeks after the onset of ARI* (swab B)

Cluster (n = 155)	**B1 (n = 96)**	**B2 (n = 34)**	**B3 (n = 25)**	***P* value**
Age, months	7.1 [4.9-8.7]	8.0 [4.9-9.3]	6.0 [3.8-8.5]	0.142
Seasons				
Spring	24 (25.0)	7 (20.6)	5 (20.0)	0.892†
Summer	9 (9.4)	4 (11.8)	**0 (0.0)** ↓	0.216†
Autumn	22 (22.9)	6 (17.6)	6 (24.0)	0.852†
Winter	41 (42.7)	17 (50.0)	14 (56.0)	0.438†
Sex, male	54 (56.3)	17 (50.0)	**18 (72.0)** ↑	0.471‡
Siblings	65 (67.7)	15 (44.1)	18 (72.0)	0.095‡
Cesarean section	12 (12.5)	**9 (26.5)** ↑	***1 (4.0)*** ↓	0.085†
Atopy	67 (69.8)	20 (58.8)	15 (60.0)	0.699‡
Childcare	**24 (25.0)** ↑	***3 (8.8)*** ↓	***2 (8.0)*** ↓	0.030†
Breastfeeding	55 (57.3)	19 (55.9)	**20 (80.0)** ↑	0.199‡
ETS§	12 (12.5)	6 (17.7)	5 (20.0)	0.814†

*ARI, acute respiratory tract infection. Data are presented as median [interquartile range] or n (%). Statistical analyses were performed using a Kruskal Wallis test for age and Fisher's exact test† or Chi-square test‡ for categorical variables. Significant overrepresentation (bold letters, ↑) and significant underrepresentation (bold/italic letters, ↓) of presence of symptoms in the clusters was assessed by enrichment analysis. §ETS, environmental tobacco smoke exposure.

Supplementary table E4. Infant clusters and detected viruses at the onset of ARI* (swab A)

Cluster	**A1**	**A2**	**A3**	**A4**	**A5**	***P* value**
(n = 165)	**(n = 56)**	**(n = 51)**	**(n = 31)**	**(n = 14)**	**(n = 13)**	
Any virus	26 (46.4)	***20 (39.2)*** ↓	**20 (64.5)** ↑	7 (50.0)	7 (53.8)	0.267†
HRV/EV	8 (14.3)	5 (9.8)	6 (19.4)	2 (14.3)	2 (15.4)	0.773‡
RSV	8 (14.3)	11 (21.6)	5 (16.1)	3 (21.4)	3 (23.1)	0.808‡
HMPV	3 (5.4)	***0 (0.0)*** ↓	**5 (16.1)** ↑	1 (7.1)	1 (7.7)	0.030‡
HCoV	4 (7.1)	4(7.8)	1 (3.2)	***0 (0.0)*** ↓	***0 (0.0)*** ↓	0.836‡
HPIV	7 (7.1)	***2 (3.9)*** ↓	5 (16.1)	2 (14.3)	1 (7.7)	0.293‡
> 1 virus	3 (5.4)	2 (3.9)	2 (6.5)	1 (7.1)	0 (0.0)	0.914‡

*ARI, acute respiratory tract infection. Data are presented as n (%). Statistical analyses were performed using a Chi-square† or Fisher's exact test‡. Significant overrepresentation (bold letters, ↑) and significant underrepresentation (bold/italic letters, ↓) of virus detection in the clusters was assessed by enrichment analysis.

Supplementary table E5. Infant clusters and detected viruses three weeks after the onset of ARI* (swab B)

Cluster	**B1**	**B2**	**B3**	***P* value**
(n = 155)	**(n = 96)**	**(n = 34)**	**(n = 25)**	
Any virus	33 (34.4)	6 (17.7)	9 (36.0)	0.162[†]
HRV/EV	**14 (14.6)** ↑	***1 (2.9)*** ↓	***0 (0.0)*** ↓	0.030[‡]
RSV	11 (11.5)	***2 (5.9)*** ↓	**5 (20.0)** ↑	0.256[‡]
HMPV	3 (3.1)	1 (2.9)	1 (4.0)	1.000[‡]
HCoV	4 (4.2)	***0 (0.0)*** ↓	**5 (20.0)** ↑	0.007[‡]
HPIV	4 (4.2)	1 (2.9)	***0 (0.0)*** ↓	0.826[‡]
> 1 virus	3 (8.3)	0 (0.0)	2 (8.0)	0.185[‡]
New virus[§]	**28 (29.2)** ↑	***3 (8.8)*** ↓	7 (28.0)	0.042[‡]
Type of new virus				
HRV/EV	**12 (12.5)** ↑	0 (0.0)	0 (0.0)	0.017[‡]
RSV	9 (9.4)	***1 (2.9)*** ↓	3 (12.0)	0.447[‡]
HMPV	3 (3.1)	1 (2.9)	1 (4.0)	1.000[‡]
HCoV	3 (3.1)	0 (0.0)	**4 (16.0)** ↑	0.021[‡]
HPIV	4 (4.2)	1 (2.9)	0 (0.0)	0.826[‡]

*ARI, acute respiratory tract infection. Data are presented as n (%). Statistical analyses were performed using a [†]Chi-square or [‡]Fisher's exact test. [§]Positive virus detection in swab B different to result of swab A (either no or different virus detected in swab A).

Significant overrepresentation (bold letters, ↑) and significant underrepresentation (bold/italic letters, ↓) of virus detection in the clusters was assessed by enrichment analysis.

Supplementary table E6. Risk of symptom persistence by microbiota clusters three weeks after onset of ARI* (swab B)

Cluster	**B1**[†]	**B2**	**B3**
(n= 155)	**(n= 96)**	**(n = 34)**	**(n= 25)**
Symptomatic after 1 week		0.7 (0.3-1.6); 0.367	0.7 (0.3-1.7); 0.383
Symptomatic after 2 weeks		0.4 (0.1-1.9); 0.103	1.4 (0.5-3.5); 0.510
Symptomatic after 3 weeks		0.2 (0.0-0.7); 0.016	3.3 (1.3-8.0); 0.011
Continously symptomatic for ≥ 3 weeks		0.2 (0.0-1.2); 0.073	2.8 (1.0-7.5); 0.038

*ARI, acute respiratory tract infection. Data are presented as odds ratio (95 % confidence interval); *P* value. Associations were estimated using logistic regression. [†]Reference level is cluster 1.

Supplementary table E7. Association between clusters 3 weeks after the onset of infection (swab B) and presence of symptoms 3 weeks after onset of ARI* and continuous presence of symptoms for ≥ 3 weeks after onset of ARI

*ARI, acute respiratory tract infection. †Adjusted for age and season. ‡B1 is reference cluster.

	Unadjusted Model			**Adjusted model†**		
Outcome: Presence of symptoms 3 weeks after onset of ARI						
	Coeff	95 % CI	*P* value	Coeff	95 % CI	*P* value
Cluster B1‡	.	.	.	.	.	.
Cluster B2	-1.83	-3.33, -0.34	0.016	-1.91	-3.42, -0.39	0.014
Cluster B3	1.18	0.27, 2.09	0.011	1.33	0.36, 2.31	0.007
Outcome: Continuous presence of symptoms for ≥3 weeks after onset of ARI						
Cluster B1‡	.	.	.	.	.	.
Cluster B2	-1.89	-3.94, 0.17	0.073	-1.90	-3.98, 0.17	0.072
Cluster B3	1.03	0.06, 2.01	0.038	1.07	0.04, 2.10	0.041

Supplementary Table E8. Associations of detected viruses at swab A with symptom persistence

	No virus (n= 85)	**HRV/EV (n = 21)**	**RSV (n = 25)**	**HMPV (n = 9)**	**HCoV (n = 8)**	**HPIV (n = 19)**	***P* value**
Symptomatic after 1 week	57 (67.1)	17 (81.0)	19 (76.0)	8 (88.9)	6 (75.0)	12 (63.2)	0.550*
Symptomatic after 2 weeks	21 (24.7)	7 (33.3)	5 (20.0)	3 (33.3)	5 (62.5)	5 (26.3)	0.283†
Symptomatic after 3 weeks	24 (28.2)	6 (28.6)	7 (28.0)	1 (11.1)	4 (50.0)	6 (31.6)	0.687†
Continously symptomatic for ≥ 3 weeks	13 (15.3)	5 (23.8)	3 (12.0)	0 (0.0)	4 (50.0)	3 (15.8)	0.132†

Data are presented as n (%). Statistical analyses were performed using a Chi-square* or Fisher's exact test†.

