Genetic and environmental determinants of pulmonary outcome in term-born and preterm children

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"No man burdens his mind with small matters unless
he has some very good reason for doing so."

Sir Arthur Conan Doyle, M.D.

Contents

List of Abbreviations and Definitions

Abbreviation	Full term
95%CI	95% confidence interval
ATS	American Thoracic Society
AUC	Area under the curve
BILD	Bern and Basel infant lung development study
BTPS	Body temperature, ambient pressure, saturated (with water vapour)
BPD	Bronchopulmonary dysplasia
CEV	Cumulative expired volume
CF	Cystic fibrosis
CO_2	Carbon dioxide
CRIB	Clinical risk index for babies
CV	Coefficient of variation
DSR	Dead space reducer
DZL	Deutsches Lungenzentrum
ELBW	Extremely low birth weight (<1.0 kg)
ELGAN	Extremely low gestational age neonates (<28 weeks of gestation)
FeNO	Fraction of exhaled nitric oxide
ERS	European Respiratory Society
FEV_1	Forced expiratory volume in 1 second
FRC	Functional residual capacity
FRC_{MBW}	Functional residual capacity measured by multiple-breath washout
FRC_{MBW_V1}	Functional residual capacity measured by multiple-breath washout at visit 1
FRC_{MBW_V2}	Functional residual capacity measured by multiple-breath washout at visit 2
FRC_{pleth}	Functional residual capacity measured by plethysmography
FVC	Forced vial capacity

Abbreviation	Full term
He	Helium
HeMBW	Helium multiple-breath washout
ISPM	Institut für Sozial- und Präventivmedizin
IQR	Inter-quartile range
LCI	Lung clearance index, marker of overall ventilation inhomogeneity
LRT	Likelihood ratio test
MBW	Multiple-breath washout
MCh	Metacholine
MM	Molar mass
MMEF	Maximum mid-expiratory flow
N_2	Nitrogen
N_2MBW	Nitrogen multiple-breath washout
no-ELGAN	Neonates $\geq$ 28 weeks of gestation
O_2	Oxygen
OLS	Online supplement
PCR	Polymerase chain reaction
PMA	Post-menstrual age
R^2	Variance explained
Rint	Airway resistance by interrupter
Reff	Effective airway resistance
RVpTLC	Residual volume per total lung capacity

Abbreviation	Full term
SIII	Slope of alveolar phase III of nitrogen expirogram
SAB	Short-acting bronchodilator
Sacin	Marker of ventilation inhomogeneity within diffusion-convection-dependent acinar airways
Scond	Marker of ventilation inhomogeneity within convection-dependent conducting airways
SD	Standard deviation
SDS	Standard deviation score
SF_6	Sulphur hexafluoride
SF_6MBW	Sulphur hexafluoride multiple-breath washout
sRaw	Specific airway resistance
Swiss TPH	Swiss tropical and public health institute
TBFVL	Tidal breathing flow volume loop
TEF_{25}	Tidal expiratory flow at 25% of remaining tidal volume
TEF_{50}	Tidal expiratory flow at 50% of remaining tidal volume
tPTEF/tE	Time to peak tidal expiratory flow / expiratory time
UKBB	Universitätskinderspital beider Basel
USFM	Ultrasonic flow meter
VC	Vital capacity
VI	Ventilation inhomogeneity
VmaxFRC	Maximal flow at functional residual capacity
Vt	Tidal volume
V'E	Minute ventilation

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Summary

Understanding early lung growth is a prerequisite for strategies to ensure ideal lung development in childhood and to minimise lifelong respiratory morbidity. Reduced respiratory health is a major burden for many individuals across all ages and for health care systems worldwide. Chronic lung diseases in adults originate early in life, and our knowledge of the underlying mechanisms is limited. The aim of this work was to describe early lung growth from infancy to early childhood in term-born and preterm children, and to compare pulmonary outcomes and their determinants cross-sectionally and longitudinally between these populations.

Non-invasive lung function tests like multiple-breath washout (MBW) enabled us to measure lung volumes and ventilation homogeneity in relatively large populations in infancy and early childhood during normal breathing.

As a first step, we challenged our equipment. We performed an in-vitro study using a lung model with quasi-physiologic conditions, testing one setup for MBW in infants and one setup for MBW in young children. Both were based on ultrasonic flow meter technology. Tracers were sulfur hexafluoride and helium respectively. We investigated the agreement of the functional residual capacity (FRC) that was measured by the equipment to the generated FRC in the model by calculating the absolute and relative differences between the two. In the infant setup, mean (95%CI) absolute difference between model and measured FRC was 1.9 (0.3-3.5) ml, corresponding to a relative difference of 0.7 (-0.6 – 2.0) %, 96% of runs met the criteria of measuring FRC within 10% accuracy. In the young child setup, for FRCs between 600 and 1,400 ml, absolute difference was -17 (-25 – -8.3) ml corresponding to a relative difference of -2.0 (-3.0 – -0.9) %. We observed results within 10% accuracy for 98% of these trials. In conclusion, we observed a measurement error for FRC within an acceptable range for the lung volumes expected in our target age groups.

Using this reliable and valid equipment, we measured FRC and lung clearance index (LCI), an early marker of small airway pathologies, repeatedly in term-born and preterm children from an ongoing observational study in Switzerland, the so-called BILD study (Bern & Basel Infant Lung Development). In addition, we performed MBW using nitrogen as a

tracer, and routine lung function tests like spirometry and plethysmography. We collected information on pre- and postnatal exposures, as well as on respiratory symptoms during infancy and young childhood.

In a population of 124 term-born children, we tested the potential association of FRC measured by MBW at the age of 5 weeks (visit 1), and other potential predictors, with FRC measured by plethysmography (FRC_{pleth}) at 6 years (visit 2) by linear regression analysis, primary outcome of which was FRC_{pleth}. We observed only a weak association of FRC over time, the univariable model explained only 4.1% of the variance of FRC_{pleth} (coefficient 2.7 ml; 95%CI 0.6 – 4.8). In the multivariable model including height and age at visit 2, male gender, and maternal smoking during infancy, the variance explained was higher (coefficient 2.0 ml; 95%CI 0.02 – 3.97, R^2 22%, $p<0.001$). We found no association of lung volume with function of larger airways, which indicated disproportionate growth of lung parenchyma and large airways.

In a group of 166 preterm infants, we investigated whether lung function at the corrected age of 44 weeks of gestation predicts respiratory morbidity in the first year of life. No improved prediction was found compared to standard clinical tools, but we observed an altered breathing pattern in the subgroup that developed wheeze in the first year of life. Tidal volume was positively associated with wheeze (adjusted odds ratio (aOR) 1.40 (95%CI 1.04-1.90) per 5 ml increase), respiratory rate was negatively associated with wheeze (aOR 0.69 (95%CI 0.50-0.96) per 10 breath per min increase). This might reflect an adaptive change in breathing pattern as a consequence of the underlying structural pathology.

Later in childhood, at the age of approximately 9 years, we compared ventilation homogeneity in 77 preterm children with 49 term-born children of the same age. We measured LCI and slopes of nitrogen expirogram reflecting ventilation in the acinar regions (Sacin), and conducting airways (Scond). We found no differences on the overall and acinar level, but Scond was significantly elevated in preterm compared to term-born participants (mean difference 1.74 z-scores, 95%CI 1.17 - 2.30, $p<0.001$). Scond was elevated in 54% of the preterm children. Younger gestational age was the strongest predictor of having an elevated Scond (R^2 37%).

When we analysed FRC in 80 preterm children over time, i.e. from infancy to early school

age, we found an even weaker association of lung volume over time than in our study in term-born children. After adjustment for height and age at visit 2, and for immaturity of the lungs, we found a very weak association between FRC at visit 1 and FRC measured by MBW at visit 2 (beta = 2.6 ml per ml, 95%CI 0.02 to 5.22, R^2 55.7%), with the highest proportion of variance explained by the variables for which we had adjusted. Younger gestational age was associated with stronger relative lung volume increase, i.e. increase in FRC standard deviation score from visit 1 to visit 2 (beta = -0.18 after adjustment, 95%CI -0.27 to -0.08, p <0.001). Compared to the term-born children, preterm children at visit 2 showed significantly higher values for FRC_{pleth} standard deviation score, and for trapped gas calculated as the difference between FRC_{pleth} and FRC measured by MBW at the same visit. We interpret these findings as excessive catch-up growth after prematurity.

In the overall view, respiratory health in infancy and lung growth seemed to be determined by prematurity per se, and not by potentially harmful or beneficial therapeutic interventions, but the latter were not randomly assigned in these observational studies. Though it was incomplete, we observed a recovery from interrupted structural growth after prematurity.

1 Introduction

1.1 Development of human lungs before and after birth

For better respiratory health in children and adults now and in the future it is crucial to improve our understanding of early lung development. Respiratory morbidity across all ages is a major public health concern, as incidence and prevalence of chronic lung diseases are increasing [World Health Organization, 2015]. Existing preventive and therapeutic strategies need further improvement, and new approaches need to be developed. Researchers worldwide are contributing to that.
There is epidemiological evidence that lung development before and after birth has an impact on pulmonary outcome in adulthood, and that environmental influences and gene-environment interactions predispose for chronic disease [Barker et al., 1991, von Mutius, 2001, Silverman and Kuehni, 2007, Bush, 2008, Frey and Suki, 2008, Postma et al., 2014]. The term asthma, despite its use as a diagnosis, is an overall term for more than one disease [Silverman, 1995], and this explains why some therapies are only beneficial for subgroups of patients. A deeper understanding of the different disease entities would allow us to identify subjects at risk and to improve therapies.

1.1.1 Lung development in term-born children

Human lungs undergo very complex physiological changes before and after birth. The most important window is during the first few years of life [Stocks et al., 2013], but lung growth has been shown even beyond [Narayanan et al., 2012].
Fetal lung development starts during week 4 of gestation, when a single airway starts to grow from the foregut. During the pseudoglandular stage, from week 5 to 17, the conducting airways branch, forming a respiratory tree. The next stage is the canalicular stage, from week 16 to 27. Here, the respiratory tree expands in length and diameter, accompanied by blood vessels. The respiratory airways form and surfactant appears. During the saccular stage, from week 28 onwards, the interstitium becomes thinner, saccules

and alveoli appear. The alveolar stage is reached around week 38. By birth, a term-born infant has 100 million alveoli approximately, and the number increases during childhood [Baraldi and Filippone, 2007, Stocks et al., 2013].
Multiple intrinsic and extrinsic factors have an impact on early lung development. Healthy infants, for example, show alterations in lung function and longer duration of infectious episodes after exposure to outdoor air pollution [Latzin et al., 2011, Stern et al., 2013].

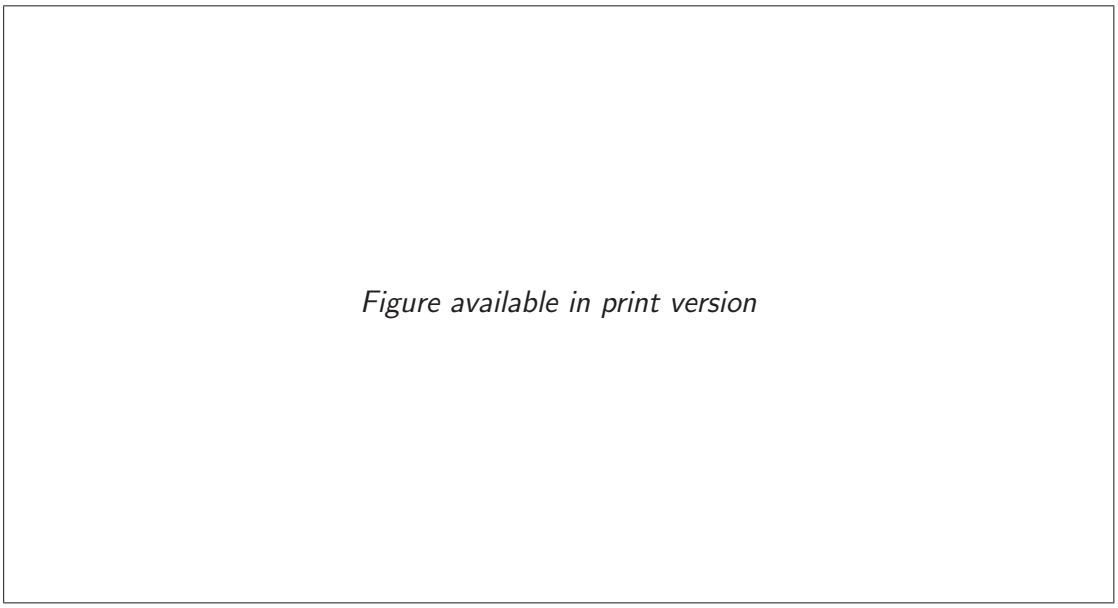

Figure 1.1.
Lung development before and after birth
[Baraldi and Filippone, 2007]

1.1.2 Prematurity disturbs alveolar growth

Premature birth, i.e. birth <37 weeks of gestation, has immediate and long-term consequences on respiratory health, as normal intrauterine maturation of the lungs is interrupted. By the end of a normal pregnancy, the final key features of a healthy lung begin to emerge: millions of alveoli grow, allowing gas exchange, and surfactant reduces

surface tension thereby allowing the alveoli to expand. The timing of birth is relevant for adaptation to extra-uterine life. The earlier a child is born, the more likely it is to develop respiratory distress (see figure 1.1.) [Baraldi and Filippone, 2007].
Worldwide, the number of preterm births was estimated at 12.9 million for the year 2005. The highest rates were observed in Africa (11.9%) and North America (10.6%). In Switzerland, numbers comparable to the rest of Europe were observed. The percentage of preterm births in 2013 was 6.2% for a gestational age from 32 to 36 weeks and 1% for a gestational age $<$32 weeks [Beck et al., 2010, Schweizer Bundesamt für Statistik, 2015].
A well known immediate consequence after preterm birth is bronchopulmonary dysplasia (BPD). The disease was initially described by Northway et al. in 1967 [Northway et al., 1967]. It occurs in approximately 30% of preterm infants with a birth weight of less than 1000 g. Characteristics of BPD at the time of its first description included structural damage like patchy bronchoalveolar scarring, overexpansion and atelectases. With the introduction of new strategies such as prenatal corticosteroids, lung protective ventilation and artificial surfactant, the clinical characteristics of lung disease following prematurity changed. The so called new BPD is mainly characterised by fewer and larger alveoli [Kinsella et al., 2006]. However, the incidence of BPD is not expected to decrease [Baker and Alvira, 2014], as continuous improvement in reproductive medicine and of survival in neonatology contribute to keep numbers of affected children high.
It was shown that prematurity does not only have short-term consequences on respiratory health, i.e. requirement of ventilation and oxygen supplementation, to name just a few. Long-term sequelae can occur in children with and without BPD [Hoo et al., 2002a, Stocks et al., 2013]. Common observations include a higher incidence of hospitalisation due to respiratory symptoms during infancy, partially reversible airflow obstruction and abnormal thoracic imaging at school age and reduced spirometric lung function results in adulthood [Greenough, 2013]. The risk of chronic obstructive pulmonary disease (COPD) is elevated after prematurity [Stocks et al., 2013, Tai et al., 2014, Bolton et al., 2015]. Phenotypes of respiratory diseases might differ between term-born and preterm individuals [Martin and Fanaroff, 2013].