Supplementary Table E9. Associations of detected viruses at swab B with symptom persistence

	No virus (n = 115)	**HRV/EV (n = 16)**	**RSV (n = 17)**	**HMPV (n = 4)**	**HCoV (n = 10)**	**HPIV (n = 5)**	***P* value**
Symptomatic after 1 week	78 (67.8)	13 (81.3)	13 (76.5)	4 (100.0)	7 (70.0)	4 (80.0)	0.760
Symptomatic after 2 weeks	28 (24.3)	3 (18.8)	6 (35.3)	2 (50.0)	5 (50.0)	2 (40.0)	0.268
Symptomatic after 3 weeks	30 (14.0)	5 (31.3)	2 (11.8)	1 (25.0)	6 (60.0)	1 (20.0)	0.367
Continously symptomatic for ≥ 3 weeks	16 (13.9)	2 (12.5)	4 (23.5)	1 (25.0)	4 (40.0)	1 (20.0)	0.236

Data are presented as n (%). Statistical analyses were performed using a Fisher's exact test.

Supplementary Table E10. Associations of new viruses* at swab B with symptom persistence

	No virus (n = 124)	**HRV/EV (n = 16)**	**RSV (n = 17)**	**HMPV (n = 4)**	**HCoV (n = 10)**	**HPIV (n = 5)**	***P* value**
Symptomatic after 1 week	85 (68.5)	10 (62.5)	10 (58.8)	4 (100.0)	6 (60.0)	4 (80.0)	0.871
Symptomatic after 2 weeks	30 (24.2)	3 (18.8)	5 (29.4)	2 (50.0)	4 (40.0)	2 (40.0)	0.308
Symptomatic after 3 weeks	31 (25.0)	5 (31.3)	5 (29.4)	1 (25.0)	5 (50.0)	1 (20.0)	0.214
Continously symptomatic for ≥ 3 weeks	17 (13.7)	2 (12.5)	4 (23.5)	1 (25.0)	3 (30.0)	1 (20.0)	0.209

Data are presented as n (%). Statistical analyses were performed using a Fisher's exact test.

*Viruses defined as new if different to result of swab A (either no or different virus present at swab A)

6.3 Aerobic exercise capacity in cystic fibrosis – does CFTR genotype matter?

Radtke T, Hebestreit H, Gallati S, Schneiderman JE, Stevens D, Hulzebos EHJ, Takken T, Boas SR, Urquhart DS, Lands LC, Tejero S, Sovtic A, Dwyer T, Petrovic M, Harris RA, Karila C, Savi D, **Usemann J**, Mei-Zahav M, Ratjen F, Kriemler S, for the CFTR-Exercise study group.

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Title: Aerobic exercise capacity in cystic fibrosis – does CFTR genotype matter?

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Appendix

Running title: CFTR genotype and aerobic fitness in cystic fibrosis

Take home message

In CF, lung function and nutritional status but not CFTR genotype is associated with exercise capacity.

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Abstract

Cystic fibrosis (CF) transmembrane conductance regulator (CFTR) is expressed in human skeletal muscle cells and CFTR dysfunction may present an important determinant of aerobic exercise capacity in CF. Previous studies on the relationship between CFTR genotype and aerobic exercise capacity are scarce and contradictory.

This study was designed to explore factors influencing aerobic exercise capacity, expressed as peak oxygen consumption (VO_{2peak}) with a specific focus on CFTR genotype in children and adults with CF.

In an international, multicentre cross-sectional study we collected data on CFTR genotype and cardiopulmonary exercise tests (CPET) in patients with CF age 8 years and older. CFTR mutations were classified into functional classes I-V.

726 patients from 17 CF centres in North America, Europe and Asia had both valid maximal CPET and complete CFTR genotype data and were included in the analysis. Overall, patients had reduced VO_{2peak} (mean±SD, 77.3 ± 19.1% predicted), but values were comparable among different CFTR classes. Using linear regression analysis adjusted for relevant confounders, lung function and body mass index, but not CFTR genotype were the main predictors of VO_{2peak},

Lung disease severity and reduced nutritional status rather than CFTR genotype are the major determinants of aerobic exercise capacity in CF patients.

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Appendix

Introduction

Cystic fibrosis (CF) is a disorder with autosomal recessive inheritance that ultimately leads to respiratory failure and premature death. The disease is caused by mutations in the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) gene coding for the CFTR protein that functions as a channel, mainly for chloride and bicarbonate [1]. CFTR mutations are grouped into different classes based on their effect on CFTR protein production, trafficking, function and stability [1]. CFTR is expressed in cell membranes of epithelial cells. It can also be found at the level of the sarcoplasmic reticulum in airway smooth muscle [2] and human skeletal muscle cells [3, 4] and myocardium [5], but the precise physiological role of CFTR in these tissues is not fully understood [6]. Previous studies suggest intrinsic functional abnormalities in muscles lacking functional CFTR [3, 4], possibly based on a disturbed calcium homeostasis in the muscle and increased systemic inflammation [3]. It is therefore plausible, that dysfunctional CFTR in human skeletal muscle could be a contributing factor to peripheral muscle weakness [7, 8] and reduced exercise capacity (e.g., peak oxygen uptake, VO_{2peak}) in CF [8, 9].

As VO_{2peak} is one of the best predictors for mortality in CF [10], the knowledge of important correlates of aerobic performance may help to guide patients' care. Few predominantly small studies have previously investigated relationships between CFTR genotype and VO_{2peak} in patients with CF [11-14]. These studies show controversial findings. In only one prominent study, Selvadurai and colleagues [12] found that children with CF carrying a F508del gene mutation on one allele and a class I or class II CFTR mutation on the second allele had strikingly lower VO_{2peak} (by about 30-45%) and peak anaerobic exercise capacity (by about 10-17%) compared to patients with a class III, IV or V mutation on the second CFTR allele. However, Selvadurai et al. [12] applied univariate analysis and, thus, did not control for relevant determinants of exercise capacity such as nutritional status and chronic infection with *Pseudomonas aeruginosa* [8, 15, 16] that could have influenced their

study findings and interpretation.

The current study was designed to investigate factors predicting VO_{2peak} (primary endpoint) and maximum work rate ($Watt_{max}$, secondary endpoint) with a specific focus on CFTR genotype in a large international cohort of children and adults with CF. We aimed to compare aerobic exercise capacity among patients with CFTR mutations resulting in severely reduced function (combinations of class I-III mutations) to patients with combinations of class IV-V mutations characterised by some residual CFTR function at the cell surface [17].

Methods

Study design and patients

We invited researchers from of the Exercise Working Group of the European Cystic Fibrosis Society to participate in the study. We also searched for publications in PubMed on exercise testing in CF to identify centres conducting clinical studies including cardiopulmonary exercise testing (CPET). Inclusion and exclusion criteria can be found in the online supplemental material as well as detailed information on CPET.

The classification of CFTR genotype [18, 19] was performed by a geneticist (SG) who was blinded for exercise testing data. Details about the functional classification of CFTR alleles are shown in Table S1 of the online supplements. We investigated effects of CFTR genotype on maximal exercise capacity (% predicted) in patients with both CFTR alleles in either class I, II or III (corresponding to severely reduced CFTR function) compared to patients with at least one mutant allele in class IV or class V (corresponding to some residual CFTR function) [17]. In an exploratory analysis, we compared maximal exercise capacity between patients with at least one copy of the F508del mutation and categorised them into five groups based on their second CFTR mutation class [12, 18].

We collected data on anthropometric characteristics, CF-related comorbidities (e.g., exocrine pancreatic insufficiency, CF-related diabetes (CFRD), colonisation with

Pseudomonas aeruginosa), pulmonary function, CPET related data and genetic data. Chronic *Pseudomonas aeruginosa* infection was considered to be present when >50% of at least 4 sputum samples collected in the previous year were positive [20]. We calculated % predicted values for spirometry [21], VO_{2peak} [22], $Watt_{max}$ [23] and peak heart rate [24] as well as z-scores for body mass index (BMI) based on WHO criteria [25]. Percent body fat was calculated using sex and age-specific prediction equations [26] and lean body mass was derived from body fat and weight. The primary outcome measure was VO_{2peak}, expressed as % predicted [22].

Ethical approval for this study was obtained from the cantonal ethical committee of Zurich, Switzerland (2015-0109). All centres obtained ethical approval (if required) for the use of their anonymized patient data according to national and local policies.

Statistical analysis

Data are presented as means±SD, median (inter-quartile range) or N (%). The Kolmogorov-Smirnov Test was used to verify normal distribution of data. The vast majority of data were not normally distributed. Descriptive analysis was performed using a non-parametric Mann-Whitney U test. The χ^2 test was used to compare categorical variables between groups. Statistical comparisons between the five groups based on CFTR classes were performed using the non-parametric Kruskal-Wallis test.

Simple linear regression analysis with study center adjustment as random intercept was used to determine predictors of VO_{2peak} and $Watt_{max}$ (% predicted). Multilevel mixed-effects models with a random intercept for each study centre were used to determine associations between CFTR genotype-based group and VO_{2peak} (%predicted, primary endpoint) and $Watt_{max}$ (%predicted, secondary endpoint) and adjusted for the following predictor variables: age, sex, BMI z-score, FEV_1 (% predicted), CF-related diabetes (CFRD), exocrine pancreatic insufficiency and infection with *Pseudomonas aeruginosa*.

To crosscheck and confirm our approach to define the exercise capacity outcome variables as percent-predicted values, we additionally performed linear mixed-models using a multiplicative, allometric approach to account for potential effects of body size and pulmonary function on the relationship between CFTR genotype and VO_{2peak} (see online data supplement).

All statistical analyses were performed with the statistical software package SPSS version 23 (IBM Corp. Armont, NY, USA) and Stata statistical software version 12 (StataCorp. 2012, College Station, Texas, USA). The significance level was set at $P<0.05$.

Results

We contacted 32 centres in North America, Europe and Asia by email of which 17 provided data for this study. Included centres were from Canada (N=3, 293 patients), US (N=2, 110 patients) and one centre each from Australia (N=30), France (N=59), Germany (N=69), Greece (N=39), Israel (N=37), Italy (N=34), Netherlands (N=93), Spain (N=51), Serbia (N=64), Switzerland (N=42), UK (N=39), and Vienna (N=30). A flow chart of included patients is shown in Figure 1 and patient characteristics are shown in Table 1. Patients that did not perform maximally during the CPET (N=112) were on average older, had lower FEV_1, lower exercise capacity and more CF-related comorbidities (Table 1). The lower exercise capacity in patients not performing maximally was not explained by a pulmonary function limitation (i.e., a lower breathing reserve at end-exercise (VE_{peak}/MVV_{pred})) compared to the group demonstrating maximal effort (Table 1). Table 2 shows data for maximal exercise capacity, lung function, nutritional status and CPET-related variables grouped by CFTR class.

Predictors of aerobic exercise capacity in patients with CF

In univariate analyses with study center adjustment, age, FEV_1 % predicted, forced vital capacity (FVC, % predicted), BMI z-score, CFRD and chronic *Pseudomonas aeruginosa*

infection were associated with VO_{2peak} and $Watt_{max}$ (expressed as % predicted). Exocrine pancreatic insufficiency was only associated with $Watt_{max}$ (% predicted). FVC was not included in the final multilevel model due to a high collinearity with FEV_1 (R=0.89, *P*=0.001). The CFTR functional class (I-V) was not associated with either VO_{2peak} or $Watt_{max}$ (% predicted). We noticed differences in VO_{2peak} and $Watt_{max}$ between the study centres. Consequently, the centre was included as random intercept in the multilevel mixed-effects models. In fully adjusted models, FEV_1 % predicted (Beta-coefficient (95% CI)) 0.413 (0.356 to 0.471), *P*<0.001 and BMI z-score 1.724 (0.727 to 2.771), *P*=0.001 were significantly associated with VO_{2peak} (% predicted). In the model for $Watt_{max}$ (% predicted), FEV_1 % predicted 0.459 (0.395 to 0.524), *P*<0.001 and BMI z-score 1.241 (0.122 to 2.361), *P*=0.030 remained the only significant predictors.

Exercise capacity between patients with severely reduced CFTR function versus patients with residual CFTR function

The prevalence of CF-related comorbidities was higher for patients with only class I-III CFTR mutations compared to patients with at least one CFTR allele associated with residual function (class IV or class V) (Table 3). Univariate analysis revealed no differences in aerobic exercise capacity (absolute and % predicted VO_{2peak} and $Watt_{max}$) between the two groups. In mixed-effects models, lung function and nutritional status were the only predictors for VO_{2peak} and $Watt_{max}$ (% predicted) independent of CFTR function (Table 4). In accordance, the results were confirmed when allometric modelling was applied (see Table S2 in the online data supplement).

Exercise capacity among patients with at least one copy of the F508del-CFTR mutation

In 653 patients with at least one copy of the F508del-CFTR (class II) mutation, no differences between CFTR groups were found for either VO_{2peak} or $Watt_{max}$ (absolute values or %

predicted, see Table S3). In linear regression analyses, patients with one copy of a class V mutation had a significantly lower VO_{2peak} compared to patients with two copies of a class II mutation (Beta-coefficient (95% CI)) -10.64 % (-17.03 to -4.24), *P*<0.001, see Table S4). Furthermore, $Watt_{max}$ values were lower for the group with one copy of a class V mutation (Beta-coefficient (95% CI)) -9.95 % (-17.31 to -2.59) *P*=0.008). In addition, the same association was found for (ln) VO_{2peak} (Beta-coefficient (95% CI)) -0.14 L (-0.22 to -0.06), *P*=0.001 and (ln) $Watt_{max}$ (Beta-coefficient (95% CI)) -0.12 W (-0.21 to -0.04), *P*=0.005 in the allometric models (Table S5).

Discussion

This international multicentre study evaluated predictors of aerobic exercise capacity with particular focus on CFTR genotype in patients with CF. We found that severity of CFTR genotype using different CFTR categorisations, combinations and analytic approaches, was not predictive for aerobic exercise capacity. In the present study, pulmonary function and nutritional status were strongly associated with exercise capacity. Our data support previous studies showing that pulmonary function and nutritional status are (among others) strong predictors of aerobic exercise capacity in CF [9, 13, 27-30].

Our main aim was to compare maximal exercise capacity between patients with CF carrying only minimal function CFTR mutations (classes I-III) and those with at least one residual function (class IV or V) CFTR mutation. In the primary analysis, our data showed no differences in aerobic exercise capacity between groups in both unadjusted and adjusted analyses. These results were confirmed by the use of allometric models that were computed to exclude potential limitations of the prediction equations for VO_{2peak} and $Watt_{max}$ [31]. Irrespective of what analytic approach was used – including the adjustments for important confounders, CFTR genotype was not related to aerobic exercise capacity in patients with CF.

Appendix

In an exploratory analysis, we could not reproduce the findings of a study by Selvadurai et al. [12] that showed a strong relationship between the severity of CFTR functional impairment and reduced exercise capacity using univariate analysis in children with CF aged 8-17 years with at least one copy of the F508del-CFTR mutation. In univariate analysis, we noticed no between group differences in either absolute or % predicted values for VO_{2peak} or $Watt_{max}$. In the adjusted analysis, patients with one copy of a class V mutation had significantly lower exercise capacity (about 10% predicted for both VO_{2peak} and $Watt_{max}$) compared to patients homozygous for the F508del-CFTR mutation. Patients with one copy of a class V mutation had the same ethnical background and comparable lung function but better nutritional status and lower prevalence of exocrine pancreatic insufficiency compared to patients homozygous for the F508del-CFTR mutation. Moreover, there was no evidence for ventilatory limitation (i.e., lower breathing reserve) during exercise. Altogether, these factors do not explain the lower exercise capacity in these patients.