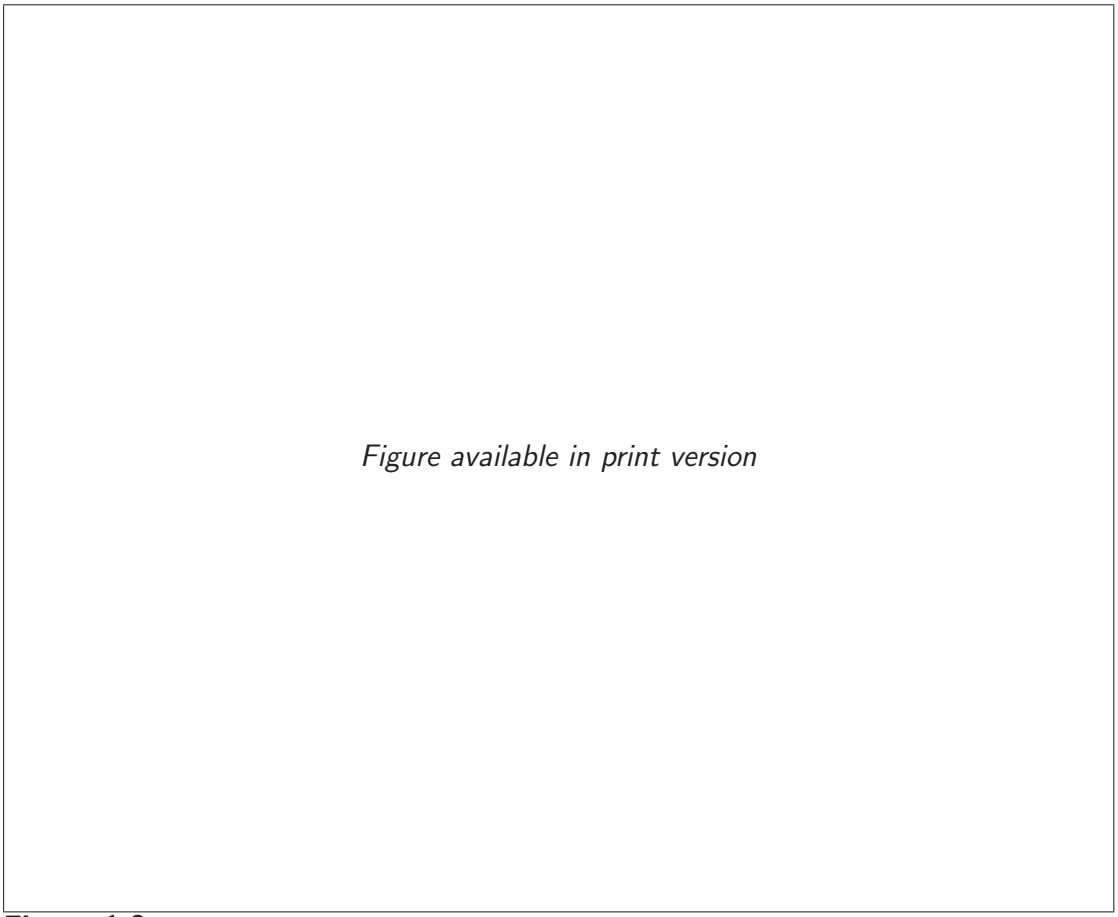

Figure 1.2.
The life course of lung function
Black line indicates term individuals, grey solid line indicates preterm-born individuals and grey dashed line indicates possible rates of decline in lung function in preterm-born individuals [Bolton et al., 2015]

1.1.3 Respiratory health in the context of Public Health

Respiratory morbidity is a burden for health care systems worldwide. In the US, for example, 10.000 premature infants are affected annually by BPD and its impact on overall and pulmonary health [McEvoy et al., 2014]. Especially in very young children, acute

respiratory infections are likely to cause symptoms that require hospitalisation [Pramana et al., 2011], and one of the parents is often admitted with the child. Compared with adults, children have higher minute ventilation in relation to their body size, so inhaled agents are likely to harm them even more, especially because of the limited ability to leave areas with exposures. There is a potential window of increased vulnerability. At the same time, this might be a window of opportunity where protection from disturbing factors might be very beneficial for long-term respiratory health.

Thus, improved respiratory health in childhood has the potential not only to reduce short-term costs, as has been observed after introducing smoke-free legislation [Been et al., 2014]; it can additionally be expected that long-term costs for health care systems will also be reduced.

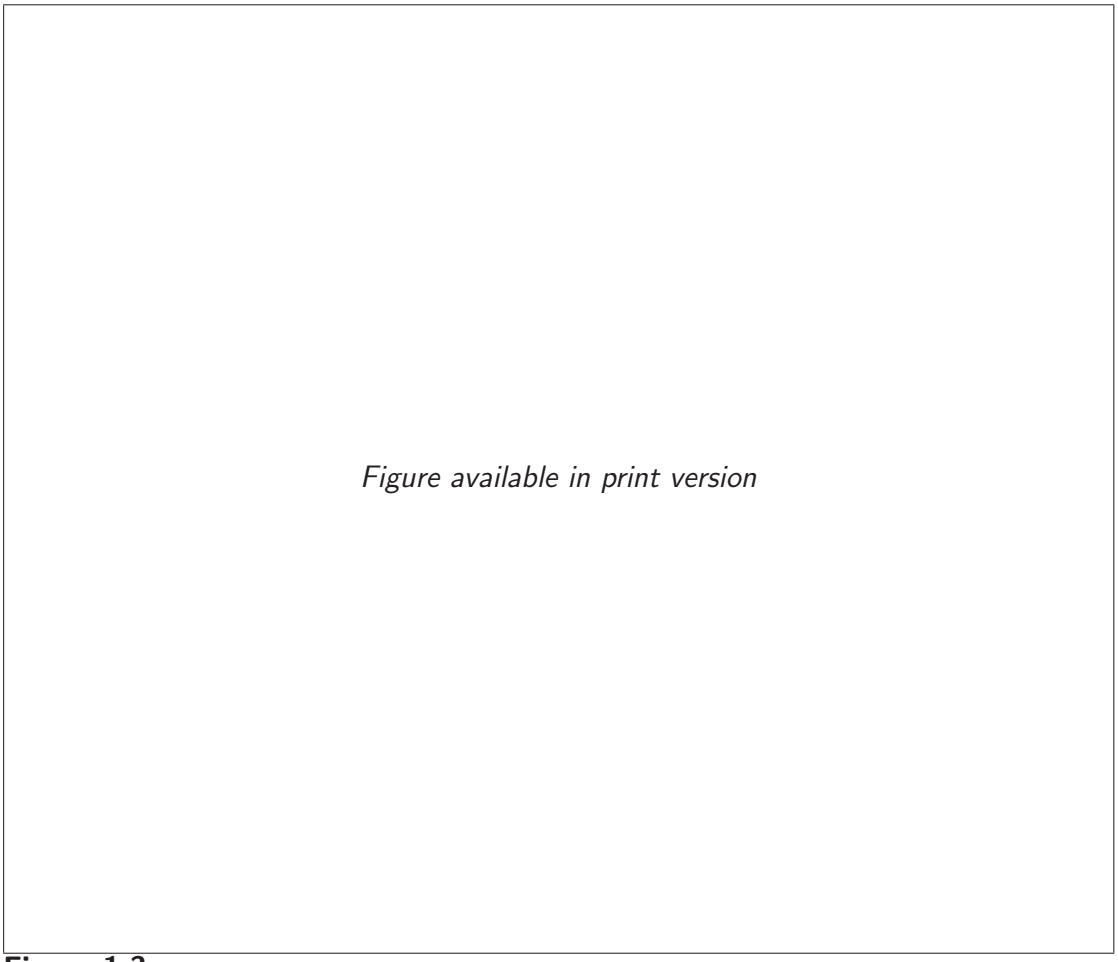

Figure 1.3.
Risk Factors for Chronic Obstructive Pulmonary Disease (COPD)
[Postma et al., 2014]

1.2 What do classic lung function tests tell us?

Classic lung function tests like spirometry measure expiratory flow in the larger airways. A forced exhalation is often performed for that purpose. Some of the advantages of spirometry are high reproducibility, low variability and availability of reference values for different ages throughout childhood and for different ethnic groups [Quanjer et al., 2012].

Valuable information on airway growth has been collected over many years [Kotecha et al., 2013]. One limitation of the method is the necessity of sedating uncooperative children, i.e. infants. It is worth noting that information on flow in the central airways is not the same as information on small airway function or volume of the lung parenchyma. Lungs and airways might grow disproportionately, a concept known as dysanapsis [Green et al., 1974, Martin et al., 1988]. Recently, new tidal-breathing lung function tests became available which do not require sedation, and which deliver additional information.

1.3 Options for measurements during normal breathing

A paediatric setting needs tailored lung function tests [Rosenfeld et al., 2013]. Here in particular, lung function measurements that can be performed during quiet, normal breathing are of great interest. For infants, these are only available in research settings, as they require specific equipment and experienced operators [Frey et al., 2000a, Frey et al., 2000b]. For older children, these tests are becoming more widely available, although experience is still essential [Robinson et al., 2013].

1.3.1 Multiple-breath washout measures lung volume and small airway function

Multiple-breath washout (MBW) is a tidal breathing lung function test of increasing importance and popularity, especially in paediatric respiratory medicine. The principle was described as far back as 1940 [Darling et al., 1940]. MBW allows detection of early lung disease, for example in cystic fibrosis, long before pathologies in the larger airways are detectable by spirometry [Gustafsson et al., 2008]. A tracer is added to the inhaled air and, breath by breath, washed into the lungs. Once it is equally distributed, the washout to 1/40th of the initial concentration can begin. Functional residual capacity (FRC) is calculated as cumulative expired volume (CEV) of tracer gas divided by the difference between end-tidal tracer concentrations at the start and end of washout. The lung clearance index (LCI), calculated as the number of turnovers of FRC needed to reach the end of

washout, is an established parameter of overall ventilation homogeneity. An international consensus on how to perform and analyse MBW was published recently [Robinson et al., 2013]. In infants, sulfur hexafluoride (SF_6) is the age-appropriate tracer. In children beyond infancy, helium (He) or nitrogen (N_2) can be used. N_2MBW allows analysis of the slopes of alveolar phase III (SIII) of the N_2 expirogram. The first SIII reflects the diffusion-convection-dependent acinar airways (Sacin), and SIII values from lung turnover 1.5 to 6.0 reflect ventilation homogeneity within convection-dependent conducting airways (Scond).

Initially, a mass spectrometer was required for direct determination of the tracer gas concentration. Nowadays, tracer concentration can be determined indirectly using an ultrasonic flow meter (USFM), which allows widespread application in specialised research laboratories.

At our paediatric lung function laboratory, we specialised in MBW in children of all age groups. While older children usually cooperate, measurements in infants require sleep phases without dreaming in order to ensure quiet breathing. We regularly perform measurements in infants as young as 5 weeks in natural sleep, i.e. without sedation. Analyses were optimised [Latzin et al., 2007b, Anagnostopoulou et al., 2015], and normative values for FRC and LCI were published [Fuchs et al., 2011a]. A typical MBW measurement in an infant at our paediatric lung function laboratory is shown in figure 1.4.

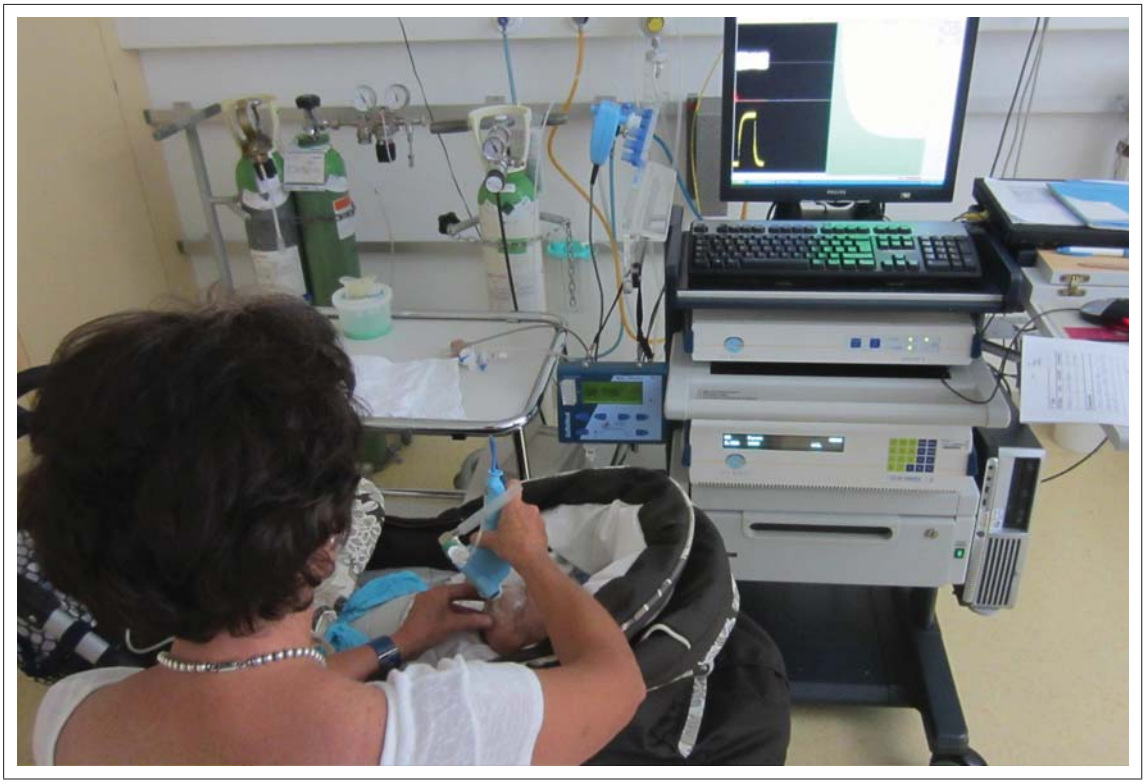

Figure 1.4.
Multiple-breath washout in an infant sleeping naturally

1.3.2 Tidal breathing parameters

In infants, tidal breathing flow volume loops (TBFVL) and the fraction of exhaled nitric oxide (FeNO) can be measured in unsedated sleep using a USFM. Normative values for tidal volume, respiratory rate, minute ventilation and time to peak tidal expiratory flow from our paediatric lung function laboratory are available [Fuchs et al., 2011a].

1.3.3 Plethysmography

Plethysmography is an established technique which allows measurements of intra-thoracic lung volume. It is based on the law of Boyle-Mariotte [Criée et al., 2011]. Most young children are able to cooperate and perform the procedure successfully. In an air-tight chamber, intra-thoracic gas volume is calculated from relative changes in pressure. The child breathes through a mouthpiece which is the only opening of the chamber. A very short occlusion of this opening is induced, and the child attempts to continue breathing. During the attempted breathing motion, the intra-thoracic air is compressed and expanded, which leads to changes of pressure in the chamber. The latter can be measured, and allows calculation of lung volume at the end of expiration. The measurement is performed in triplicates. For adults, variability of single trials should be within 5%. This seems a reasonable approach for older children as well, but for younger children, a greater variability certainly needs to be accepted.

1.4 BILD study - Basel & Bern Infant Lung Development

As early lung development is only poorly understood, an observational study was initiated in Switzerland to investigate predisposing and modifying factors of respiratory morbidity in early childhood. The so-called Basel & Bern Infant Lung Development (BILD) study began at the Children's University Hospital at Inselspital Bern in 1999, and recruitment has been ongoing ever since. Recently, the study expanded to a second site, the Children's University Hospital Basel (UKBB). The study was described in details by Fuchs et al. [Fuchs et al., 2012a].
In this population-based study, it was essential to use non-invasive lung function tests, where variability can be higher than in tests using sedation and forced manoeuvres.
Term-born and preterm children were recruited and information on family history, exposures, and respiratory symptoms was collected using standardised interviews and questionnaires. Whenever possible, we obtained DNA samples from cord blood or buccal swabs. Many results were previously published, including new recommendations for non-invasive lung function testing [Frey, 2001, Latzin et al., 2007b], and interesting comparisons of

respiratory morbidity between term-born and preterm infants [Roiha et al., 2007, Latzin et al., 2009b, Pramana et al., 2011].
The aim of this cohort study was to improve our understanding of early lung development and to create new strategies to improve the respiratory health of individuals at risk and the general population.