Chronic systemic inflammation is known to be associated with reduced exercise capacity due to its deleterious effect on skeletal muscle function [32]. Unlikely, but one could argue that these patients have higher chronic systemic inflammation. Unfortunately, we do not have data on the level of inflammation on our patients, but chronic *Pseudomonas aeruginosa* infection is known to induce inflammatory processes in CF [33] that could negatively impact on exercise capacity. Van de Weert-van Leeuwen et al. [13] have previously been shown that presence of *Pseudomonas aeruginosa* in adolescents with CF is a predictor of a steeper decline in VO_{2peak} over time, independent of age, nutritional status, pulmonary function and immunoglobulin G levels. However, relatively fewer patients with a group V mutation were colonized with *Pseudomonas aeruginosa* than patients with solely CFTR class II mutations (reference group) despite a lower VO_{2peak} in the former compared to the latter group. Inflammation is, thus, unlikely causing the reduced VO_{2peak} in the patients with group V-mutations. Although, systemic inflammation has been shown to be associated with severity of

lung impairment [34, 35], lung function among groups was comparable. The differences between our study and the study by Selvadurai et al. [12] might be explained by the much better preserved lung function in all of our patients and our rigorous adjustment for important clinical confounders in the statistical models. Our data do not support a substantially higher exercise capacity in patients with mild CFTR mutations. Finally, we can only speculate that lower habitual physical activity, a determinant of VO_{2peak} in CF [9] contributes to the reduced exercise capacity in patients with one copy of a class V mutation.

Our data do not support a relevant role of CFTR genotype on aerobic exercise capacity in patients with CF. However, some theoretical considerations deserve further explanation to shed light on potential underlying molecular mechanisms of the CFTR defect and its potential consequences on peripheral muscle function and exercise capacity. Recently, the expression of functional CFTR channels in human skeletal muscle was demonstrated [3, 4]. Lamhonwah and colleagues [4] speculated that a dysfunction in sarcoplasmic reticulum CFTR channels might affect calcium release channels and thus ATP-mediated actin-myosin interaction that is essential for muscle contraction. Moreover, Divangahi et al. [3] suggested that excessive systemic inflammation initiates a process in which the abnormal vulnerability of CFTR-deficient muscle to pro-inflammatory mediators could play a key role in the development of skeletal muscle weakness observed in individuals with CF [7]. Of note, these observations are based on animal experiments and have not yet been confirmed in humans. Based on the in vitro experiments, suggesting that systemic inflammation plus CFTR dysfunction may impair muscle function, one could speculate that potential detrimental effects of CFTR genotype on aerobic exercise capacity would only be detected with high levels of inflammation. Overall, our patients had moderately impaired pulmonary function (mean±SD FEV_1 77.3 ± 23 % predicted) and a sub analysis including only patients with severe lung disease (FEV_1<50% predicted) yielded similar results for the comparison between minimal-function and residual function mutations.

Appendix

In the aggregate, in our study the severity of CFTR functional class had no negative influence on VO_{2peak}, a strong and independent predictor of survival in CF [10, 36]. Previous work demonstrated differential effects of CFTR genotype and mortality [37], independent of lung function, nutritional status, pancreatic insufficiency or *Pseudomonas aeruginosa* colonization [17]. Whether the predictive value of VO_{2peak} differs among groups with varying CFTR genotype remains to be investigated in longitudinal studies. Moreover, since regular exercise training has the potential to improve VO_{2peak} in CF patients [38], future studies might explore whether there is a different responsiveness of VO_{2peak} to exercise training according to CFTR genotype.

In comparison to previous studies on the genetic contribution to aerobic exercise capacity in CF, our study with more than 700 patients provides by far the largest dataset on CPET-derived measures of aerobic exercise capacity in CF. We collected CPET data from 17 specialised CF centers among Europe, North America and Asia including patients with a broad age range (8 to 61 years) and disease severity (FEV_1 16 to 123 % predicted) providing a quite representative study sample for the overall CF population.

This study has several limitations. Firstly, we collected data from different international study centres. Thus, we cannot rule out differences in treatment strategies and treatment quality possibly affecting the health status of the patients thereby introducing bias. Secondly, the groups classified according to CFTR classes were unevenly distributed with a high number of patients in the group with the most common F508del-CFTR mutation [39, 40] compared to patients carrying a CFTR class III, IV or V mutation. Despite the large sample size of 726 patients from 17 different CF centres worldwide, group sizes with CFTR class III-V mutations were relatively small because of the generally low prevalence of these gene mutations [41]. For these reasons, the explanatory analysis should be interpreted with caution. Moreover, we were not able to consider all known confounders impacting exercise capacity such as physical activity [9] and inflammatory markers [13] that were either not routinely

assessed or not available for this retrospective analysis. Nevertheless, strong predictors of VO_{2peak} such as pulmonary function [9, 13, 27-29] and nutritional status [30] as well as proxy measures of inflammation (i.e., *Pseudomonas aeruginosa* status), were included in our statistical analysis. Finally, we acknowledge the limitation of the retrospective study design and the collection of data over a large time period. However, it seems practically impossible to acquire such a large dataset on CPET variables in a prospective study that would overcome the limitations of the current study.

In summary, in our large, international cohort of children, adolescents and adults with CF, we found that pulmonary function and nutritional status were strong predictors of exercise capacity without evidence of an association between the severity of CFTR genotype and reduced maximal exercise capacity. Future studies might explore whether there is a different responsiveness of VO_{2peak} to exercise training according to CFTR genotype.

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Appendix

Figure

Figure 1. Flow chart of included patients. CFTR, cystic fibrosis transmembrane conductance regulator; CPET, cardiopulmonary exercise test.

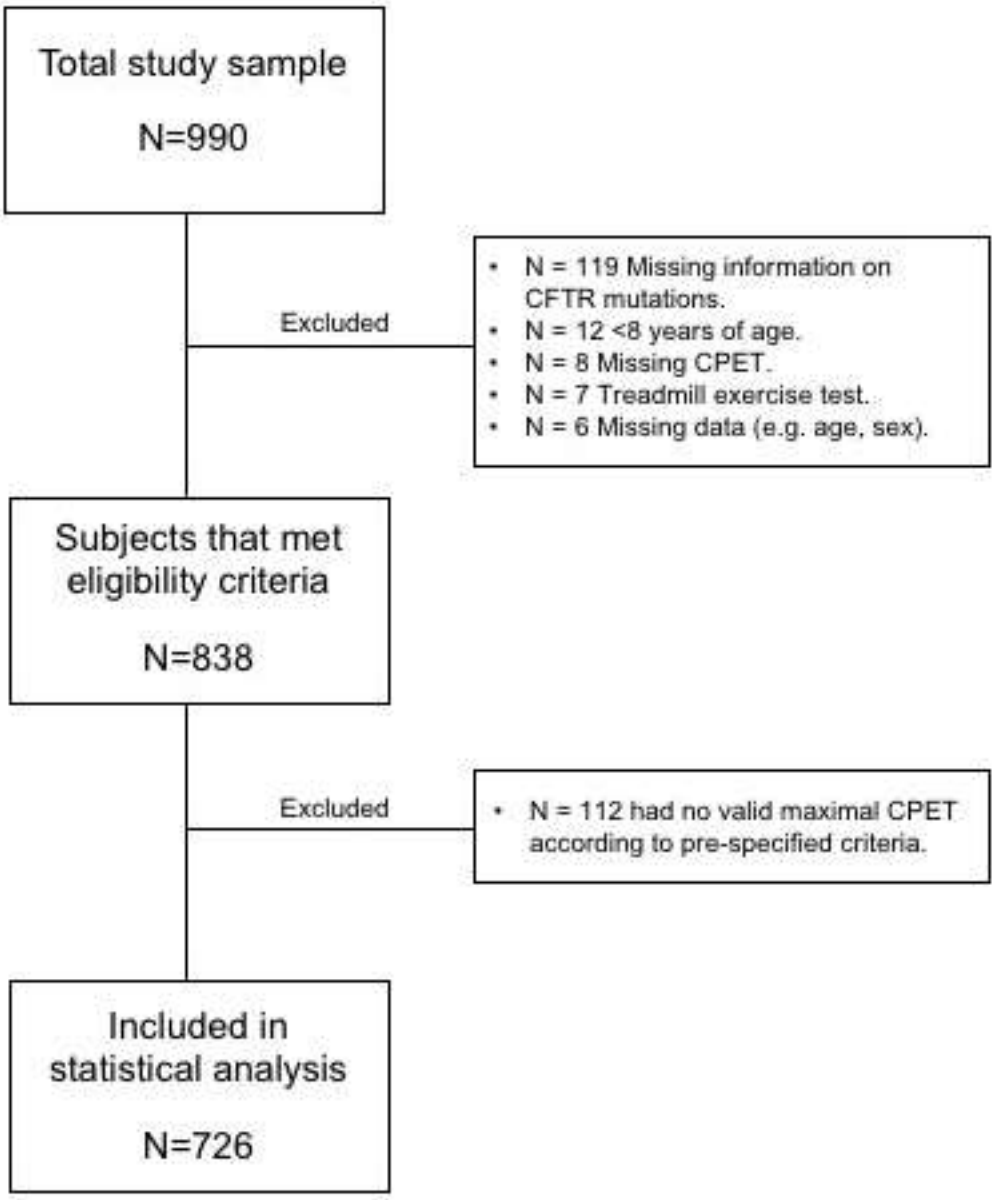

Table 1. Clinical characteristics and cardiopulmonary exercise testing data for patients with valid versus non-valid maximal exercise tests.