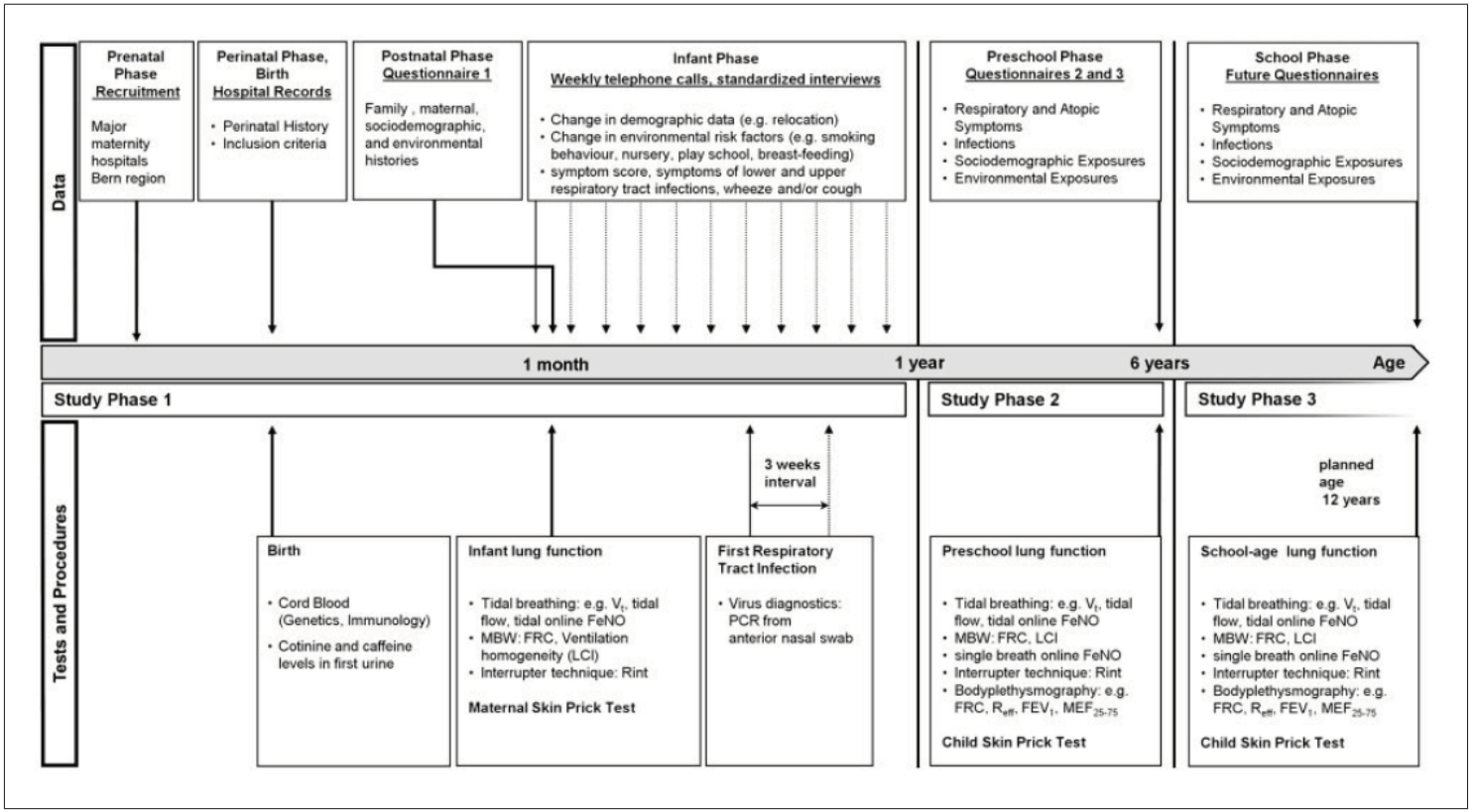

Figure 1.5.
Bern & Basel Infant Lung Development (BILD) study
The BILD cohort: time-flow of recorded data, as well as of tests and procedures performed during the follow-up. Vt: tidal volume; FeNO: fraction of exhaled nitric oxide; MBW: multiple breath washout; FRC: functional residual capacity; LCI: 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; MEF25-75: midexpiratory flow [Fuchs et al., 2011a]

1.5 Aims of this work

The accuracy of the relatively new setups for MBW using SF_6 in infants and using helium in young children was not tested previously. However, using an improved lung model with quasi-physiologic conditions, this was now possible. Such validation studies were requested by the current consensus on inert gas washout [Robinson et al., 2013], they ensure that measurements are performed with high quality. We hypothesised that both setups measure FRC reliably and accurately.

Using these newly validated and other established lung function tests in a prospective cohort study, we were able to investigate early lung volume increase in unselected term-born children. A big advantage for application in a population-based study is that MBW is non-invasive. Information on lung volume increase early in childhood was lacking, and this new knowledge would allow a better understanding of early lung development. We hypothesised to find tracking of lung volume.

In preterm infants, early biomarkers for later respiratory morbidity are needed to identify infants at risk and to improve clinical care. We hypothesised that prediction might be more accurate when infant lung function results were added to established clinical tools, and that infant lung function parameters were altered in individuals with respiratory symptoms like wheeze during the first year of life.

School-age children after premature birth show higher respiratory morbidity and altered spirometric lung function results compared to their peers, but until now, small airway function was not described in detail in a larger population with heterogeneous neonatal characteristics. Nitrogen MBW enabled us to measure small airway function in a relatively large population, including ventilation on the overall, conductive and acinar level. We hypothesised to find inhomogeneous ventilation in preterm children.

Whether or not physiological lung volume increase is altered in preterm children was previously unknown. Data on spirometry showed tracking over time, but data on FRC was not available. As MBW can be performed without sedation in individuals of all ages, we were able to assess FRC over time in a population of preterm children. We hypothesised to find different lung growth characteristics in preterm children compared to term-born controls, and to find catch-up growth.

The following specific aims will be addressed in this work:

1. Investigate the accuracy of multiple-breath washout equipment using the tracers SF_6 and helium for measuring lung volumes in infants and young children
2. Test for tracking of lung volume from infancy to early childhood in term-born children, i.e. for an association of lung volumes in infancy with lung volumes in early childhood; test which factors were associated with lung volume and whether or not airways and lung tissue grow disproportionately
3. Test whether lung function at the corrected gestational age of 44 weeks in preterm infants, using physiological breathing tests, helps to predict respiratory symptoms, and whether altered infant lung function was associated with respiratory symptoms during 1st year of life
4. Test for abnormal ventilation distribution at school age in preterm children, especially in the peripheral lung regions
5. Test for tracking and catch-up growth of lung volumes from infancy to early childhood in preterm children, and investigate which factors have an impact on early lung growth in preterm children

2 Validation of multiple-breath washout equipment for infants and young children

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2.1 SUMMARY

Introduction: The new ATS/ERS consensus report recommends in vitro validation of multiple-breath inert gas washout (MBW) equipment based on a lung model with simulated physiologic conditions. We aimed to assess accuracy of two MBW setups for infants and young children using this model, and to compare functional residual capacity (FRC) from helium MBW (FRC_{MBW}) with FRC from plethysmography (FRC_{pleth}) in vivo.
Methods: The MBW setups were based on ultrasonic flow meter technology. Sulphur hexafluoride and helium were used as tracer gases.We measured FRC in vitro for specific model settings with and without carbon dioxide and calculated differences of measured to generated FRC. For in vivo evaluation, difference between FRC_{MBW} and FRC_{pleth} was calculated in 20 healthy children, median age 6.1 years. Coefficient of variation (CV) was calculated per FRC.
Results: In the infant model (51 runs, FRC 80 – 300 ml), mean (SD) relative difference between generated and measured FRCs was 0.7 (4.7) %, median CV was 4.4% for measured FRCs. In the young child model, one setting (8 runs, FRC 400 ml) showed a relative difference of up to 13%. For the remaining FRCs (42 runs, FRC 600 – 1,400 ml), mean (SD) relative difference was -2.0 (3.4) %; median CV was 1.4% for measured FRCs. In vivo FRC_{pleth} exceeded FRC_{MBW} values by 37% on average.
Conclusions: Both setups measure lung volumes in the intended age group reliably and reproducibly. Characteristics of different techniques should be considered when measuring lung volumes in vivo.

2.2 INTRODUCTION

Multiple-breath inert gas washout (MBW) is of special interest in infants and children. This tidal breathing pulmonary function test allows measurement of lung clearance index (LCI), a marker of ventilation heterogeneity that is of growing relevance for researchers and clinicians working with children with conditions such as cystic fibrosis (CF) and primary ciliary dyskinesia. Originally, this method required a mass spectrometer [Robinson et al., 2009, Gustafsson, 2005, Aurora et al., 2005a]. Nowadays, devices based on ultrasonic flow meters (USFM) that measure the molar mass (MM) in order to derive the respired tracer gas concentration are commercially available, and allow widespread application. Several investigators have found the technique to be reliable and feasible in healthy and diseased children with a regular breathing pattern [Schibler et al., 2002, Pillow et al., 2004, Fuchs et al., 2012b]. LCI measurement, calculated as cumulative expired volume (CEV) over functional residual capacity (FRC), directly relies on correctly measured lung volumes. Therefore the current ATS/ERS consensus for MBW recommends in vitro validation of MBW derived lung volumes [Robinson et al., 2013]. Most previous attempts for validation of sulfur hexafluoride (SF_6) and helium MBW equipment were based on in vivo data or on in vitro data without simulated physiologic conditions regarding temperature, humidity, or carbon dioxide (CO_2) [Schibler et al., 2002, Fuchs et al., 2012b, Wauer et al., 2003, Scalfaro et al., 2000, Proquitte et al., 2006, Fuchs et al., 2009]. However a new rigid lung model was developed recently, simulating physiologic conditions encountered during clinical testing, such as body temperature, pressure, and humidity (BTPS) over the breath cycle. This model has been successfully used to validate a nitrogen MBW setup [Singer et al., 2012a]. The aims of this study were twofold: first, to use this lung model to test accuracy of two available MBW setups using age-appropriate tracer substances: one setup using SF_6 as a tracer gas for MBW in infants (MBW_{SF6}), and one setup using helium for MBW in young children (MBW_{He}); and second, to gain more physiological insight by comparing FRC from MBW_{He} (FRC_{MBW}) in vivo with FRC derived by plethysmography (FRC_{pleth}) in young children. We hypothesized that the difference between generated FRC and measured FRC would be within 10%.

2.3 MATERIALS AND METHODS

Study Design

First, MBW setups for both infants and young children were tested in vitro for accuracy of FRC determination using a realistic lung model [Singer et al., 2012a]. Second, FRC_{MBW} was compared with FRC_{pleth} in 20 young children. The children's age ranged from 5.9 to 6.9 years; further characteristics are shown in Table 1. Inclusion criteria were Caucasian ethnicity and term delivery. General exclusion criteria were severe maternal health problems or drug abuse other than nicotine, major birth defects, or perinatal disease, previously abnormal spirometry, respiratory tract infection within 2 weeks prior to the study, and any other known conditions likely to affect lung function. This study was approved by the Ethics Committee of the Canton of Bern, Switzerland. The children's assent was obtained and parents or caregivers provided written informed consent for this study. Primary outcome was the difference between measured FRC and the known nominal FRC generated with the model. Secondary outcomes were intra-test repeatability of FRC measurements, and the difference between FRC_{MBW} and FRC_{pleth}.

Table 2.1.
Characteristics of Children for Helium MBW and Plethysmography In Vivo Studies

		20 Healthy children
Age	Median (range) in years	6.1 (5.9–6.9)
Female	Number (%)	13 (65)
Body weight	Median (range) in kg	22.5 (17.7–35.8)
Height	Median (range) in cm	119 (107–138)
White ethnicity	Number (%)	20 (100)
Tobacco smoke exposure	Number (%)	3 (15)

Hardware and Software

A commercially available apparatus containing USFM sensors (Exhalyzer D, Eco Medics AG, Duernten, Switzerland) was used. We used established tracer gases for MBW in infants and children that is, for the infant setup 4% SF_6, and for the young child setup 20% helium. Both are medical gases produced by Carbagas, Bern, Switzerland (details in Supplemental online figures). Availability of inert test gases may vary between countries. Approval for testing with SF_6 depends on national regulations [Robinson et al., 2013]. Apparatus and procedure for infants have been described previously [Schibler et al., 2002, Latzin et al., 2007b, Fuchs et al., 2011a]. For young children, the same apparatus was used in a slightly different setup as provided by the manufacturer. Schematic layout of both setups can be found in the online supplement (Supplementary Figs. 1 and 2). In both setups, tracer concentration was derived by measuring MM of in- and exhaled gases. Mainstream MM was the primary signal used in infants and side stream MM in young children. Flow and volume were measured by the mainstream USFM in both setups. In the young child setup, CO_2 concentration was measured in addition to other signals via side stream. Stable end-tidal CO_2 concentrations were used as a quality control proxy for sustained regular tidal breathing.

Daily calibration of volumes, two-point calibration of CO_2 sensor, and verification of adequate MM change and flow when adding tracers were performed. All MBW tests were recorded and analyzed using commercially available software (WBreath, ndd Medical Technologies, Zurich, Switzerland). Corrections for BTPS conditions and signal alignment were conducted as previously described [Latzin et al., 2007b]. FRC was calculated as CEV of tracer gas divided by the difference between end-tidal tracer gas concentrations at the start and end of MBW.

Lung Model

The lung model established by Singer et al. [Singer et al., 2012a], an improvement on an existing model without BTPS conditions [Brunner et al., 1985], is built of two water tanks. The inner tank contains two compartments: one lung compartment, that is open and can be set to different specific lung volumes and another communicating compartment that enables mechanical ventilation.We used two different inner tanks (Soloplex,

Tidaholm, Sweden), a smaller infant and a larger young child tank. The outer tank serves to heat the inner chamber to 37°C using a thermostat in order to generate BTPS conditions. The inner chamber was filled with water until the desired FRC was achieved, measured as the end-expiratory water level using a vertical tape measure. Each FRC was determined geometrically: 1mm change of water level corresponds to 4.8 ml in the infant lung model and 18.2 ml in the young child lung model. A continuous flow, pressure-controlled infant ventilator (Acutronic, Medical Systems AG, Hirzel, Switzerland) was used to ventilate the lung compartment and thus generate specific FRCs with physiological ranges for tidal volumes, and respiratory rates (see Table 2). All in vitro measurements were performed by A.S. and supervised by T.R. and F.S. In vitro assessment of the infant MBW_{SF6} system was undertaken across six different nominal FRCs ranging from 80 to 300 ml (n=51, minute ventilation 900 – 3,600 ml). The young child MBW_{He} system was evaluated across six different FRC settings, ranging from 400 to 1,400 ml (n=50, minute ventilation 3,250 – 6,000 ml). FRC settings were measured with and without CO_2 in the model. For runs with CO_2, pure CO_2 from cylinders (Carbagas) was bubbled at a flow of 0.7 – 2.7 ml/s in order to establish stable and quasi-physiological end-tidal CO_2 fractions ranging from 2.4% to 4.4%. Added CO_2 volume in the infant model was corrected by applying standard flow baseline correction. In the larger model, added CO_2 volume was estimated by the product of test duration and CO_2 flow and subtracted from measured FRC.

Table 2.2.
In Vitro Settings of the Infant and Young Child Setup

Approximate age group [a]	Generated FRC	Respiratory rate	Tidal volume
Infant model			
Newborn	80	60	35
2 months	95	30	50
3 months	110	40	25
8 months	160	30 and 60	60
1 year	215	30 and 60	30
2 years	300	30 and 40	45
Young child model			
3 years	390	25	130
5 years	590	25	170
6 years	810	20	220
7 years	1,000	20	240
8 years	1,200	15	400
10 years	1,430	15	400

Generated FRC (ml) is the functional residual capacity that was determined from the known dimensions of the lung compartment within the inner tank. Respiratory rate (min^{-1}) and tidal volume (ml) were regulated using a ventilator.
[a] Age estimates of FRC values were based on published reference values [Fuchs et al., 2011a, Lum et al., 2011].

Comparison of Lung Volume Measurements In Vivo

To gain more physiological insight in vivo, we compared lung volumes measured using different pulmonary function tests in healthy children aged 5 – 6 years participating in an ongoing study at the University Children's Hospital Bern [Fuchs et al., 2012a]. All children performed MBW in triplicate using the helium setup described earlier in this manuscript, MBW_{He} was conducted according to recent consensus [Robinson et al., 2013]. After

MBW_{He} measurements, children performed plethysmography using JAEGER® MasterScreen® Body (CareFusion, Wuerzburg, Germany) according to standards, applied by a trained operator [Wanger et al., 2005]. MBW_{He} and plethysmography were both performed with the child sitting upright. Means of FRC_{MBW} and FRC_{pleth} were calculated from two to three technically acceptable tests.

Statistical Methods

We assessed accuracy of FRC measurements in vitro by calculating absolute (ml) difference (measured FRC generated FRC), relative (%) difference (absolute difference * 100/generated FRC), and limits of agreement (mean difference ± 1.96 SD of differences). Differences were assessed according to Bland and Altman [Bland and Altman, 1986]. Intra-test variability of both generated and measured FRCs was calculated as intra-test coefficient of variation (CV = SD/ mean *100) per specified lung volume. Median values and interquartile range (IQR), that is, the range from percentile 25 to 75, of CV are reported. In vitro parallax error, that is, the possible error of reading water levels, was assumed to be one mm corresponding to 4.8 ml for the smaller and 18.2 ml for the larger model. For the in vivo part, the difference between FRC measurement techniques was assessed as absolute and relative difference between FRC_{pleth} and FRC_{MBW}. CV of FRC_{MBW} and FRC_{pleth} were calculated per individual. As currently no consensus exists regarding acceptable differences in pediatric MBW, we arbitrarily defined error cut-offs of 10% in vitro and expected difference of 20% in vivo. All analyses were performed using Stata^{TM} (Stata Statistical Software: Release 11. College Station, TX: StataCorp LP).

2.4 RESULTS

Infant Lung Model

Accuracy of FRC measurement of the MBW_{SF6} setup with six generated FRCs between 80 and 300 ml is displayed in Figure 1A and B. We found high accuracy in the infant setup with a mean (95%CI) absolute difference between model and measured FRC of 1.9 (0.3 – 3.5) ml, corresponding to a relative difference of 0.7 (-0.6 to 2.0) %. Median CV was 2.2 (IQR 1.9 – 2.8) % for generated FRCs and 4.4 (IQR 3.1 – 6.8) % for measured FRCs. CV for runs with and without CO_2 is displayed in Table 3.