Variable	**N**	**Patients with valid maximal CPET**	**N**	**Patients with non-valid maximal CPET**
Age (years)[†]	726	16.4 (13.0, 22.1)	112	20.9 (13.9, 28.8)
Sex (% females)	726	330 (45)	112	45 (40)
BMI z-score[*]	726	-0.24 (-0.92, 0.47)	112	-0.01 (-0.74, 0.70)
FEV_1 (% predicted)[*]	724	79 (66, 91)	111	74.3 (56.9, 85.9)
CFRD (N, %)[*]	723	79 (11)	109	20 (18)
Pseudomonas aeruginosa (N, %)[†]	716	371 (52)	105	83 (79)
Pancreatic insufficiency (N, %)	724	611 (84)	112	90 (80)
VO_{2peak} (% predicted)[†]	726	79 (66, 91)	112	68 (58, 76)
$Watt_{max}$ (%predicted)[†]	724	84 (70, 100)	112	74 (61, 90)
HR_{peak} (% predicted)[†]	725	93 (88, 96)	112	87 (80, 92)
RER[†]	639[‡]	1.16 (1.10, 1.23)	36[‡]	0.99 (0.96, 1.01)
VE_{peak} ($L.min^{-1}$)[†]	726	68.0 (53.3, 86.3)	112	56.9 (45.1, 75.6)
VE_{peak}/MVV_{pred}[†]	725	86 (70, 102)	111	76 (63, 87)

Data are median (interquartile range) or N (%). BMI; body mass index; CFRD, Cystic Fibrosis Related Diabetes; CPET, cardiopulmonary exercise test; FEV_1, forced expiratory volume in one second; RER, respiratory exchange ratio; VO_{2peak}, peak oxygen consumption; $Watt_{max}$, maximum work rate; Statistical comparisons between groups were performed using the non-parametric Mann-Whitney U test or the χ^2 test as appropriate. [*] $P<0.05$; [†] $P<0.001$; [‡] RER data were not available from two study centers.

Table 2 Lung function, nutritional status and exercise capacity in 726 patients according to CFTR mutation (functional classes I-V) based on the functional defect of the milder of the two mutations.

	CFTR class I/I	**CFTR class ≤ II/II**	**CFTR class ≤ III/III**	**CFTR class ≤ IV/IV**	**CFTR class ≤ V/V**
N	32	550	39	63	42
Age	14.6 (12.7, 18.3)	16.2 (12.9, 21.3)	16.7 (12.1, 25.0)	16.6 (12.1, 25.0)	19.0 (14.8, 26.5)
Sex (% female)	14 (44)	244 (44)	16 (41)	34 (54)	21 (50)
CFRD, (N, %)*	7 (22)	64 (12)	4 (10)	2 (3)	1 (2)
Pancreatic insufficiency (N, %)†	35 (97)	529 (93)	34 (89)	15 (24)	10 (24)
Pseudomonas aeruginosa (N, %)*	32 (100)	519 (95)	21 (55)	23 (37)	15 (36)
BMI ($kg.m^{-2}$)†	18.8 (16.9, 20.1)	19.3 (17.3, 21.5)	20.4 (17.5, 24.2)	20.6 (18.8, 23.0)	22.3 (19.2, 25.0)
BMI z-score*	-0.35 (-1.05, 0.39)	-0.28 (-0.97, 0.35)	0.26 (-0.53, 1.04)	-0.20 (-0.76, 0.63)	0.08 (-0.79, 1.14)
Lean body mass (kg)*	38.2 (30.0, 46.4)	41.3 (32.9, 50.3)	43.5 (32.1, 55.2)	42.3 (36.7, 52.8)	47.0 (40.4, 61.1)
Body fat (%)†	18.0 (15.5, 20.1)	18.0 (14.1, 22.1)	20.2 (16.9, 22.5)	21.0 (17.2, 25.2)	21.8 (18.7, 25.4)
FEV_1 (%pred)	80 (45, 93)	79 (60, 94)	78 (50, 90)	86 (72, 96)	80 (62, 94)
VO_{2peak} ($L.min^{-1}$)	1.6 (1.3, 1.8)	1.7 (1.4, 2.3)	1.8 (1.3, 2.2)	1.8 (1.5, 2.3)	1.7 (1.3, 2.4)
VO_{2peak} (%pred)	77 (60, 86)	79 (65, 92)	77 (65, 96)	82 (70, 94)	74 (49, 94)
VO_{2peak} <82 %pred (N, %)	21 (66)	313 (57)	23 (59)	32 (51)	28 (67)
$Watt_{max}$ (W)	111 (83, 140)	127 (98, 170)	130 (95, 163)	124 (95, 170)	130 (85, 180)
$Watt_{max}$ (%pred)	77 (64, 88)	85 (70, 101)	83 (74, 102)	81 (69, 104)	80 (62, 102)
$Watt_{max}$ <93 %pred (N, %)	27 (84)	358 (65)	23 (59)	44 (70)	31 (74)
HR_{max} (%pred)	92 (86, 96)	93 (88, 97)	91 (87, 97)	92 (87, 96)	92 (86, 96)
RER	1.18 (1.12, 1.25)	1.16 (1.10, 1.23)	1.16 (1.11, 1.24)	1.14 (1.09, 1.20)	1.17 (1.08, 1.23)
VE_{peak} ($L.min^{-1}$)	62.3 (50.8, 81.7)	68.1 (53.9, 81.7)	68.0 (54.0, 85.0)	68.0 (51.7, 86.0)	68.0 (48.5, 82.7)
VE_{peak}/MVV_{pred} (%)*	81 (72, 106)	88 (71, 103)	95 (72, 118)	75 (64, 92)	80 (61, 101)

Data are median (interquartile range) or N (%). BMI; body mass index; CFRD, Cystic Fibrosis related diabetes; FEV_1, forced expiratory volume in one second; HR_{max}, maximum heart rate; MVV_{pred}, predicted maximum voluntary ventilation (calculated as FEV_1*35); RER, respiratory exchange ratio; VE_{peak}, peak minute ventilation; VO_{2peak}, peak oxygen consumption; $Watt_{max}$, maximum work rate. Reduced exercise capacity (VO_{2peak} <82%pred and $Watt_{max}$ <93%pred) according to [10]. Statistical comparisons between different CFTR classes were performed using the non-parametric Kruskal-Wallis test or χ^2 test for categorical variables. *P<0.05, †P<0.001 significantly different between CFTR classes.

Table 3 Clinical characteristics and cardiopulmonary exercise testing data between patients with two CFTR mutations in class I, II or III compared with patients with at least one mutation in class IV or V.

Variable	**CFTR classes I-III**	**CFTR classes IV-V**
N	621	105
CFTR modulator therapy (N, %)	9 (1)	3 (3)
Age	16.2 (12.9, 21.6)	18.0 (13.0, 25.5)
Sex, (% female)	273 (44)	55 (52)
CFRD, (N, %)*	75 (12)	3 (3)
Pancreatic insufficiency, (N, %)*	585 (95)	25 (24)
Pseudomonas aeruginosa, (N, %)†	332 (54)	38 (36)
BMI z-score*	-0.25 (-0.95, 0.42)	-0.11 (-0.77, 0.74)
Body fat (%)†	18.1 (14.3, 22.2)	21.5 (17.4, 25.2)
Lean body mass (kg)	41.1 (32.5, 50.3)	44.1 (36.4, 54.0)
FEV_1 (% predicted)	79 (59, 93)	84 (68, 96)
VO_{2peak} (L. min^{-1})	1.74 (1.4, 2.2)	1.78 (1.4, 2.4)
VO_{2peak} (% predicted)	79 (65, 91)	79 (67, 91)
$Watt_{max}$ (W)	125 (95, 168)	130 (94, 176)
$Watt_{max}$ (% predicted)	84 (70, 100)	82 (69, 96)
HR_{max} (% predicted)	93 (88, 96)	92 (87, 96)
RER	1.16 (1.10, 1.23)	1.15 (1.09, 1.23)
VE_{peak} ($L.min^{-1}$)	68.0 (53.9, 87.0)	68.0 (51.6, 85.0)
VE_{peak}/MVVpred (%)*	88 (71, 104)	78 (63, 96)

Data are median (interquartile range) or N (%). BMI; body mass index; CFRD, Cystic Fibrosis related diabetes; CFTR, cystic fibrosis transmembrane conductance regulator; FEV_1, forced expiratory volume in one second; HR_{max}, maximum heart rate; MVV_{pred}, predicted maximum voluntary ventilation (calculated as FEV_1*35); RER, respiratory exchange ratio; VE_{peak}, peak minute ventilation; VO_{2peak}, peak oxygen consumption; $Watt_{max}$, maximum work rate. Statistical comparisons between the two groups were performed using the non-parametric Mann-Whitney U test or the χ^2 test, as appropriate. *P<0.05, †P<0.001 significantly different between CFTR classes.

Table 4. Linear regression models on the association between patients with two CFTR mutations in class I, II or III (group I-III) compared to patients with at least one mutation in class IV or V (group IV-V).

VO_{2peak} (%pred)	**B-coefficients (95% CI's)**	**SE**	***P*-value**
Age	-0.14 (-0.32 to 0.04)	0.09	0.127
Sex	-1.32 (-3.49 to 0.85)	1.11	0.234
CFRD	-1.29 (-4.88 to 2.30)	1.83	0.353
Pseudomonas aeruginosa	-1.69 (-4.21 to 0.83)	1.28	0.189
Pancreatic insufficiency	-3.50 (-7.69 to 0.70)	2.14	0.130
BMI z-score	1.78 (0.78 to 2.77)	0.51	<0.001
FEV_1 (% predicted)	0.41 (0.35 to 0.47)	0.03	<0.001
CFTR group	-3.51 (-7.86 to 0.83)	2.22	0.113
$Watt_{max}$ (%pred)			
Age	0.01 (-0.20 to 0.23)	0.11	0.898
Sex	0.23 (-2.20 to 2.67)	1.24	0.852
CFRD	-1.48 (-5.53 to 2.57)	2.06	0.473
Pseudomonas aeruginosa	-2.52 (-5.36 to 0.32)	1.45	0.082
Pancreatic insufficiency	-4.47 (-9.20 to 0.26)	2.41	0.064
BMI z-score	2.02 (0.55 to 3.50)	0.75	0.007
FEV_1 (% predicted)	0.48 (0.40 to 0.57)	0.04	<0.001
CFTR group	-4.65 (-9.56 to 0.25)	2.50	0.063

BMI, body mass index; CFRD, Cystic Fibrosis related diabetes; FEV_1, forced expiratory volume in one second; SE, standard error; VO_{2peak}, peak oxygen consumption, $Watt_{max}$, maximum work load. The categorical variables CFRD, Pancreatic insufficiency, *Pseudomonas aeruginosa* are coded as '0' = no; '1' = yes. Sex is coded as '0' for females and '1' for males. CFTR groups are coded as '0' (class I-III combined) and '1' (class IV-IV combined).

ONLINE DATA SUPPLEMENT

Aerobic exercise capacity in cystic fibrosis – does CFTR genotype matter?

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Appendix

Methods

Inclusion and exclusion criteria

Study centres that could provide data on at least 20 patients, aged 8 years or older who completed a maximal cardiopulmonary exercise test (CPET) between January 1999 and December 2014 were eligible. We excluded patients with missing or incomplete information on cystic fibrosis transmembrane conductance regulator (CFTR) genotype and those who did not achieve the criteria for a maximal effort during CPET.

Cardiopulmonary exercise test

All patients performed a cycle ergometer test employing the Godfrey cycle protocol [1] or a modification thereof. Patients were tested during stable clinical conditions and exercise tests were performed as part of routine annual assessments and/or exercise studies. In case of exercise studies, baseline data were used.

We excluded patients who did not achieve the criteria for a maximal effort during CPET: 1) a respiratory exchange ratio (RER) ≥1.05 for adults and ≥ 1.03 for children [2]; 2) peak heart rate that reached or exceeded predicted peak heart rate (for children ≥195 beats.min^{-1} [3]), for adults based on published equations in [4]); 3) ventilation at peak exercise (VE_{peak}) exceeding estimated maximal voluntary ventilation (MVV=forced expiratory volume in 1s (FEV_1) x 35) [5]; 4) the patient achieved the predicted peak oxygen uptake (VO_{2peak}); or 5) the patient achieved the predicted maximum work rate ($Watt_{max}$) [2]. At least one of five criteria had to be fulfilled for the test to be considered maximal. In case of missing data (e.g., RER or heart rate), the respective criterion was considered as not fulfilled.

Body composition

Body fat (BF, in %) was calculated using age and sex-specific prediction formulas (≤ 15 years; >15 years) by Deurenberg et. al.[6] Lean body mass (LBM) was calculated from body weight and body fat. We did a cross-validation between predicted values for BF (%) and LBM (kg) with dual-energy absorptiometry (DXA) measured BF (%) and LBM (kg) from the Swiss cohort (39 patients with CF; 20 females) aged 15 to 39 years with a median (IQR) FEV_1 of 62 (46, 75) % predicted)). The mean bias between predicted and

measured BF was 0.20% (95% CI, -1.39 to 1.43). The mean bias between predicted and measured LBM was -3.32 kg (95% CI, -4.30 to -2.50).

Statistical analysis

We performed regression analysis using a multiplicative, allometric approach [7, 8] to eliminate potential effects of body size and pulmonary function on the relationship between CFTR genotype and VO_{2peak}. This approach is based on the assumption that VO_{2peak} is not linearly related to measures of body size, e.g. body mass M, but is proportional to power functions of those measures, for example M to the power of an exponent x. This approach has been used previously to determine predictors of VO_{2peak} in cystic fibrosis (CF) patients [8]. For the allometric model, we computed the natural logarithm (ln) of VO_{2peak} (primary outcome measure) and $Watt_{max}$ (secondary outcome measure), which entered the regression as dependent variable. We considered the following covariates in the model: age, ln(weight), ln(height), ln(FEV_1), CF-related diabetes, Pancreatic insufficiency, presence of *Pseudomonas aeruginosa* and CFTR genotype. We performed two separate analyses with ln(VO_{2peak}) (in L) and ln($Watt_{max}$) (in Watt) as dependent variables.

Appendix

Supplementary Tables

Table S1 Functional classification of CFTR alleles

CFTR classes (I-V)	CFTR nomenclature by legacy names
Class I	S4X, CFTRdele2,3, Q39X, E60X, 405+ 1G->A, 306delTAGA, 365-366insT, 489delC, 547insGA, 557delT, 574delA, 621+1G->T, Y109X, 711+1G->T, Q220X, L218X, C276X, 1154insTC, 1461ins4, 1525-1G->A, S466X, S489X, Q493X, 1677delTA, E479X, Q525X, 1717-1G->A, G542X, G550X, R553X, E585X, 1898+1G->A, 1259insA, 2183AA->G, R709X, 2347delG, 2184insA, E822X, R785X, Q781X, 2896insAG, W882X, 2991del32, 3120+1G->A, 3121-1G->A, V1108L, E1104X, R1070Q, Y1092X, E1104X, R1162X, 3791delC, 3659delC, 3724delG, 3732delA, 3876delA, 3905insT, R1283M, W1274X, W1282X, 4040delA, 4021insT, CFTRdele10, CFTRdele19-21, CFTRdele16-18, CFTRdele14b-17b, c.3874-1G>A, 1154insTC
Class II	G85E, G178R, F508del, I507del, L1077P, M1101K, R1162X, N1303K
Class III	M1V, G551D, S549R, R560T, S549N, Y569D, H609R, H1085R, S1251N
Class IV	P5L, P67L, R74W, G126D, I148T, R117H, D192G, L206W, V232D, L320V, L365P, R334W, Q359K/T360K, R347P, T338I, N369Y, V562I, 1717- 8G->A, Y914C, L997F, D1152H, S1235R, S18I, S898R, A457P, G567A, V109C, G1244V
Class V	1249-30delAT, A455E, 1811+1.6kbA->G, 2789+5G>A, 2789+ 5G>A, 3272-26A->G, 3849+10kbC->T, 5T, 5T-12TG, 5T-13TG

CFTR, cystic fibrosis transmembrane conductance regulator.