Young Child Lung Model

Accuracy of FRC measurement of the MBW_{He} setup with generated FRCs between 400 and 1,400 ml is displayed in Figure 2A and B. For the lowest FRC setting in the model (n=8, FRC 400 ml), accuracy was beyond predefined error cut-off ($\pm 10\%$) in most runs. Relative difference was between -12% and $+13\%$. For all other settings of the model (n=42, FRC 600 – 1,400 ml), mean (95%CI) absolute difference was -17 (-25 to -8.3) ml corresponding to a relative difference of -2.0 (-3.0 to -0.9) %. For runs without CO_2, absolute difference was -8.1 (-15 to -1.6) ml and relative difference was -1.3 (-2.3 to -0.3) %. In runs with CO_2, absolute difference was -26 (-41 to -10) ml; relative difference was -2.7 (-4.6 to -0.8) %. Median CV was 0.2 (IQR 0–0.6) % for generated FRCs and 1.4 (IQR 0.6– 2.1) % for measured FRCs. CV for runs with and without CO_2 is displayed in Table 3.

Table 2.3.
In Vitro Intra-Test Variability of FRC

		Measured FRC		
	Generated FRC	All runs	Runs without CO_2	Runs with CO_2
Infant model	2.2 (1.9–2.8)	4.4 (3.1–6.8)	1.2 (1.2–1.6)	6.0 (3.1–6.5)
Young child model	0.2 (0–0.6)	1.4 (0.6–2.1)	0.6 (0.4–0.8)	2.1 (1.8–2.7)

Median (IQR) coefficient of variation in % for all in vitro multiple-breath washout (MBW) runs (n=51) of the infant MBW_{SF6} setup and for runs with functional residual capacity (FRC) between 600 and 1,400 ml (n=42) of the young child MBW_{He} setup.

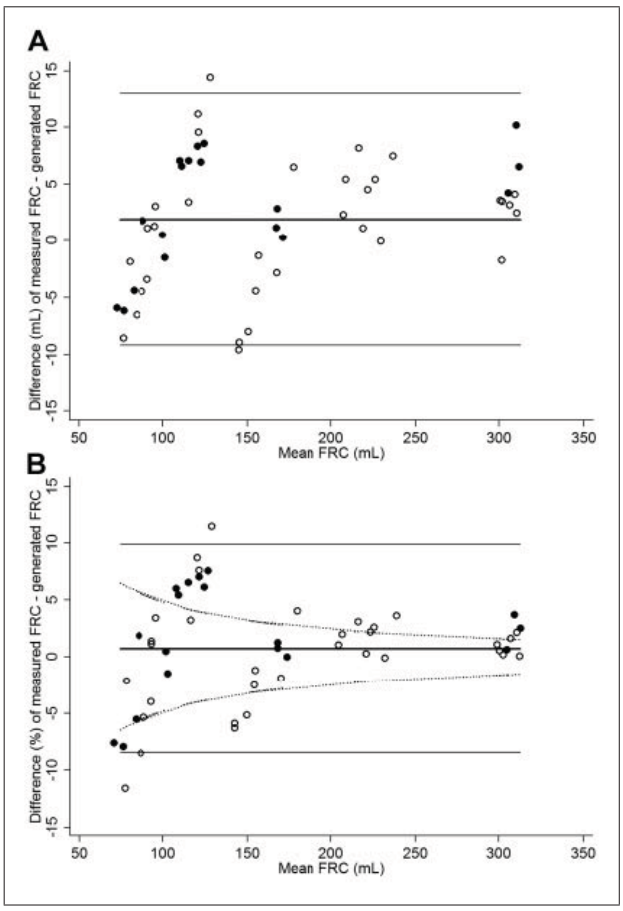

Figure 2.1. A and B
Accuracy of in vitro functional residual capacity (FRC) measurement for the infant setup using sulfur hexafluoride (n=51)
Bland Altman plot [Bland and Altman, 1986] of measured FRC minus generated FRC plotted versus mean of measured and generated FRC. Closed circles represent runs without CO_2; open circles represent runs with CO_2. The thick line shows mean difference, thin lines show upper and lower limits of agreement. A: Mean (95%CI) absolute difference was 1.9 (0.3 – 3.5) ml, upper and lower limits of agreement were 13 and -9.2 ml. B: Mean (95%CI) relative difference was 0.7 (-0.6 to 2.0) %, upper and lower limits of agreement were 9.9% and -8.4%. The dotted line represents possible nominal volume bias due to reading parallax error (1mm, i.e., ± 4.8 ml).

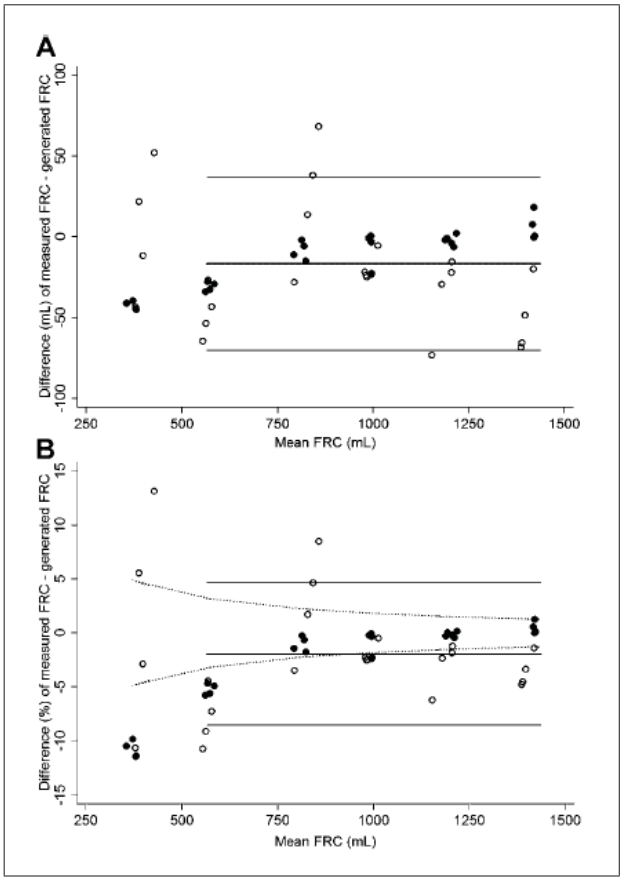

Figure 2.2. A and B
Accuracy of in vitro functional residual capacity (FRC) measurement for the young child setup using helium (n=50)
Bland Altman plot [Bland and Altman, 1986] of measured FRC minus generated FRC plotted versus mean of measured and generated FRC. Closed circles represent runs without CO_2, open circles represent runs with CO_2. The thick line shows mean difference for FRCs between 600 and 1,400 ml, thin lines show upper and lower limits of agreement. A: Mean (95%CI) absolute difference for FRCs from 600 to 1,400 ml was -8.1 (-15 to -1.6) ml in runs without CO_2, and -26 (-41 to -10) ml in runs with CO_2. Upper and lower limit of agreement for runs with and without CO_2 were +37 and -70 ml. B: Mean (95%CI) relative difference for FRCs from 600 to 1,400 ml was -1.3 (-2.3 to -0.3) % for runs without CO_2, and -2.7 (-4.6 to -0.8) % for runs with CO_2. Upper and lower limit of agreement for runs with and without CO_2 were 4.6% and -8.6%. The dotted line represents possible nominal volume bias due to reading parallax error (1mm, i.e., $\pm 18.2ml$).

Lung Volume Measurements In Vivo

FRC_{pleth} exceeded FRC_{MBW} in all children. Mean (SD) FRC_{pleth} and FRC_{MBW} were 1,075 (141) and 676 (119) ml, respectively. The relation of FRC_{pleth} and FRC_{MBW} is displayed in Figure 3. Difference was beyond the expected range ($\pm 20\%$). Mean absolute (relative) difference was 399 ml (37%), upper limit of agreement was 596 ml (52%), and lower limit of agreement was 201 ml (22%).

In vivo intra-test variability of FRC measured by MBW_{He} and plethysmography was higher compared to in vitro intra-test variability of FRC measured by MBW. In vivo median CV for FRC_{MBW} and FRC_{pleth} was 7.4 (IQR 4.4–11.4) %and 6.5 (IQR 3.8–13.4) %, respectively. No adverse events occurred in our studies.

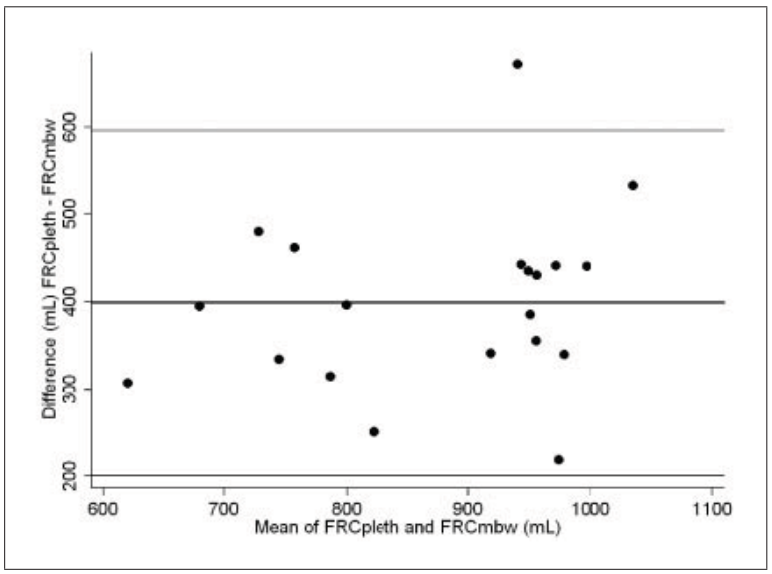

Figure 2.3.
Comparison of functional residual capacity (FRC) measured by helium multiple-breath washout (FRC_{MBW}) and by plethysmography (FRC_{pleth}) in 20 healthy young children
Bland Altman plot [Bland and Altman, 1986] of FRC_{pleth} minus FRC_{MBW} plotted versus mean of both measurements. FRC_{pleth} values exceeded FRC_{MBW} values in all children. The thick line represents the mean absolute difference of 399 ml (corresponding to a mean relative difference of 37%), the thin lines show upper limit of agreement (596 ml resp. 52%), and lower limit of agreement (201 ml resp. 22%).

2.5 DISCUSSION

This is the first in vitro study assessing accuracy of USFM setups for MBW_{SF6} and MBW_{He} according to the current consensus on inert gas washout testing [Robinson et al., 2013]. We found that FRC can be measured within 10% accuracy over FRC volumes between 80 and 300 ml with the infant setup and between 600 and 1,400 ml with the young child setup. Intra-test repeatability of measured FRC in vitro is 4.4% in the infant setup and 1.4% in the young child setup. We confirm that lung volumes and realistic breathing patterns can be simulated in this lung model and that it is suitable for bench-testing USFM setups, as recommended by the consensus statement. As expected, FRC measured by MBW_{He} and plethysmography in young children differ systematically, but to an unexpected extent, suggesting considerable influence from physiological FRC variability, trapped gas and different measurement techniques [Pillow et al., 2004, Miller et al., 1995, Jensen et al., 2013].

Accuracy of MBW for FRC Measurements and Comparison With Other Setups
Our in vitro results show good agreement for generated and measured infant lung volumes. Forty-nine out of 51 runs (96%) met the criteria of measuring FRC within 10% relative difference from generated FRC; mean relative difference between generated and measured volumes was not statistically significant, although the mean absolute difference was significant. Accuracy of FRC measurement for FRCs between 600 and 1,400 ml using the helium setup was also good with 41 out of 42 runs (98%) meeting the criteria of measuring within predefined error cut-off. In the helium trials, runs without CO_2 show a linear trend from discrete under-estimation of FRC in lower lung volumes towards almost no measurement error in higher lung volumes. Ventilator settings are a likely cause, especially the ratio of dead space and tidal volume. For detailed analysis of the single factors contributing to this slight linear trend, a new study with varying ventilator settings would be required. Both absolute and relative differences were small but significant for the helium setup. We assume that none of the significant differences are clinically relevant: Bland Altman plots show that in both setups, measurement error

included over- and under-estimation of the true FRC values; and the mean offset for absolute values was less than ml per kg body weight in the intended age groups. Altogether, the measurement error was within acceptable range for lung volumes between 80 and 300 ml, and between 600 and 1,400 ml.

We can only speculate on possible reasons for the higher relative error observed in the smallest nominal FRCs of 400 ml in the young child model. In all in vitro trials, error might be caused by inaccurate determination of dead space and water level; the impact on relative error is higher in smaller lung volumes. Non-linear flow dependent temperature and humidity fluctuations, and volume drift due to CO_2 administration are likely to contribute as well. The error introduced by fixed (and not flow-dependent) delay corrections for side stream MM is higher in smaller lung volumes. Bland Altman plots show that relative error decreases with increasing FRC in both setups. It has been shown previously that accuracy of FRC measurements is lower in smaller lung volumes [Singer et al., 2012a].

Many prior studies validating MBW_{SF6} and MBW_{He} equipment were based on in vivo studies or on in vitro models with neither BTPS conditions nor CO_2 [Schibler et al., 2002, Fuchs et al., 2012b, Wauer et al., 2003, Scalfaro et al., 2000, Proquitte et al., 2006, Fuchs et al., 2009]; our study evaluates in vitro FRC data derived in simulated physiologic conditions using SF_6 and helium. Comparable but preliminary in vitro FRC data using a similar model come from other USFM setups (EasyOne Pro, ndd Medical Technologies) and customized photo-acoustic sensors (Innocor, Innovision, Odense, Denmark) as well as mass spectrometers (AMIS 2000, Innovision) [Fuchs et al., 2011b, Gonem et al., 2012, Singer et al., 2012b]. The technical error from the current USFM setup is, however, higher than that published for an available nitrogen MBW setup (Exhalyzer D, Eco Medics AG) where error was within 5% for 98% of the runs in the large lung model and within 7% for the small model. Comparable to our study, tidal flow was measured using a mainstream USFM. Nitrogen concentration was measured indirectly via oxygen and CO_2 sensors [Singer et al., 2012a]. A limitation of our study is that results from the USFM were not compared to results derived via mass spectrometry. Other investigators compared in vivo MBW results derived by mass spectrometry to those derived using an USFM in infants [Pillow et al., 2004] and adults [Fuchs et al., 2006] and found signifi-

cant differences. Possible Limitations of the Current Setup The conditions of the in vitro model are quasi physiologic and may have some limitations. Even though we monitored the temperature within the model in order to assure stable BTPS conditions we cannot rule out, for example, different temperature dynamics compared to in vivo conditions. The low variability of FRC measurements without CO_2 indicates stable model conditions regarding generated lung volumes, temperature, and humidity. Intra-test variability of FRC measurements is slightly higher when CO_2 is added in the model, and it is known that regard should be paid to CO_2 levels in MBW [Fuchs et al., 2006]. The impact of CO_2 bubbled into the model was higher in the larger helium setup which is why we subtracted additional CO_2 volume. We assume that the additional gas in the model limits complete washin of the tracer and thus influences measurement of FRC. This phenomenon might be caused by varying CO_2 volumes added, different diffusivity of the tracer gases, and a consecutively uneven gas distribution within the helium model. In vivo, irregular CO_2 levels, for example, hyperventilation, may also influence FRC and LCI measurements, as it is the expired CO_2 fraction that mainly determines the MM of expired air. This is unlikely to happen in sleeping infants, but more likely in older children due to limited cooperation. BTPS correction algorithms might also need refinement (Singer F 2012). Some of the breathing parameters we chose to bench test the MBW setups were less physiological with rather low tidal volumes and high respiratory rates. Extreme breathing patterns, for example, high respiratory rate, can impact on FRC measurements in most MBW setups as delay and BTPS correction factors are fixed. Such extreme breathing patterns combined with fixed corrections would cause an under-estimation of the true accuracy encountered during quiet tidal breathing. Bench-testing MBW setups with these extreme settings enabled us to observe technical limitations and posit that flexible corrections would be desirable. The small but significant mean absolute differences between generated and measured FRC are clinically irrelevant for both setups. Software alignments for parameters influencing the MM signal are crucial for correct calculation of FRC. We conducted data analysis with appropriate care, but had only restricted insight to details of internal calculations of the software.