Table S2 Linear regression models on the association between patients with two CFTR mutations in class I, II or III compared with patients with at least one mutation in class IV or V.

ln(VO_{2peak}) (L)	**B-coefficients (95% CI's)**	**SE**	***P*-value**
Age	-0.00 (-0.00 to 0.00)	0.15	0.150
Sex	0.16 (0.14 to 0.19)	0.01	<0.001
ln(weight) (kg)	0.37 (0.25 to 0.48)	0.06	<0.001
ln(height) (m)	0.51 (0.16 to 0.85)	0.17	0.004
ln(FEV_1) (L)	0.39 (0.34 to 0.43)	0.02	<0.001
CFRD	-0.03 (-0.08 to 0.01)	0.02	0.169
Pancreatic insufficiency	-0.03 (-0.09 to 0.02)	0.03	0.208
Pseudomonas aeruginosa	-0.02 (-0.05 to 0.01)	0.02	0.224
CFTR class	-0.42 (-0.96 to 0.01)	0.03	0.123
ln($Watt_{max}$) (W)			
Age	-0.00 (-0.00 to 0.00)	0.00	0.199
Sex	0.14 (0.11 to 0.17)	0.06	<0.001
ln(weight) (kg)	0.29 (0.18 to 0.42)	0.06	<0.001
ln(height) (m)	0.98 (0.61 to 1.35)	0.19	0.004
ln(FEV_1) (L)	0.39 (0.34 to 0.44)	0.03	<0.001
CFRD	-0.01 (-0.06 to 0.03)	0.02	0.551
Pancreatic insufficiency	-0.04 (-0.10 to 0.01)	0.03	0.119
Pseudomonas aeruginosa	-0.03 (-0.06 to 0.00)	0.02	0.064
CFTR class	-0.05 (-0.11 to 0.00)	0.03	0.063

CFRD, Cystic Fibrosis related diabetes; CFTR, cystic fibrosis transmembrane conductance regulator; FEV_1, forced expiratory volume in one second; ln, natural logarithm; SE, standard error; VO_{2peak}, peak oxygen consumption, $Watt_{max}$, maximum work load. The categorical variables CFRD, exocrine Pancreatic insufficiency and *Pseudomonas aeruginosa* infection are coded as '0' = no; '1' = yes. Sex is coded as '0' for females and '1' for males. CFTR groups are coded as '0' for CFTR classes I-III and '1' for CFTR classes IV-V.

Table S3 Clinical characteristics and cardiopulmonary exercise testing data according to the class of second CFTR mutation in patients with at least one copy of the F508del mutation.

	CFTR class II/I	CFTR class II/II	CFTR class II/III	CFTR class II/IV	CFTR class II/V
N	154	394	35	43	27
Age	15.4 (12.7, 20.0)	16.4 (13.0, 22.0)	17.8 (13.2, 25.6)	16.9 (12.0, 22.0)	16.4 (13.7, 24.0)
Sex (% female)	81 (53)	161 (41)	15 (43)	20 (47)	9 (33)
CFRD, (N, %)	14 (9)	50 (13)	3 (9)	1 (2)	0 (0)
Pancreatic insufficiency (N, %)[†]	147 (95)	371 (95)	30 (88)	8 (22)	8 (30)
Pseudomonas aeruginosa (N, %)[*]	79 (52)	213 (55)	18 (53)	17 (39)	7 (26)
BMI ($kg.m^{-2}$)[*]	19.2 (17.2, 21.4)	19.3 (17.4, 21.6)	21.6 (17.5, 24.7)	20.6 (18.8, 22.1)	22.6 (19.2, 25.1)
BMI z-score	-0.37 (-1.02, 0.33)	-0.25 (-0.94, 0.41)	0.35 (-0.49, 1.04)	-0.25 (-0.77, 0.63)	0.04 (-0.73, 0.68)
Body fat (%)[*]	18.6 (14.5, 22.4)	17.8 (14.0, 22.0)	21.2 (17.0, 23.0)	20.1 (14.9, 24.1)	21.2 (16.2, 24.7)
Lean body mass (kg) [*]	41.0 (33.3, 49.4)	41.3 (32.7, 50.7)	45.2 (34.6, 55.3)	43.5 (35.9, 52.8)	48.7 (41.9, 61.7)
FEV_1 (% predicted)	82 (64, 96)	78 (58, 93)	78 (49, 90)	85 (72, 96)	82 (63, 99)
VO_{2peak} ($L.min^{-1}$)	1.75 (1.4, 2.2)	1.75 (1.4, 2.3)	1.85 (1.3, 2.2)	1.89 (1.5, 2.4)	1.96 (1.4, 2.5)
VO_{2peak} (% predicted)	78 (67, 91)	80 (65, 92)	77 (63, 93)	84 (70, 96)	75 (61, 87)
$Watt_{max}$ (W)	123 (92, 161)	129 (100, 175)	135 (97, 172)	117 (93, 175)	136 (112, 195)
$Watt_{max}$ (% predicted)	83 (68, 101)	86 (71, 101)	83 (72, 105)	81 (69, 95)	83 (74, 96)
HR_{max} (% predicted)	93 (89, 96)	93 (88, 97)	93 (87, 98)	92 (86, 95)	92 (87, 96)
RER	1.14 (1.11, 1.20)	1.16 (1.09, 1.24)	1.16 (1.09, 1.24)	1.14 (1.09, 1.20)	1.17 (1.08, 1.23)
VE_{peak} ($L.min^{-1}$)	67.6 (54.1, 84.0)	68.7 (53.8, 88.3)	68.5 (55.9, 87.1)	68.2 (52.0, 88.2)	69.5 (53.5, 86.0)
VE_{peak}/MVV_{pred} (%)[*]	83 (66, 100)	89 (73, 105)	95 (72, 118)	75 (65, 96)	78 (60, 89)

Data are median (interquartile range) or N (%). BMI; body mass index; CFRD, Cystic Fibrosis related diabetes; FEV_1, forced expiratory volume in one second; HR_{max}, maximum heart rate; MVV_{pred}, predicted maximum voluntary ventilation (calculated as FEV_1*35); RER, respiratory exchange ratio; VE_{peak}, peak minute ventilation; VO_{2peak}, peak oxygen consumption; $Watt_{max}$, maximum work rate. Statistical comparisons between different CFTR classes were performed using the non-parametric Kruskal-Wallis test or χ^2 test for categorical variables. [*]$P<0.05$; [†] $P<0.001$ significantly different between CFTR classes.

Table S4 Linear regression models on the association between class of second CFTR mutation and maximal exercise capacity in patients with at least one copy of the F508del mutation.

VO_{2peak} (%pred)	**B-coefficients (95% CI's)**	**SE**	***P*-value**
Age	-0.12 (-0.31 to 0.08)	0.10	0.240
Sex	-1.17 (-3.47 to 1.12)	1.17	0.317
BMI z-score	2.01 (1.02 to 3.14)	0.54	<0.001
FEV_1 (%predicted)	0.41 (0.35 to 0.47)	0.03	<0.001
CFRD	-1.96 (-5.78 to 1.86)	1.95	0.315
Pseudomonas aeruginosa	-2.52 (-5.16 to 0.12)	1.35	0.061
Pancreatic insufficiency	-2.64 (-7.26 to 1.97)	2.35	0.262
CFTR class II/I	-1.53 (-4.30 to 1.25)	1.41	0.280
CFTR class II/II	-	-	-
CFTR class II/III	-2.87 (-7.99 to 2.25)	2.61	0.272
CFTR class II/IV	0.33 (-5.51 to 6.17)	2.99	0.912
CFTR class II/V	-10.64 (-17.03 to -4.24)	3.26	0.001
$Watt_{max}$ (%pred)			
Age	0.05 (-0.18 to 0.28)	0.12	0.673
Sex	1.04 (-1.59 to 3.68)	1.34	0.437
BMI z-score	1.33 (0.11 to 2.55)	0.62	0.033
FEV_1 (% predicted)	0.46 (0.40 to 0.53)	0.04	<0.001
CFRD	-1.44 (-5.85 to 2.98)	2.25	0.523
Pseudomonas aeruginosa	-2.80 (-5.84 to 0.25)	1.55	0.072
Pancreatic insufficiency	-3.62 (-8.94 to 1.71)	2.72	0.181
CFTR class II/I	-1.19 (-4.38 to 2.01)	1.63	0.466
CFTR class II/II	-	-	-
CFTR class II/III	-0.49 (-6.44 to 5.46)	3.03	0.872
CFTR class II/IV	-2.84 (-9.58 to 3.89)	3.44	0.408
CFTR class II/V	-9.95 (-17.31 to -2.59)	3.75	0.008

CFRD, Cystic Fibrosis related diabetes; CFTR, cystic fibrosis transmembrane conductance regulator; FEV_1, forced expiratory volume in one second; SE, standard error; VO_{2peak}, peak oxygen consumption, $Watt_{max}$, maximum work load. The categorical variables CFRD, exocrine Pancreatic insufficiency and *Pseudomonas aeruginosa* infection are coded as '0' = no; '1' = yes. Sex is coded as '0' for females and '1' for males. CFTR classes were coded as class II/II = 1; class I/II = 2; class II/III = 3; class II/IV = 4 and class II/V =5. The CFTR class II/II was chosen as reference class.