Comparison of Lung Volume Measurements In Young Children

We applied plethysmography and MBW_{He} to explore physiological and technical factors influencing FRC measurements. FRC values from plethysmography are within expected normal range (Hibbert ME 1989, Rosenthal M 1993). FRC_{pleth} shows consistently higher results than FRC_{MBW}. Intra-individual discrepancy of FRC measurements of 20% and more using different techniques has been described earlier for infants and older children [Pillow et al., 2004, Jensen et al., 2013, Owens et al., 2011]. Our findings confirm previous data in children: mean differences between FRC_{MBW} using SF_6 and FRC_{pleth} were 28% in healthy children and 39% in children with CF aged – years 20; and 13% in healthy children and 40% in children with CF aged 6-10 years [Owens et al., 2011]. In healthy school-aged children, mean FRC difference was 8.5 ml/kg body weight [Lum et al., 2011]. There are only few published studies evaluating in vivo tests of MBW_{He} and unfortunately FRC values were not reported [Wang et al., 2013, Vermeulen et al., 2013]. One group of investigators used a different setup with quartz tuning forks to detect density of the air, the main outcome was accuracy of measuring gas concentrations [Wang et al., 2013]. Another group compared LCI results from washin and washout in 130 children [Vermeulen et al., 2013]. LCI showed rather poor agreement, possibly due to technical and physiological factors. It is noteworthy that mainstream instead of side stream MM was analyzed, the latter being more appropriate in children beyond infancy [Robinson et al., 2013]. Helium dilution technique is frequently used for FRC measurements in infants and young children. Due to technical differences, values cannot be directly compared to values from MBW_{He}. However, it is worth mentioning that published FRC values derived by helium dilution were lower than those derived by plethysmography [Stocks and Quanjer, 1995]. It is likely that the high mean difference observed in our study group is explained by a combination of physiologic intra-subject variability of FRC as well as differences in measurement techniques and trapped gas detected. The relative difference is slightly higher than previously observed in healthy children [Jensen et al., 2013, Owens et al., 2011, Lum et al., 2011]. This might be explained by the different age ranges assessed, the use of different tracer gases and customized setups. Whether MBW_{He} or plethysmography technique is more accurately measuring the true range of physiologically variable FRC is unknown and cannot be disentangled based on the current data.

Relevance and Implications

Accuracy of FRC determination needs to be assessed prior to measurement and interpretation of, for example, LCI, an increasingly acknowledged measure of lung function. Knowledge of technical reliability of lung function techniques is fundamental for conducting early intervention studies, for example, in children with CF, and longitudinal studies on lung development in healthy and diseased children.

We tested the setups with age-appropriate tracer substances. This is relevant because tracer gas characteristics differ [Robinson et al., 2013], and the preferred substance depends on the age of the child. For infants, SF_6 is currently preferred as nitrogen MBW may change breathing patterns and MBW_{He} is susceptible to gas leaks [Robinson et al., 2013, Singer et al., 2014a]. In older children, helium compares preferably to SF_6, as it is non-polluting and air-tight setups are available. For routine clinical use in children beyond infancy in the future, nitrogen MBW currently seems very promising [Singer et al., 2014a, Singer et al., 2013, Yammine et al., 2013, Schulzke and Frey, 2013]. However, different rheological characteristics of the tracers might lead to systematic differences in MBW results [Jensen et al., 2013]. Values derived from MBW_{He} might elucidate different aspects of lung physiology than those derived by other tracers, which may hamper interpretation of serial MBW measurements at different time points during childhood. As differences across age-groups will remain in the near future, age- and setup-specific z-scores should be used to overcome this issue, at least partially.

The current software algorithm may need further improvement to better account for variable conditions during MBW, for example, CO_2 level and flow.

2.6 CONCLUSION

The two tested MBW systems allow accurate and reproducible measurement of FRC in the intended age group: the SF_6 setup is appropriate for FRCs between 80 and 300 ml; the helium setup is appropriate for FRC between 600 and 1,400 ml.Accuracy of both systems may be further improved by more adaptive software algorithms. As expected, FRC values measured in young children by MBW_{He} and plethysmography differ, suggesting considerable influence from physiological FRC variability and different measurement techniques. The two systems are appropriate for MBW in infants and in young children of 5 years and more, and serve as valuable tools for clinical and research applications.

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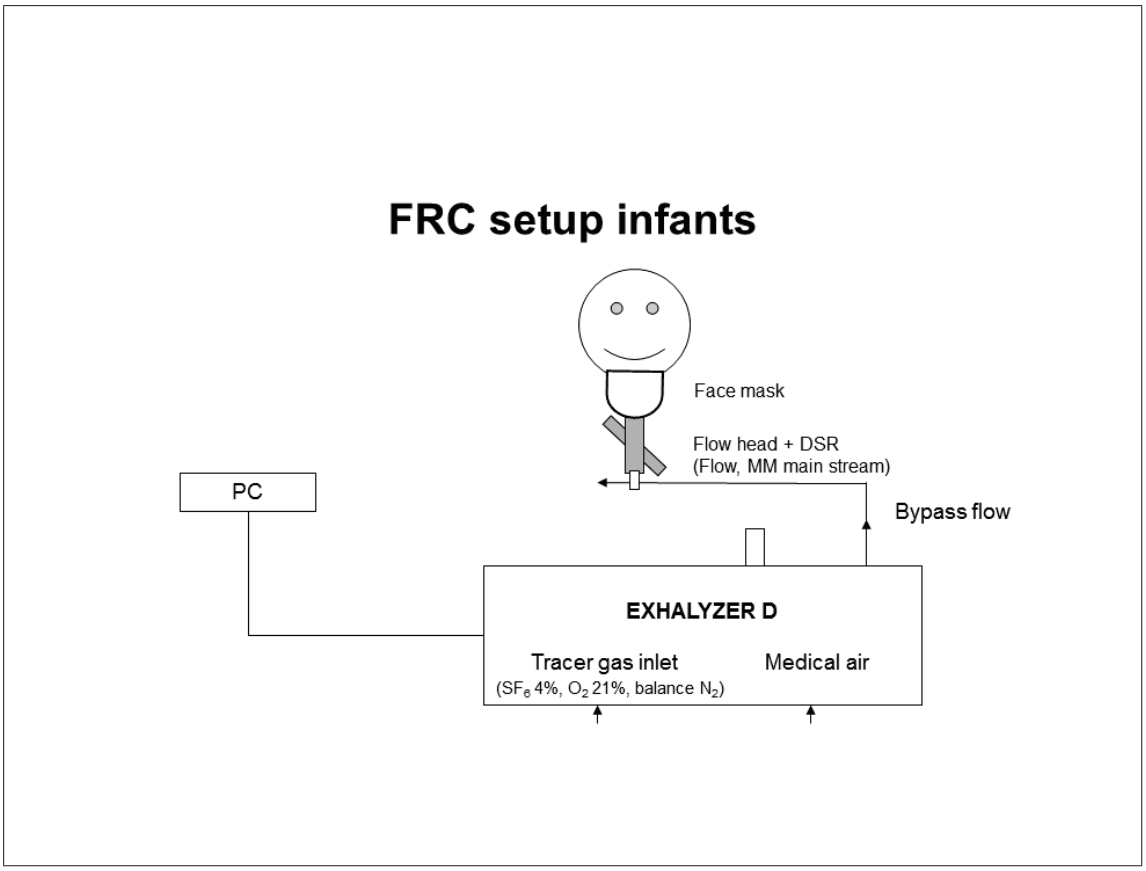
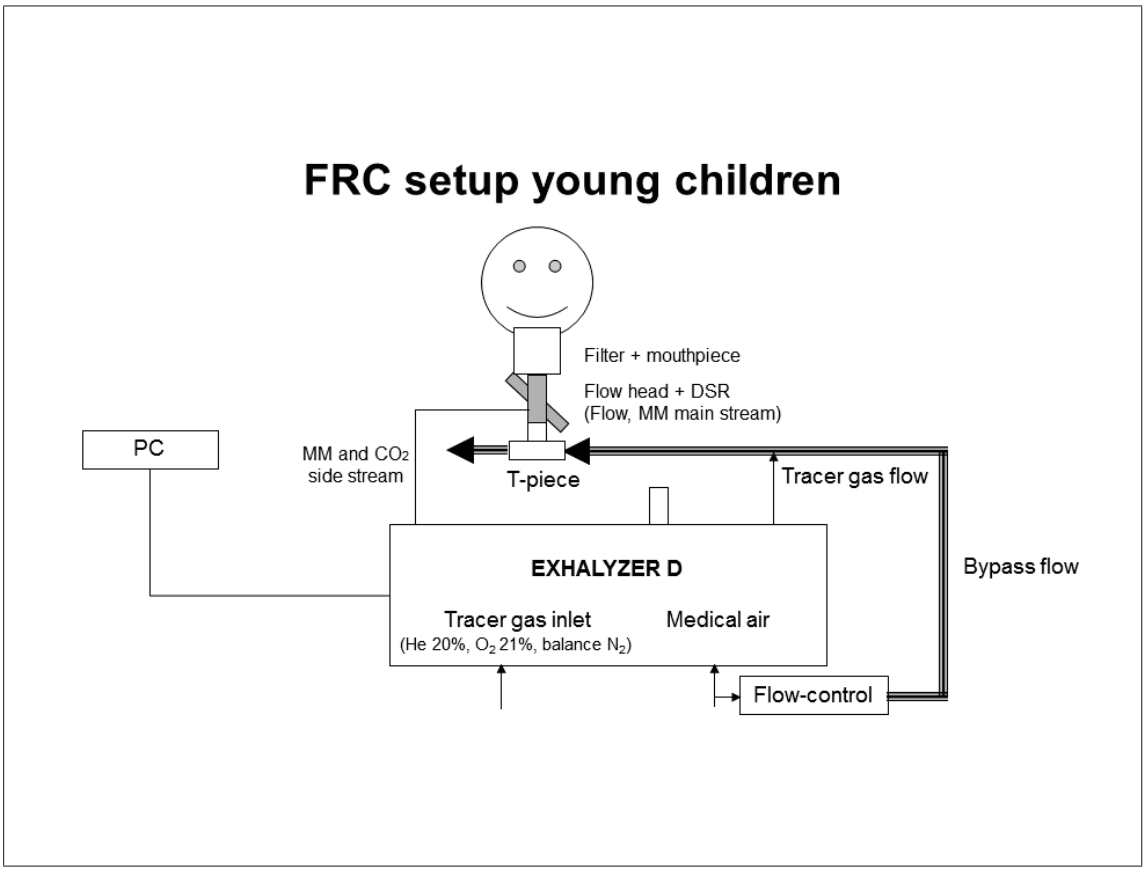
SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher's web-site

2.7 Validation of multiple-breath washout equipment for infants and young children ONLINE SUPPLEMENT



Supplemental online figure 1
Multiple breath washout setup for infants
In the setup for infants, flow and molar mass are measured via the main stream ultrasonic flow meter (USFM). The apparatus (Exhalyzer D, Eco Medics AG, Duernten, Switzerland) and software (WBreath, ndd medical technologies, Zurich, Switzerland) are available. The flow head with dead space reducer (DSR) is applied using a face mask. Sulfur hexafluoride (SF_6) is used as tracer gas produced by Carbagas, Bern, Switzerland (SF_6 4%, oxygen (O_2) 21%, balance nitrogen (N_2). All signals are processed using a personal computer (PC). The figure was provided by the manufacturer.



Supplemental online figure 2
Multiple breath washout setup for young children
In the setup for young children, flow is measured via the main stream ultrasonic flow meter (USFM). In addition, a side stream USFM and infrared carbon dioxide (CO_2) sensor measure molar mass and CO_2, respectively. Temperature and humidity of exhaled air for side stream USFM are equilibrated with room air due to characteristics of the tube (Nafion, Perma Pure LLC, Toms River, USA). The apparatus (Exhalyzer D, Eco Medics AG, Duernten, Switzerland) and software (WBreath, ndd medical technologies, Zurich, Switzerland) are available. The flow head with dead space reducer (DSR) is applied using a bacterial filter and snorkel mouth piece. Helium is used as tracer gas produced by Carbagas, Bern, Switzerland: A mixture of 22% oxygen (O_2) and 78% helium is blended with medical air containing balance nitrogen (N_2) to achieve a helium concentration of 20%. All signals are processed using a personal computer (PC). The figure was provided by the manufacturer.

3 Early lung growth in term-born children: Weak tracking of end-expiratory lung volume from infancy to early childhood

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Manuscript ready for submission

3.1 ABSTRACT

Background

Chronic lung disease may originate in early childhood. Understanding physiological lung growth and its influencing factors facilitates the development of preventive strategies for respiratory health in children and adults. We investigated whether functional residual capacity (FRC) tracks throughout childhood, and which factors have an effect on lung growth.

Methods

We performed longitudinal measurements of FRC without sedation at the ages of 5 weeks and 6 years in term-born children from a prospective birth cohort. We used multiple-breath washout (MBW) at the first visit, and plethysmography at the second visit. Main outcome was plethysmographic FRC (FRC_{pleth}) at 6 years. We tested tracking of lung volumes, i.e. association of FRC at both visits, and factors potentially influencing lung growth, by regression analysis.

Results

In 124 children, we observed weak tracking of FRC values. The univariable model explained only 4.1% of the variance of FRC_{pleth} (coefficient 2.7 ml FRC at visit 2 per 1 ml FRC at visit 1; 95%CI 0.6 – 4.8). The explained variance was higher (22%, $p<0.001$) in the multivariable model including height and age at visit 2, male gender, and maternal smoking during infancy (coefficient 2.0; 95%CI 0.02 – 3.97).

Conclusions

As previous studies reported lung function data from forced manoeuvres, this investigation based on more physiological manoeuvres adds new information to the field of early lung development. Our study showed weak tracking of FRC in term-born children from infancy to early childhood.

3.2 INTRODUCTION

Understanding physiological lung growth during childhood and its influencing factors holds the potential for preventive strategies: Lung morbidity is a major health issue [World Health Organization, 2015]; and obstructive lung diseases in adults originate partly in childhood [Postma et al., 2014, Henderson, 2014, Silverman and Kuehni, 2007]. The pathogenesis of respiratory disease is complex and multi-factorial [Frey and Suki, 2008, Barker et al., 1991], and, especially in early childhood, is only poorly understood. Lung growth was observed even beyond childhood [Narayanan et al., 2012]. The most important window for the complex physiological lung development were the first few years of life [Stocks et al., 2013].

Data on early lung development were published from different types of studies, mostly reporting airway function. Studies reporting lung function data throughout childhood in term-born children focused on forced manoeuvres [Hopper et al., 1991, Turner et al., 2014], where results were mostly determined by fixed mechanical properties, providing valuable information on growth of the larger airways. Children with severe asthma were at risk of having chronic obstructive pulmonary disease in adulthood [Tai et al., 2014].

Within an organ, physiologically normal growth can be disproportionate, a concept known as dysanapsis [Green et al., 1974]. Dysanapsis of airways and lung tissue seems to originate early in childhood [Martin et al., 1988]. Thus, data on lung volume is necessary to fill in the gap of knowledge regarding growth of the entire organ. This study is the first to provide longitudinal data on increasing lung volume, i.e. functional residual capacity (FRC), in unselected children between the ages of 5 weeks and 6 years. In addition to what was known from previous publications using forced manoeuvres, our study focused on measurements of intrapulmonary gas volume using techniques without sedation, and with more natural breathing. We used multiple-breath washout (MBW) for FRC determination in infancy [Fuchs et al., 2011a], and plethysmography [Criée et al., 2011] at the age of 6 years.

The main aim of this study was to investigate how strongly FRC tracks from infancy to early childhood, and to investigate the effect of different factors on lung growth in an unselected population. The secondary aim was to test disproportionate growth of airways

and lung tissue. Main outcome was lung volume at the age of 6 years, we hypothesised to find an association of lung volumes over time.

3.3 METHODS

Participants

Term-born, unselected Caucasian children from a prospective birth cohort were recruited antenatally for longitudinal lung function measurements using MBW and plethysmography. The entire study protocol was previously described,[Fuchs et al., 2012a] and relevant details for this publication are provided in the Online Supplement (OLS). The study was approved by the Ethics Committee of the Canton of Bern, Switzerland. Parents or caregivers provided written informed consent for this study. At the second visit, the child's assent was obtained.

Lung function measurements

End-expiratory lung volume was measured at the age of 5 weeks (visit 1) using MBW (FRC_{MBW}) during quiet natural sleep in the supine position with the Exhalyzer D (Eco Medics AG, Duernten, Switzerland) and 4% sulfur hexafluoride (SF_6). At 6 years (visit 2), FRC was measured by plethysmography (FRC_{pleth}) with the awake child sitting upright using the JAEGER Master-Screen Body (CareFusion, Wuerzburg, Germany). At visit 2, spirometry was performed before plethysmography measuring maximum mid-expiratory flow (MMEF). All measurements were performed according to current standards and consensus [Frey et al., 2000a, Robinson et al., 2013, Wanger et al., 2005, Miller et al., 2005] by experienced operators (for details see OLS).

For longitudinal analysis, participants with high quality FRC measurements at both study visits were included.

At visit 2, children performed helium MBW in addition, for details see OLS.

Assessment of exposures and health

We assessed the family history and potentially harmful exposures. We defined binary variables for any exposure to parental smoking (no vs. yes for mother and/or father/other person in the household during year one and year six, and any of those). Severe respiratory symptoms during the first year of life were assessed during weekly phone calls [Latzin et al., 2006]. At visit 2, symptoms from 5 to 6 years of age were assessed in

a standardised interview. [Kuehni et al., 2001] A skin prick test was performed on the mothers at visit 1, and on the participants at visit 2. More information on the definition of exposures is provided in the OLS.

Statistical Methods

In order to analyse tracking of lung volumes, linear regression and Pearson correlation coefficient were used, with FRC_{pleth} in ml at visit 2 being the outcome, and FRC_{MBW} in ml at visit 1 being the exposure. Variables with potential association with FRC_{pleth} were tested using univariable linear regression (see OLS for details), including gender and height at visit 2, as their impact on lung volume is well known. We selected predictors for the multivariable model according to physiological plausibility and p-value <0.10 in the univariable analysis. We ranked those by R^2 and performed a forward stepwise selection where co-linearity was tested. Variables that improved R^2 by at least 10% were included in the multivariable model. We tested non-linear correlation of exposures with FRC_{pleth} by adding quadratic transformation of predictors to the model. Regression models with and without quadratic terms were compared using the likelihood ratio test. If the latter showed no improvement of the model using the quadratic term, we used the non-quadratic term only. We calculated gender-specific z-scores for FRC and spirometric values. Details and results of regression analysis using FRC z-scores instead of absolute values are provided in the OLS. Coefficient of variation (CV) of FRC measurements was calculated as the ratio of SD and mean FRC value per child and technique.

In order to test for dysanapsis, i.e. whether airway function and lung volume were associated per individual at visit 2, the association of MMEF and FRC was tested using visual data analysis and linear regression.

Power calculation was limited due to lack of comparable investigations. The size of the population was, however, equal to a population where the association of forced expiratory flow over time was assessed [Stern et al., 2007]. Power was 80% to detect a correlation coefficient of 0.25 with a significance level of 0.05 (two-sided).

Statistical analyses were performed in STATA 11 (STATA Corporation, Texas, USA) and R 3.1.2 (http://www.r-project.org/).

3.4 RESULTS

Study participants and lung volume

We evaluated FRC measurements at study visits 1 and 2. After strict quality control, we included 124 children with a successful FRC measurement at both visits in the longitudinal analysis (figure 1). All but four of these performed spirometry successfully.

Characteristics of the study population are shown in table 1 (see OLS for comparison with non-included children). Mean (SD) gestational age at birth was 40 (1.2) weeks. At visit 1, mean (SD) FRC_{MBW} was 103 (17) ml. Median (inter-quartile range, IQR) intra-subject CV for FRC_{MBW} (n=105) was 4.2 (2.6 6.1) %. At visit 2, mean (SD) FRC_{pleth} was 1088 (206) ml. Median (IQR) intra-subject CV for FRC_{pleth} (n=123) was 5.0 (3.4 8.7) %.

In addition, we performed helium MBW at visit 2. Despite the experience in our group, success rate was poor, as leaks and other technical problems were frequent. MBW results at visit 2 are reported in the OLS. In this ongoing study, we switched to nitrogen MBW at visit 2.

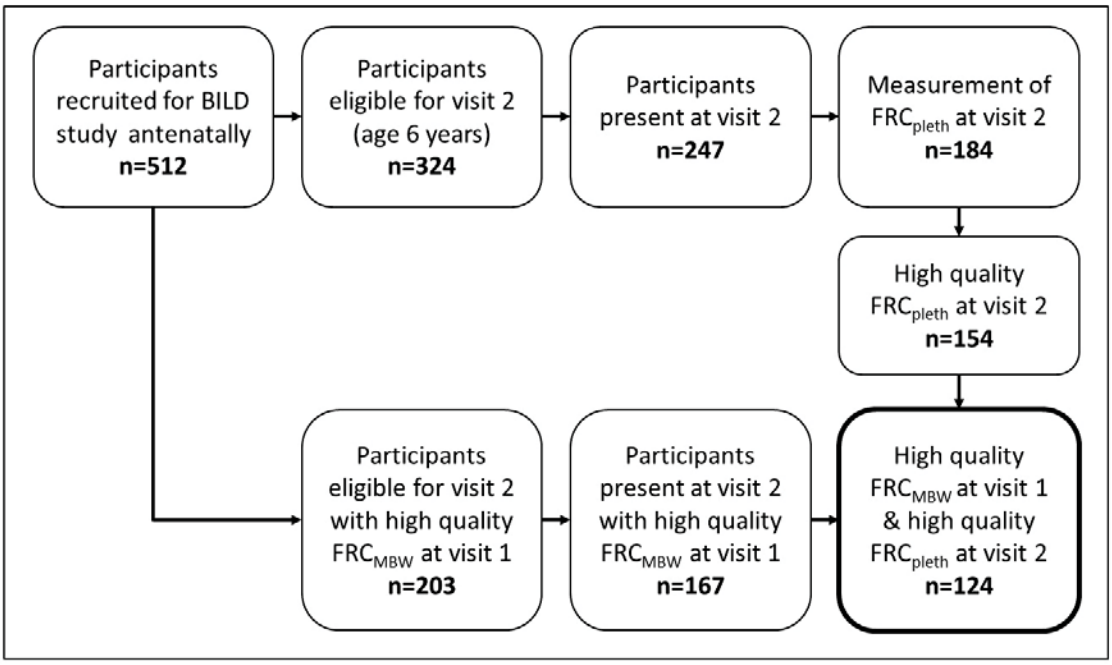

Figure 3.1.
Participants
Longitudinal measurements of functional residual capacity (FRC) in children participating in the cohort study (BILD study, Bern and Basel Infant Lung Development).[Fuchs et al., 2012a] The study is ongoing. Only children who already reached the age of 6 years were eligible for visit 2. High quality measurements of FRC from multiple breath-washout (FRC$_{MBW}$) at visit 1 (age 5 weeks), and from plethysmography (FRC$_{pleth}$) at visit 2 (age 6 years) were available in n=124 children. In the online supplement, characteristics of the entire BILD cohort, compared to the subgroup included in this study, are provided.

Table 3.1.
Population characteristics (n=124)

Variable	Population characteristics #
At birth	
Male gender, n (%)	62 (50.0)
Weight at birth, kg	3.4 (0.4)
Length at birth, cm	49.7 (2.0)
Visit 1	
Age visit 1, weeks	4.9 (0.6)
Weight Visit 1, kg	4.4 (0.6)
Length visit 1, cm	54.6 (2.2)
FRCmbw visit 1, ml	102.7 (17.0)
Weight gain from birth to visit 1, g/day	27.3 (10.8)
Smoking mother first year of life, n (%)	5 (4.0)
Visit 2	
Age visit 2 (years)	6.1 (0.2)
Weight visit 2 (kg)	22.6 (3.5)
Height visit 2 (cm)	117.8 (5.2)
Wheeze within 12 months prior to visit 2*, n (%)	8 (6.6)
Smoking mother visit 2*, n (%)	6 (5.0)
Smoking father/other person in household visit 2*, n (%)	15 (12.4)
Smoking of any person in household visit 2*, n (%)	17 (14.0)
Smoking of any parent any time of the study, n (%)	31 (25.0)
FRCpleth visit 2 (ml)	1088.0 (206.0)
FRCmbw visit 2 (ml) **	662.3 (155.2)

Values are displayed in mean (SD) of n=124 if not otherwise stated
* Some information missing in n=3
** Available in n=99

Tracking of functional residual capacity

Table 2 shows the association of FRC_{pleth} with different predictors. FRC_{MBW} at visit 1 significantly predicts FRC_{pleth} at visit 2, but only weakly: Univariable regression showed that per ml FRC at visit 1, FRC at visit 2 was 2.7 ml larger (95%CI 0.6 to 4.8), and

FRC at visit 1 explains only 4.1% of the variance of FRC at visit 2. The adjusted coefficient was 2.0 (95%CI 0.02 to 4.0), the multivariable model explains 22% of the variance. Pearson correlation coefficient for FRC_{MBW} at visit 1 with FRC_{pleth} at visit 2 was 0.22 (p=0.014).
Statistical analyses using FRC z-scores instead of absolute values showed comparable results (see OLS). Figure 2 shows trajectories of FRC z-scores from visit 1 to visit 2 for all children.

Factors associated with lung volume

As expected, larger FRC_{pleth} values at visit 2 were associated with greater height and weight, male gender, and with being older at visit 2. We found larger FRC_{pleth} in children exposed to maternal smoking during the first year of life, and to any parental smoking at the age of 6 years. No association of severe respiratory symptoms during infancy or early childhood, maternal atopy or parental asthma with FRC_{pleth} at visit 2 was found (variables without association are listed in the OLS). In a post-hoc-analysis, we found a trend towards stronger FRC increase over time in individuals with longer duration of respiratory symptoms during infancy, this trend was explained by an association of larger FRC at visit 1 with longer duration of respiratory symptoms in infancy (data not shown). Stepwise generation of the multivariable regression model showed co-linearity of anthropometric data at visits 1 and 2, and of height and weight at visit 2, with height at visit 2 being the strongest predictors of FRC_{pleth}.
The association of FRC_{MBW} at visit 1 with FRC_{pleth} at visit 2 was confirmed in the multivariable analysis after adjustment for height and age at visit 2, gender, and exposure to maternal smoking.
At visit 2, no relationship between airway function and lung volume, i.e. between MMEF in ml and FRC_{pleth} in ml was found in the study population (figure 3) (n=120; coefficient 0.04, 95%CI -0.06 to 0.15 in the univariable model; coefficient 0.02, 95%CI -0.8 to 1.1 after adjustment for height and age at visit 2, male gender, and exposure to maternal smoking in the first year of life). MMEF z-scores were not associated with FRC_{pleth} z-scores, nor with FVC z-scores (data not shown).

Table 3.2.
Results of regression analysis (n=124)

	Univariable regression outcome: FRC_{pleth} in ml at visit 2				Multivariable regression outcome: FRC_{pleth} in ml at visit 2		
Variable	Coefficient	95%CI	P-value	R2	Coefficient	95%CI	P-value
At birth							
Male gender	113.1	(42.4 to 183.8)	<0.01	0.07	75.0	(8.7 to 141.4)	0.027
Weight at birth (kg)	88.0	(6.8 to 169)	0.03	0.03			
Length at birth (cm)	30.5	(12.4 to 48.6)	<0.01	0.08			
Visit 1 at age 5 weeks							
Weight Visit 1 (kg)	120.0	(58 to 182)	<0.01	0.10			
Length visit 1 (cm)	31.3	(15.4 to 47.3)	<0.01	0.10			
FRC_{MBW} visit 1 (ml)	2.7	(0.6 to 4.8)	0.01	0.04	2.0	(0.02 to 3.97)	0.048
Weight gain from birth to visit 1 (g/day)	3.0	(-0.4 to 6.3)	0.08	0.02			
Smoking mother first year of life	158.7	(-26.1 to 343.4)	0.09	0.02	198.9	(32.9 to 365.0)	0.019
Visit 2 at age 6 years							
Age visit 2 (months)	24.6	(8.7 to 40.5)	<0.01	0.06	20.5	(5.4 to 35.6)	0.008
Weight visit 2 (kg)	14.9	(4.6 to 25.2)	0.01	0.06			
Height visit 2 (cm)	12.9	(6.3 to 19.6)	<0.01	0.10	9.3	(2.8 to 15.8)	0.006
Wheeze within 12 months prior to visit 2*	143.2	(-5.0 to 291.3)	0.06	0.01			
Smoking mother visit 2*	159.7	(-9.9 to 329.4)	0.06	0.01			
Smoking father/other person in household visit 2*	130.5	(19.6 to 241.3)	0.02	0.03			
Smoking of any person in household visit 2*	104.6	(-1.6 to 210.8)	0.05	0.02			
Smoking of any parent any time of the study*	80.5	(-3.2 to 64.2)	0.06	0.02			
					Adjusted R^2, %		**22.0**
					P-value		**< 0.001**

* Some information missing in n=3
Definition of abbreviations:
FRC_{MBW} - functional residual capacity measured by multiple-breath washout (using SF_6) at visit 1
FRC_{pleth} - functional residual capacity measured by plethysmography at visit 2

Table 3.3.a.
Overview of studies with longitudinal lung function data from infancy / preschool age to school age in selected populations

Authors	Fillipone et al.[28]	Schmalisch et al.[25]	Kieninger et al.[30]	Aurora et al.[31]
Year	2003	2012	2011	2011
Journal	Lancet	Pediatrics	J Cyst Fibros	Am J Resp Crit Care Med
Disease group	preterm children	preterm children	children with CF	children with CF
Number of lung function visits	2	3	2	2
Youngest age (mean)	24 months	50 weeks PMA	5 months	4 years
First school-age visit (mean)	9 years	.	10 years	8 years
Group size at first school-age visit	n=18	(no school age visit) n=81	n=11	n=48
Oldest age	9 years	corrected age 24 mo.	10 years	8 years
Lung function parameter first visit	V'maxFRC	FRC, tidal breathing, VmaxFRC	LCI	LCI, FEV_1, MMEF
Lung function parameter school-age visit	FEV_1, MMEF	.	LCI	LCI
FRC reported	No	Yes	No	No
Outcome of interest	correlation over time	BPD vs. non-BPD over time	tracking of LCI	tracking of LCI
Measurement technique	spirometry	MBW, plethysmography, spirometry, tidal	MBW	MBW, spirometry
Forced manoeuvre	yes	yes (partly)	no	no
Sedation in infants	yes	yes	no	.
Tracking of lung function	yes	.	yes	yes
How tracking was assessed	correlation	.	LCI course over time	LCI within ± 1.3
Coefficient of correlation / PPV	r=0.85 V'maxFRC with MMEF	.	.	PPV preschool LCI for abnormal school-age LCI 94%

Abbreviations:
BPD bronchopulmonary dysplasia
CF cystic fibrosis
MMEF maximum mid-expiratory flow
FEV_1 forced expiratory volume in 1 second
FVC forced vital capacity
r correlation coefficient
VmaxFRC maximal flow at functional residual capacity
PMA post-menstrual age
PPV positive predictive value

Table 3.3.b.
Overview of studies with longitudinal lung function data from infancy / preschool age to school age in unselected birth cohorts

Authors	Turner et al.[10]	Belgrave et al.[34]	Stern et al.[23]
Year	2014	2014	2007
Journal	Thorax	Am J Resp Crit Care Med	Lancet
Number of lung function visits	6	4	4
Youngest age (mean)	1 month	3 years	2 months
First school-age visit (mean)	6 years	8 years	11 years
Group size at first school-age visit	n=106	(n=350 at all 4 study visits)	123
Oldest age	18 years	11 years	22 years
Lung function parameter first visit	V'maxFRC	sRaw	V'maxFRC
Lung function parameter school-age visit	MMEF	sRaw	FEV_1, FVC, MMEF
FRC reported	No	No	No
Outcome of interest	change in % predicted over time	group differences over time	correlation over time
Measurement technique	spirometry	body plethysmography	spirometry, SAB, MCh challenge
Forced manoeuvre	yes	no	yes
Sedation in infants	yes	.	yes
Tracking of lung function	.	.	yes
How tracking was assessed	.	.	Pearson correlation
Explained variance	.	.	different study visits R^2 9 to 14%

Abbreviations:
MMEF maximum mid-expiratory flow
FEV_1 forced expiratory volume in 1 second
FVC forced vital capacity
MCh Metacholine
R^2 explained variance
sRaw specific airway resistance
SAB short-acting bronchodilator
VmaxFRC maximal flow at functional residual capacity

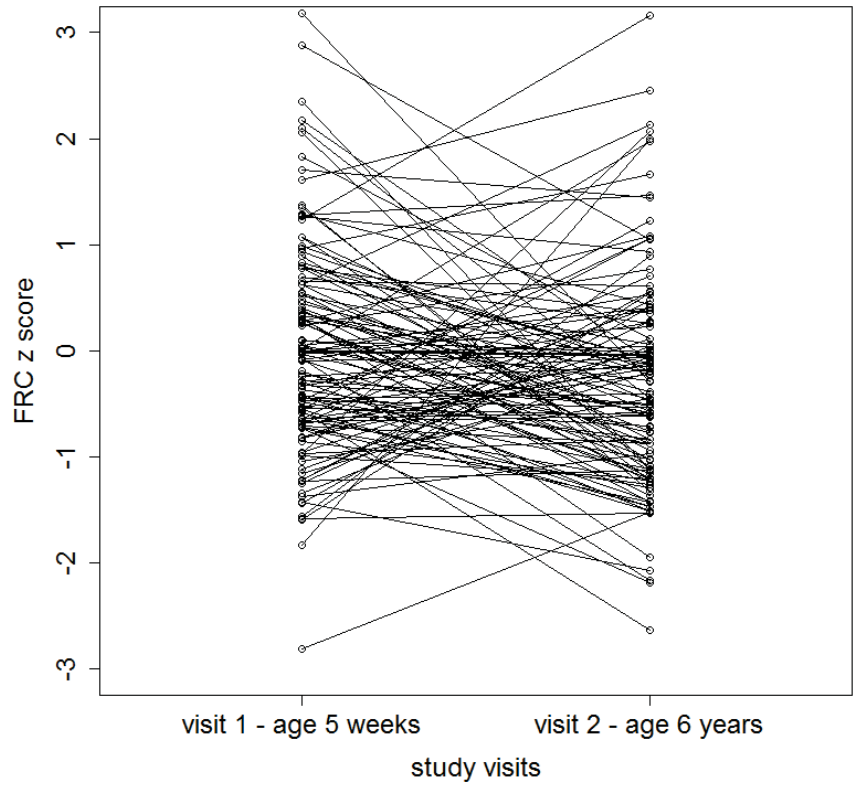

Figure 3.2:
Weak tracking of functional residual capacity (n=124)
Functional residual capacity (FRC) tracks from the age of 5 weeks to the age of 6 years. Z-scores were derived separately for males and females for FRC measured by multiple-breath washout at visit 1 and FRC measured by plethysmography at visit 2. Z-scores for FRC_{pleth} were based on published reference values [Rosenthal et al., 1993]. Regression analysis showed an association of absolute FRC values and of FRC z scores over time.