Table S5 Linear regression models on the association between class of second CFTR mutation and maximal exercise capacity in patients with at least one copy of the F508del mutation.

ln(VO_{2peak}) (L)	**B-coefficients (95% CI's)**	**SE**	***P*-value**
Age	-0.00 (-0.00 to 0.00)	0.00	0.188
Sex	0.17 (0.14 to 0.20)	0.01	<0.001
ln(weight) (kg)	0.38 (0.26 to 0.50)	0.06	<0.001
ln(height) (m)	0.49 (0.12 to 0.85)	0.19	0.009
ln(FEV_1) (L)	0.40 (0.35 to 0.45)	0.03	<0.001
CFRD	-0.04 (-0.08 to 0.01)	0.02	0.145
Pancreatic insufficiency	-0.02 (-0.08 to 0.03)	0.03	0.431
Pseudomonas aeruginosa	-0.03 (-0.06 to 0.01)	0.02	0.104
CFTR class II/I	-0.01 (-0.04 to 0.02)	0.02	0.568
CFTR class II/II	-	-	-
CFTR class II/III	-0.05 (-0.11 to 0.01)	0.03	0.111
CFTR class II/IV	0.00 (-0.07 to 0.08)	0.04	0.858
CFTR class II/V	-0.14 (-0.22 to -0.06)	0.04	0.001
ln($Watt_{max}$) (W)			
Age	-0.00 (-0.00 to 0.00)	0.00	0.423
Sex	0.15 (0.12 to 0.18)	0.02	<0.001
ln(weight) (kg)	0.30 (0.17 to 0.43)	0.07	<0.001
ln(height) (m)	0.95 (0.55 to 1.34)	0.20	<0.001
ln(FEV_1) (L)	0.40 (0.34 to 0.45)	0.03	<0.001
CFRD	-0.01 (-0.06 to 0.04)	0.03	0.657
Pancreatic insufficiency	-0.04 (-0.10 to 0.02)	0.03	0.222
Pseudomonas aeruginosa	-0.03 (-0.07 to 0.00)	0.02	0.076
CFTR class II/I	-0.02 (-0.05 to 0.02)	0.02	0.418
CFTR class II/II	-	-	-
CFTR class II/III	-0.04 (-0.11 to 0.03)	0.04	0.265
CFTR class II/IV	-0.04 (-0.12 to 0.04)	0.04	0.353
CFTR class II/V	-0.12 (-0.21 to -0.04)	0.04	0.005

CFRD, Cystic Fibrosis related diabetes; CFTR, cystic fibrosis transmembrane conductance regulator; FEV_1, forced expiratory volume in one second; ln, natural logarithm; SE, standard error; VO_{2peak}, peak oxygen consumption, $Watt_{max}$, maximum work load. The categorical variables CFRD, exocrine Pancreatic insufficiency and *Pseudomonas aeruginosa* infection are coded as '0' = no; '1' = yes. Sex is coded as '0' for females and '1' for males. CFTR classes were coded as class II/II = 1; class I/II = 2; class II/III = 3; class II/IV = 4 and class II/V =5. The CFTR class II/II was chosen as reference class.

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Curriculum vitae

Personal data

Date of birth:	25.01.1984
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Languages:	German, English, Italian, French, Spanish

Positions

08/14-07/17	PhD Student Pediatric Pneumology Research Group, UKBB, Basel
07/11-08/14	Resident physician Pädiatrische Pneumologie m. S. Immunologie, Charité, Berlin

MD-Thesis

07/09-11/14	Max-Planck Institut Bad Nauheim, University Giessen, Germany. Research Group Structure und Development of the Alveolus, Prof. Seeger and Dr. R.E. Morty. "All–trans retinoic acid recruits Smad3 to drive TGF–β signaling in lung fibroblasts: implications for the management of bronchopulmonary dysplasia with retinoids".

Internships

07/09-09/09	Internal Medicine, University Giessen
09/09-11/09	Internal Medicine/Oncology, Memorial Sloan Kettering Center, New York, USA
11/09-03/10	Surgery, Clinic Hirslanden, Zürich
03/10-07/10	Pediatrics, L'Ospedale dei Bambini Meyer, Florence
02/07-08/07	General clinical education University Padova, Italy

Education

03/04-04/10	Medical School, Justus-Liebig-Universität, Giessen
07/01-05/03	High School, Albert Schweitzer Gymnasium, Hamburg
06/00-06/01	Cranbrook-Kingswood Boarding School Detroit, Michigan, USA
07/90-05/00	Elemantary School Albert Schweitzer Schule, Hamburg

Memberships

- Gesellschaft für pädiatrische Pneumologie
- Deutsche Gesellschaft für Kinder- und Jugendmedizin
- Swiss School for Public Health (SSPH+)
- PhD Program Health Sciences

Scholarships and awards

- German- Swiss and Austrian society for pediatric pneumology fellowship
- Swiss School for Public Health (Switzerland)
- University Children's Hospital Basel Research Congress Fellowship (2016, 2017)
- University Children's Hospital Basel Research Award

Reviews for Journals

- European Respiratory Journal, PloS One, Pediatric Allergy and Immunology, British Medical Journal, Respiratory Medicine, Pediatric Research, International Journal of Public Health, Pediatric Pulmonology

Master Thesis Supervision

- Andrea Suter, MD Student University of Basel: "Can volumetric capnography predict respiratory morbidity during the first year of life in preterm infants?"

Teaching activity

- 2 years of teaching students in anatomy during medical studies
- 2 semesters of weekly teaching "Problemorientiertes Lernen" at the "Modellstudiengang Charité-Universitätsmedizin, Berlin"
- Supervision of medical master and doctorate students at the University of Basel

Publication list

Submitted/completed original Articles:

- **Usemann J**[*], Xu B[*],Delgado-Eckert E, Korten I, Anagnostopoulou P, Gorlanova O, Kuehni C, Röösli M, Latzin P, Frey U on behalf of the BILD study group. Dynamics of respiratory symptoms during infancy and associations with wheezing at school age. Under Review

- Lech Cantuaria M[*], **Usemann J**[*], Proietti E, Blanes-Vidal V, Dick B, Frey C.E, Rüedi S, Héritier H, Wunderli J.M, Latzin P, Frey U, Röösli M, Vienneau D on behalf of the BILD study group. Glucocorticoid metabolites in newborns: a marker for traffic noise related stress? Manuscript ready for submission

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Published original Articles:

- Roth M[*], **Usemann J[*]**, Bisig C, Comte P, Czerwinski J, Mayer A.C.R, Beier K, Rothen-Rutishauser B, Latzin P, Müller L. Effects of gasoline and ethanol-gasoline exhaust exposure on human bronchial epithelial and natural killer cells *in vitro.* Accepted Toxicol. in vitro

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- **Usemann J**, Korten I, Latzin P. A big step forward in understanding global differences in respiratory health: first lung function data in African infants. Respirology. 2015 Oct;20(7):1006-7

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- **Usemann J**, Yammine S, Singer F, Latzin P. Inert gas washout: background and application in different lung diseases. Accepted Swiss Med Wkly

- Frey U, Latzin P, **Usemann J**, Maccora J, Zumsteg U, Kriemler S. Asthma and obesity in children: current evidence and potential systems biology approaches. Allergy. 2015 Jan;70(1):26-40.

Book Contribution:

- Kompendium Pneumologie 2014 “State of the Art: Allergologie**” Jakob Usemann**, Philippe Stock. September 2014