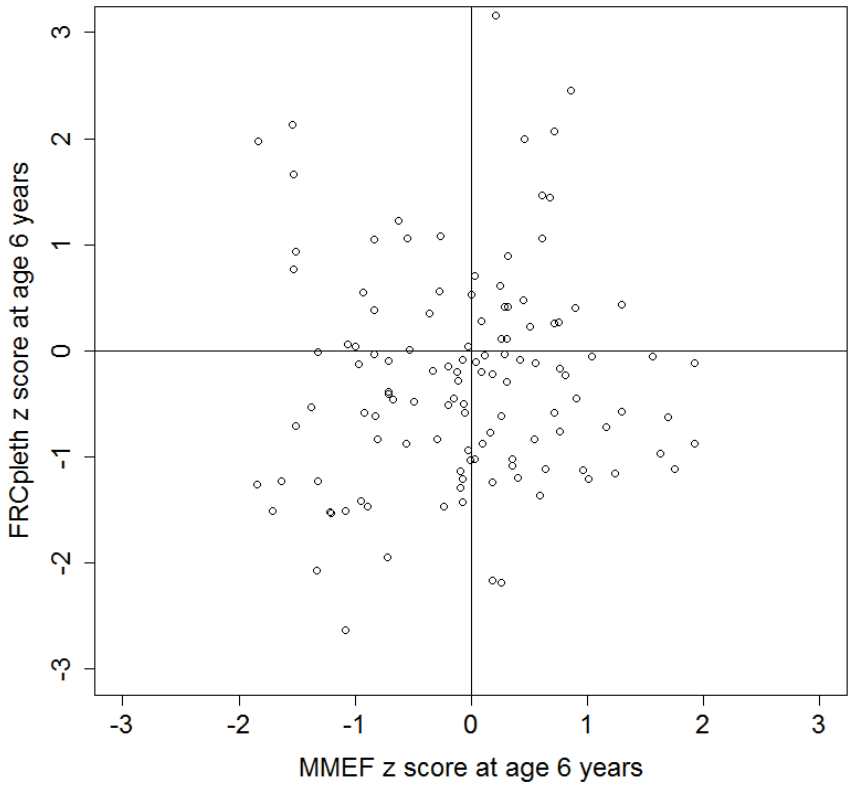

Figure 3.3
Dysanapsis - FRC_{pleth} and MMEF at age 6 years (n=120)
Maximum mid-expiratory flow (MMEF) in spirometry plotted versus functional residual capacity measured by plethysmography (FRC_{pleth}) at the age of 6 years in children with successful plethysmography and spirometry (n=120). Z-scores were derived separately for males and females, and based on published reference values [Rosenthal et al., 1993, Quanjer et al., 2012]. Lines represent z-scores of 0. Regression analysis showed no association of absolute values or z-scores.

3.5 DISCUSSION

This study was the first to examine tracking of FRC in term-born, unselected children from infancy to early childhood. Tracking of this physiological lung function parameter was weak. Male gender, height and age at visit 2, and exposure to maternal smoking during the first year of life, were associated with larger plethysmographic lung volumes at visit 2. At visit 2, MMEF and FRC_{pleth} were not associated, which was consistent with dysanapsis, and underlines the relevance of analysing longitudinal data on end-expiratory lung volume, as presented in this article.

Strengths and limitations

Strengths of this study were the prospective design with longitudinal assessment of lung volume data and potential influencing variables in an unselected population, and the use of established and validated methods for lung volume determination not requiring sedation in infants.

We applied strict quality criteria for inclusion of FRC measurements in the longitudinal analyses, which increased the quality of the data, but limited the sample size.

Limitations of this study were methodological differences between FRC techniques at different visits. We took measurements during natural sleep and in supine position at visit 1, and awake in an upright position at visit 2. At visit 2, some children's cooperation was limited. MBW and plethysmography used different principles. The first was based on dilution of a tracer gas in the lungs during tidal breathing, while the latter relied on the relationship of intra-thoracic gas volume and pressure when normal breathing was interrupted. Thus, MBW measured the gas volume that participated in ventilation; plethysmography measured trapped gas in addition.

Both techniques showed variability between the single tests, especially as we performed infant measurements without sedation. As in all comparable studies, a remaining measurement error of both techniques had to be assumed. For FRC_{MBW} at visit 1, error was quantified in an in-vitro study; it was within approximately $\pm$ 10% [Schmidt et al., 2015b]. For FRC_{pleth}, the measurement error was unknown.

A selection bias cannot be excluded for either participation or exposures. It is possible

but not given that a stronger effect of exposures would have been found indeed in a population with more and randomly - exposed children.

Interpretation and comparison with literature

Tracking of lung volumes in our study population was weak; only 4.1% of variance of FRC_{pleth} values at visit 2 was explained by FRC_{MBW} values at visit 1 in the univariable regression. The multivariable regression model, including height and age at visit 2, male gender, FRC_{MBW} and exposure to maternal smoking in the first year of life, explained 22% of the variance of FRC_{pleth}.

The remaining variance might be explained by several physiological and methodological factors. Certainly, the physiological variability of the end-expiratory level was high at both visits. Infants in particular change their FRC frequently and within a short period of time. Some measurement error was likely to contribute to the remaining non-explained variance as well. Some exposures were not included in our analysis, such as outdoor air pollution, individual susceptibility to harmful substances, or individual capacity for tissue regeneration after injury.

Comparability with previous studies was limited because, so far, investigations of tracking were based on parameters from forced manoeuvres. Results from studies in selected children of comparable ages are summarised in table 3a. The same outcome, FRC, was available from a longitudinal study in preterm children with and without bronchopulmonary dysplasia during the first 2 years of life, but not beyond, the outcome of interest was a group comparison over time [Schmalisch et al., 2012]. Hoo and colleagues showed tracking of maximal flow at FRC (VmaxFRC) early in life in term-born and preterm children [Hoo et al., 2002a, Hoo et al., 2002b]. Filippone et al. described tracking of airway function from 2 years to 9 years of age [Filippone et al., 2003]. In a longitudinal study of older children and young adults, tracking of airway function was found in full-term and preterm children [Vollsæ ter et al., 2013]. Studies in children with cystic fibrosis showed that LCI tracks from infancy to childhood [Kieninger et al., 2011] and from age 4 years to 8 years [Aurora et al., 2011] in those individuals with elevated values. Several studies of term-born children reported data from school age on only [Twisk et al., 1998, Hibbert

et al., 1990]. Recent studies with a comparable study protocol, i.e. unselected birth cohorts with longitudinal lung function measurements starting very early in childhood, are listed in table 3b. Outcomes were spirometry [Turner et al., 2014, Stern et al., 2007], and specific resistance [Belgrave et al., 2014]. The explained variance was reported by Stern and colleagues [Stern et al., 2007] it was 9 to 14%, so tracking was weak here as well. Spirometric measurements in infants were performed with sedation, and physiological variability might be lower than in our measurements without sedation.

Respiratory symptoms in infancy or early childhood showed no association with lung volume at visit 2 in the multivariable model. Despite the low number of children in this subgroup, we found larger FRC_{pleth} values in those children exposed to maternal smoking even after adjustment. As potential explanations cannot be differentiated, i.e. hyperinflation and increased lung growth, this result needs to be interpreted with caution. It would be interesting to compare lung function and respiratory health in these children in adolescence.

With the data currently available, the question as to which lung function method is most informative for understanding lung development, i.e. expiratory flow from forced manoeuvres or lung volume data from more physiological manoeuvres, cannot be answered. We found no association between MMEF and FRC at visit 2, a previous study showed a very weak association of VC and expiratory flow in healthy children and adolescents [Hopper et al., 1991]. Most likely, different techniques provide different, albeit complementary information. Studies reporting data from physiological manoeuvres, like FRC, will allow us to understand lung growth better in the future.

In order to increase the comparability of future longitudinal studies, we would like to recommend a somewhat clearer definition and use of the term tracking. We observed that it is currently used for a broad spectrum of findings, and in our opinion, this is not always appropriate. We would like to suggest avoiding the term when reporting group comparisons over time, e.g. stratified by symptoms, or other factors. Group comparisons help us indeed to understand lung development, and they are an important part of the epidemiological puzzle. The term tracking, however, should only be used when the association of longitudinal measurements in the same individuals is reported, which was done indeed by most of the authors we are citing. Interestingly, the variance explained

by tracking was only reported by some investigators.

Medical relevance and conclusions To the best of our knowledge, this was the first study reporting tracking of FRC, a parameter with high physiological variability, in unselected term-born children from infancy to early childhood. Tracking was weak which shows that by measuring lung volume after birth, lung volume in early childhood can only be predicted to a small extent. Certainly, the high physiological variability of FRC at both visits hampers detection of tracking. It is likely that many factors modify lung growth in this early period. The size of the population included in this analysis was relatively high considering the complexity of measuring FRC throughout childhood. However, observations of children exposed to potentially disturbing factors were few. This increases the challenge of interpreting our findings with regards to clinical relevance. We found no evidence for hyperinflation in children exposed to parental smoking, but it is possible that, later in life, hyperinflation will be measurable in these individuals.
Our study adds relevant information to current knowledge, as previous studies in unselected populations early in childhood focused on data from forced exhalation techniques; which reflects airway growth rather than overall lung growth.
In the future, a standardised approach for investigating lung function tracking is desirable, as this would help us to compare findings from different studies. In our opinion, studies on tracking should report associations of the individuals' parameters over time.
In conclusion, tracking of lung volume during early childhood was weak in term-born children. We hypothesise that this was due to a combination of high physiological variability and multiple influencing factors early in life.

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AUTHORS CONTRIBUTIONS

Conception and design of the study: AS, CK, UF, and PL Contributed substantially to study design, data analysis and interpretation, and the writing of the manuscript: AS, EP, OF, BE, DR, ED, CK, UF, and PL

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3.6 Early lung growth in term-born children: Weak tracking of end-expiratory lung volume from infancy to early childhood

ONLINE SUPPLEMENT #1

Additional details on methods and results

ADDITIONAL DETAILS ON METHODS

Detailed criteria for inclusion and exclusion

Inclusion criteria were Caucasian ethnicity, term delivery (at least 37 weeks), and ability of parents to speak one of the major Swiss languages (German or French). Exclusion criteria for study participation were severe maternal health problems, maternal drug abuse other than nicotine, known major birth defects or perinatal disease of the newborn, such as respiratory distress, airway malformation or other major respiratory diseases diagnosed after birth; while exclusion criteria for lung function analysis were respiratory tract infection within two weeks prior to lung function measurements or poor technical quality of FRC measurements. The entire study protocol was previously described.[Fuchs et al., 2012a]

Lung function measurements

For MBW, we used a commercially available apparatus (Exhalyzer D, Eco Medics AG, Duernten, Switzerland) with an age appropriate tracer gas, i.e. 4% sulfur hexafluoride (SF_6).[Robinson et al., 2013] We performed daily calibration of flow, volume and tracer concentration. Apparatus and procedure were described previously,[Fuchs et al., 2011a, Latzin et al., 2007b] and the setup was validated as requested by current consensus.[Schmidt et al., 2015b] Spirometry and plethysmography were performed at visit 2 only, using the JAEGER Master-Screen Body (CareFusion, Wuerzburg, Germany). Plethysmographic FRC determination was based on the law of Boyle-Mariotte.[Criée et al., 2011]

Criteria for exclusion of a single test were poor cooperation of the child, irregular breathing pattern during MBW, incomplete wash-in or washout of the tracer, deviations in molar mass, or relevant volume drift. The latter was defined as $>$3 ml*s-1 at visit 1. Maximal tracer concentration during MBW was determined from end-inspiratory molar mass [Anagnostopoulou et al., 2015]. At least 3 tests were performed for each FRC measurement. Mean FRC_{MBW} and mean FRC_{pleth} were calculated from the maximum of technically acceptable tests. Single FRC measurements beyond 25% deviation from the median of trials per child and technique were excluded [Robinson et al., 2013].

Assessment of exposures and respiratory health

Exposure to parental smoking was assessed several times and for each parent using standardised questionnaires and interviews: during pregnancy, during the first year of life, and at age 6 years. Exposure during pregnancy was verified based on children's urine cotinine levels. We defined binary variables for any exposure (no vs. yes for mother and/or father/other person in the household during year one and year six, and any of those).
Severe respiratory symptoms during the first year of life were assessed during weekly phone calls, and were defined as previously reported.[Latzin et al., 2006] At visit 2, symptoms from 5 to 6 years of age were assessed in a standardised interview, based on the ISAAC questionnaire.[Kuehni et al., 2001]
Maternal atopic disease was defined as the mother having atopic dermatitis, atopic rhinitis or asthma. Parental asthma was defined as either parent reporting doctor-diagnosed asthma.

Skin Prick Test

We performed a skin prick test (Allergomed, Switzerland) in mothers at visit 1, and in children at visit 2. We tested for dog dander, cat dander, Dermatophagoides pteronyssinus, mixed tree pollens, mixed grass pollens, Alternaria tenuis, positive control: histamine, negative control: NaCl. Tests were positive in case of hives bigger than positive control in any of the tested allergens.

Statistical Methods

Z-scores were calculated for functional residual capacity (FRC) at visit 1 based on gender specific mean and standard deviation (SD) within the study population:
z-score = (gender-specific mean within group individual value) / gender-specific SD within group Z-scores for FRC_{pleth} were calculated based on gender specific reference values. [Rosenthal et al., 1993]
Z-scores for spirometry were calculated based on gender specific reference data published by the Global Lung Initiative. [Quanjer et al., 2012]

ADDITIONAL DETAILS ON RESULTS

Participants

Of the 324 children in this ongoing study who had reached the age of eligibility for visit 2 (6 years of age), 247 participated (76%), and 184 performed plethysmography. Online Supplement Table 3.6.1 shows characteristics of all participants of this nested study (n=124), compared to children present at follow up but lacking high quality FRC measurements at visit 1 and / or visit 2 (n=123), and with children who participated but were below the age of 6 years or not present at visit 2 (n=265).

Univariable regression analysis

Online Supplement Table 3.6.2 shows variables without association with FRC_{pleth} at visit 2, and how these variables were defined.

Multivariable regression analysis using highest or lowest FRC measurement per child and visit

Online Supplement Table 3.6.3a (outcome FRC_{pleth}) shows results of multivariable regression analysis if, instead of using mean values of FRC per child and visit, only the highest or lowest FRC measurement was used.

Online Supplement Table 3.6.3b shows results when FRC from helium MBW at visit 2 was used as the outcome in 75 children, using variables for adjustment from the multivariable model regarding FRC_{pleth}.

Multivariable regression analysis if FRC z-scores were used instead of absolute values

Online Supplement Table 3.6.4 shows results of multivariable regression analysis when, instead of absolute FRC values, z-scores were used.

Online Supplement Table 3.6.1: Comparison of included population of this nested study (n=124) with non-included participants of the BILD study

Variable		Group n=124 (included)		Group n=123 (not included)		Group n=265 (not included)	
		Available in (n)		Available in (n)		Available in (n)	
At birth							
Male gender	n (%)	124	62 (50.0)	123	68 (55.3)	265	142 (53.6)
Maternal atopy	n (%)	124	44 (35.5)	123	47 (38.2)	263	74 (28.1)
Parental asthma	n (%)	124	25 (20.2)	123	23 (18.7)	262	49 (18.7)
Gestational age at birth (weeks)		124	39.8 (1.2)	123	39.8 (1.1)	265	39.6 (1.2)
Weight at birth (kg)		124	3.4 (0.4)	123	3.4 (0.4)	265	3.4 (0.4)
Length at birth (cm)		124	49.7 (2.0)	123	49.5 (1.9)	265	49.5 (2.1)
Visit 1 and first year of life							
Age at visit 1 (weeks)		124	4.9 (0.6)	119	5.2 (0.8)	216	5.3 (0.8)
Weight SDS visit 1		124	-0.2 (0.9)	119	-0.2 (0.9)	216	-0.2 (0.9)
Length SDS visit 1		124	0.1 (0.9)	119	0.2 (1.0)	216	0.0 (0.9)
Maternal smoking first year of life	n (%)	124	5 (4.0)	123	19 (15.4)	264	25 (9.5)
Paternal smoking first year of life	n (%)	124	20 (16.1)	123	33 (26.8)	261	43 (16.5)
Any respiratory symptoms during first year of life	n (%)	124	106 (85.5)	123	117 (95.1)	265	228 (86.0)
Severe respiratory symptoms during first year of life	n (%)	124	41 (33.1)	123	56 (45.5)	265	112 (42.3)
Visit 2							
Age at visit 2 (years)		124	6.1 (0.2)	123	6.0 (0.4)		
Weight at visit 2 (kg)		124	22.6 (3.5)	123	22.1 (3.1)		
Weight SDS visit 2		124	0.5 (1.0)	123	0.4 (0.9)		
Height at visit 2 (cm)		124	117.8 (5.2)	123	116.7 (5.8)		
Height SDS visit 2		124	0.3 (1.0)	123	0.3 (1.1)		
Parental smoking at visit 2	n (%)	121	17 (14.0)	117	31 (26.5)		
Parental smoking during study period	n (%)	124	31 (25.0)	121	47 (38.8)		
Wheeze ever reported at visit 2	n (%)	124	26 (21.0)	121	37 (30.6)		
Wheeze within 12 months prior to visit 2	n (%)	124	8 (6.5)	121	10 (8.3)		
Doctor diagnosed asthma at visit 2	n (%)	124	10 (8.1)	106	3 (2.8)		

Data displayed in mean (SD) unless otherwise stated
Children in group n=124 had successful measurements of FRC at both visits, and were included in this study.
Children in group n=123 lacked successful measurements of FRC at one or both visits, and were not included in this study.
Children in group n=265 were too young for visit 2 or not present at visit 2, and were not included in this study

Online Supplement Table 3.6.2: Variables without association with FRC_{pleth} in univariable regression analysis

variable	definition of variable (if applicable)
gestational age at birth	post-menstrual age at birth (weeks)
oligohydramnios	
duration of any breastfeeding, Wk.	
absence of older siblings	
maternal atopy	either maternal history of allergic rhinitis, allergic asthma or atopic dermatitis
parental asthma	parent reported maternal or paternal doctor diagnosed asthma
skin prick test in mother positive	performed in n=119, valid in n=116, positive in n=38
any respiratory symptoms present first year	assessed in weekly phone calls during first year of life
severe respiratory symptoms present first year	definition of severe respiratory symptoms during first year of life as previously published:[8]3] respiratory symptoms causing repeated sleep interruptions during night time or GP consultations during daytime
wheeze during first year	reported by parents, doctor diagnosed, or inhalation of bronchodilators
relative increase in body length and in body weight	z-score visit 2 - z-score visit 1 (z-scores for anthropometric values were derived using the zanthro package in STATA which is based on data published by the World Health Organization [12]
wheeze ever reported at visit 2	questions were based on ISAAC questionnaire[13]
asthma at visit 2	questions were based on ISAAC questionnaire[13], defined as either ever doctor diagnosed asthma or current inhalation of bronchodilators and / or steroids
skin prick test in child positive at visit 2	performed in n=116, valid in n=112, positive in n=28
atopy of child at visit 2	history of allergic rhinitis, allergic asthma or atopic dermatitis

12 [World Health Organization, 2006]
13 [Asher et al., 1995]

Online Supplement Table 3.6.3a: Outcome FRC from plethysmography at visit 2 (n=124) Comparison of multivariable models, when highest or lowest of all FRC_{pleth} measurements per visit and technique were used

Exposure	Outcome FRCpleth visit 2 in ml Mean of all trials			Outcome FRCpleth visit 2 in ml Highest of all trials			Outcome FRCpleth visit 2 in ml Lowest of all trials		
		95%CI	P		95%CI	P		95%CI	P
FRCmbw visit 1 in ml:									
Mean of all trials	**1.99**	(0.02 - 3.97)	0.048	1.87	(-0.25 - 4.00)	0.083	**2.24**	(0.33 - 4.16)	0.022
adj.R2 (%)	22.1			18.9			24.2		
P value model	< 0.001			< 0.001			< 0.001		
Highest of all trials	**1.84**	(0.02 - 3.66)	0.048	1.74	(-0.22 - 3.70)	0.081	**2.07**	(0.3 - 3.83)	0.022
Adjusted R2 (%)	22.1			18.9			24.2		
P value model	< 0.001			< 0.001			< 0.001		
Lowest of all trials	**2.13**	(0.08 - 4.17)	0.042	1.97	(-0.23 - 4.18)	0.078	**2.38**	(0.40 - 4.37)	0.019
Adjusted R2 (%)	22.3			18.9			24.4		
P value model	< 0.001			< 0.001			< 0.001		

Bold: Coefficients for the association of FRC at visit 1 in ml with FRC at visit 2 in ml.
Each model was adjusted for height and age at visit 2, male gender, and maternal smoking during the first year of life.

Online Supplement Table 3.6.3b: Outcome FRC from helium MBW at visit 2 (n=75) Comparison of multivariable models, when highest or lowest of all FRC measurements per visit and technique were used.

Exposure	Outcome FRCmbw visit 2 in ml Mean of all trials			Outcome FRCmbw visit 2 in ml Highest of all trials			Outcome FRCmbw visit 2 in ml Lowest of all trials		
		95%CI	P		95%CI	P		95%CI	P
FRCmbw visit 1 in ml:									
Mean of all trials	**1.22**	(-0.78 – 3.23)	0.228	1.41	(-0.66 – 3.47)	0.179	**0.99**	(-1.09 – 3.07)	0.345
adj.R2 (%)	25.8			29.4			20.0		
P value model	< 0.001			< 0.001			< 0.001		
Highest of all trials	**1.17**	(-0.73 – 3.06)	0.222	1.33	(-0.62 - 3.28)	0.179	**0.98**	(-0.98 – 2.94)	0.323
Adjusted R2 (%)	25.8			29.4			20.1		
P value model	< 0.001			< 0.001			< 0.001		
Lowest of all trials	**1.11**	(-0.92 – 3.14)	0.280	1.30	(-0.79 – 3.39)	0.219	**0.87**	(-1.24 – 2.98)	0.412
Adjusted R2 (%)	25.5			29.1			19.7		
P value model	< 0.001			< 0.001			0.001		

Bold: Coefficients for the association of FRC at visit 1 in ml with FRC at visit 2 in ml.
Each model was adjusted for height and age at visit 2, male gender, and maternal smoking during the first year of life.

Online Supplement Table 3.6.4:
Multivariable regression using z scores of FRC_{pleth}

	Multivariable regression		
	Outcome FRC_{pleth} in z-scores at visit 2		
Variable	Coefficient	95%CI	P-value
Male gender	0.77	(0.44 to 1.09)	<0.001
FRC_{MBW} visit 1 (z-score)	0.17	(0.01 to 0.34)	0.043
Smoking mother first year of life	1.11	(0.29 to 1.93)	0.008
Height visit 2 (cm)	-0.01	(-0.04 to 0.03)	0.738
Age visit 2 (months)	0.07	(-0.01 to 0.14)	0.079
	Adjusted R2, %		21.2
	P-value		< 0.001

Results of multivariable regression analysis when, instead of absolute FRC values, z-scores were used. Z-scores were calculated for functional residual capacity (FRC) measured by multiple-breath washout (FRC_{MBW}) at visit 1 based on gender specific mean and standard deviation (SD) within the study population. Z-scores for FRC measured by plethysmography (FRC_{pleth}) at visit 2 were calculated based on gender and height specific published reference values.[Rosenthal et al., 1993]

3.7 Early lung growth in term-born children: Weak tracking of end-expiratory lung volume from infancy to early childhood

ONLINE SUPPLEMENT # 2

Details on helium multiple-breath washout at visit 2 – Successful measurements, exclusion criteria, and implications for MBW using helium

Helium multiple-breath washout performed insufficiently in young children

At the second visit of the longitudinal study, in addition to plethysmography, we measured functional residual capacity (FRC) using multiple-breath washout (MBW, FRC_{MBW}). We used helium which is an appropriate tracer for young children [Robinson et al., 2013]; and when follow-up was initiated helium MBW was a promising approach. The setup was based on an ultrasonic flow meter (USFM); its validity was described in detail previously [Schmidt et al., 2015b]. Operators in our lung function laboratory were experienced with MBW in infants and children of all ages. Surprisingly, many of the results were not within the expected range for healthy children at the age of six years. Thus, we tested the credibility of the FRC_{MBW} values in order to ensure their suitability as an outcome for longitudinal analysis. In the process, many measurements had to be excluded. From our experience, helium is not suitable for MBW in young children, at least when tracer concentration is measured indirectly using molar mass signal. Details about the problems we encountered, and a discussion of those, are presented in this online supplement.

Equipment for helium MBW and daily calibration

We used a commercially available apparatus (Exhalyzer D, Eco Medics AG, Duernten, Switzerland) and software (WBreath, ndd medical technologies, Zurich, Switzerland). A main stream USFM measured flow, a side stream USFM sensor measured molar mass (MM). For the side-stream MM signal, temperature and humidity of exhaled air were equilibrated with the surrounding air via a nafion tube (Nafion, Perma Pure LLC, Toms River, USA). A side-stream infrared carbon dioxide (CO_2) sensor measured CO_2. Helium was produced by Carbagas, Bern, Switzerland: A mixture of 22% oxygen (O_2) and 78% helium was blended with medical air containing balance nitrogen (N_2) to achieve a helium concentration of 20%. Tracer concentration was measured indirectly via MM of in- and exhaled air. We performed daily calibration of volumes, CO_2 signal, and controlled appropriate MM change when adding the tracer. Stable CO_2 levels were used as a quality control for regular tidal breathing. FRC was calculated as cumulative expired volume (CEV) of tracer gas divided by the difference between end-tidal tracer gas concentrations at the start and end of MBW. Lung Clearance Index (LCI) as a measure of ventilation homogeneity was calculated as CEV over FRC.

Initial inclusion of n=99 helium MBW measurements for longitudinal analysis

For inclusion of helium MBW measurements in the longitudinal analysis, we performed visual control for quiet breathing, appropriate wash-in; and for absence of leak, sensor issues, or hyperventilation. The latter is problematic in helium MBW using an USFM: tracer concentration is indirectly measured via molar mass of inhaled and exhaled air, and CO_2 is one of the most important determinants of molar mass of exhaled air. A change in CO_2 levels during MBW can interfere with correct calculation of tracer concentration. After a first visual control, 66% of the 209 helium MBW measurements we performed showed acceptable quality. In 99 out of the 137 children, a high quality FRC measurement at birth was available, so these children were included in the detailed analysis.
After analysis of the initially included 99 measurements according to standards [Robinson et al., 2009], LCI results were not within the range we expected in a group of unselected children at the age of six years [Lum et al., 2013]. (Online Supplement Table 3.7.1)
Thus, we performed several post-hoc analyses to test credibility of the helium MBW results.

Online Supplement Table 3.7.1:
Results from helium MBW (n=99) at visit 2

	mean	SD
FRC_{MBW} at visit 2, ml	662	155
LCI at visit 2	9	1.4

Statistical methods to test quality of helium MBW measurements

Comparison with normative data was not possible, because reference values for FRC measured by helium MBW were not available. Most children (n=79) had a success-

ful measurement of FRC_{pleth} at the same visit, so we calculated the absolute (FRC_{pleth} FRC_{MBW}) and relative differences (absolute difference * 100 / FRC_{pleth}) between the two measurements and displayed the agreement of the two techniques according to Bland and Altman [Bland and Altman, 1986].
FRC_{pleth} values were compared to normative values published by Hibbert and by Rosenthal [Hibbert et al., 1989, Rosenthal et al., 1993]. Coefficient of variation (CV) was calculated for children with 2 or 3 successful measurements for each FRC technique. We tested a potential association of FRC_{MBW} values with LCI values in the same individuals by graphical and linear regression analysis. Statistical analyses were performed in STATA 11 (STATA Corporation, Texas, USA) and R 3.1.2 (http://www.r-project.org/).

Results of the credibility analyses

We found larger values in FRC_{pleth} compared to FRC_{MBW} with a mean (SD) relative difference of 39 (13) % (see Online Supplement Table 3.7.2). Plethysmography showed FRC results within expected range (Online Supplement Table 3.7.3). Intra-subject CV was slightly higher for FRC_{MBW} than for FRC_{pleth} (Online Supplement Table 3.7.4).
Bland-Altman plots show agreement of both techniques (Online Supplement Figure 3.7.1). LCI values plotted versus FRC values from helium MBW (Online Supplement Figure 3.7.2) showed an association of both, especially in FRC values below 800 ml. Linear regression analysis showed that both parameters were associated, an increase of 1 ml FRC reduced LCI by 0.004 (95%CI -0.006 to -0.002; $p<0.001$; R^2 19%).

Online Supplement Table 3.7.2:
Difference of functional residual capacity (FRC) from helium MBW at visit 2 (FRC_{MBW}) to FRC from plethysmography at visit 2 (FRC_{pleth}) (n=79)

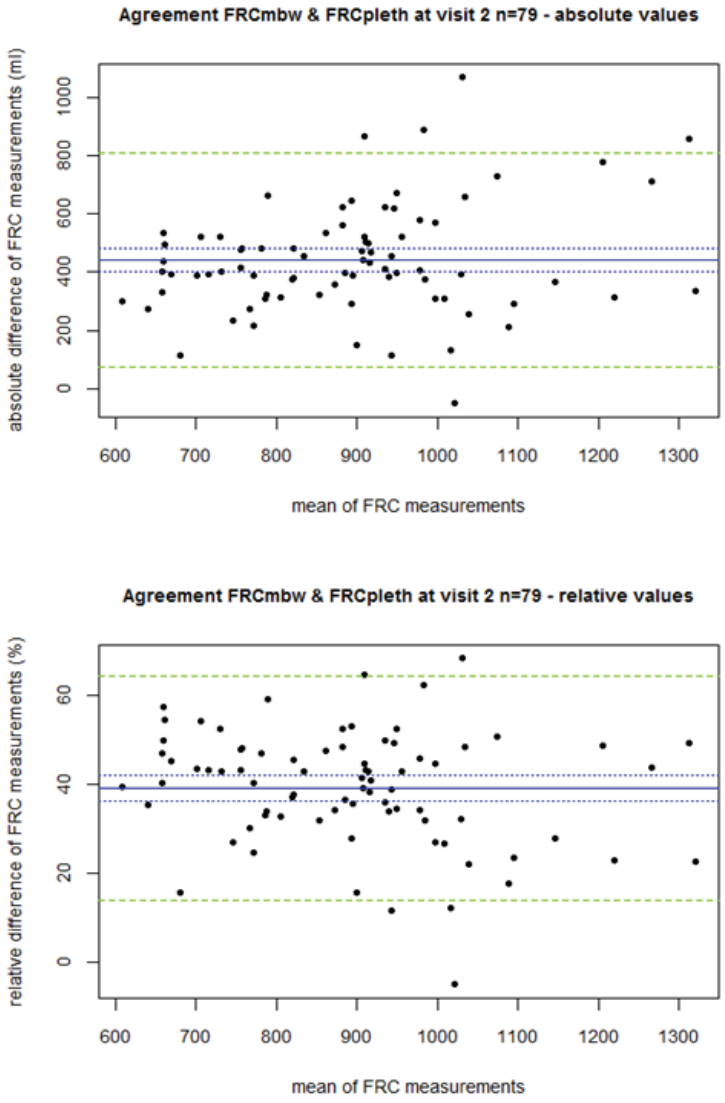
		mean	SD
Absolute difference from FRC_{pleth}	ml	442	187
Relative difference from FRC_{pleth}	%	39	12

Online Supplement Table 3.7.3
Functional residual capacity from plethysmography (FRC_{pleth}) in % of predicted values from published reference data (n=101)[Hibbert et al., 1989, Rosenthal et al., 1993]

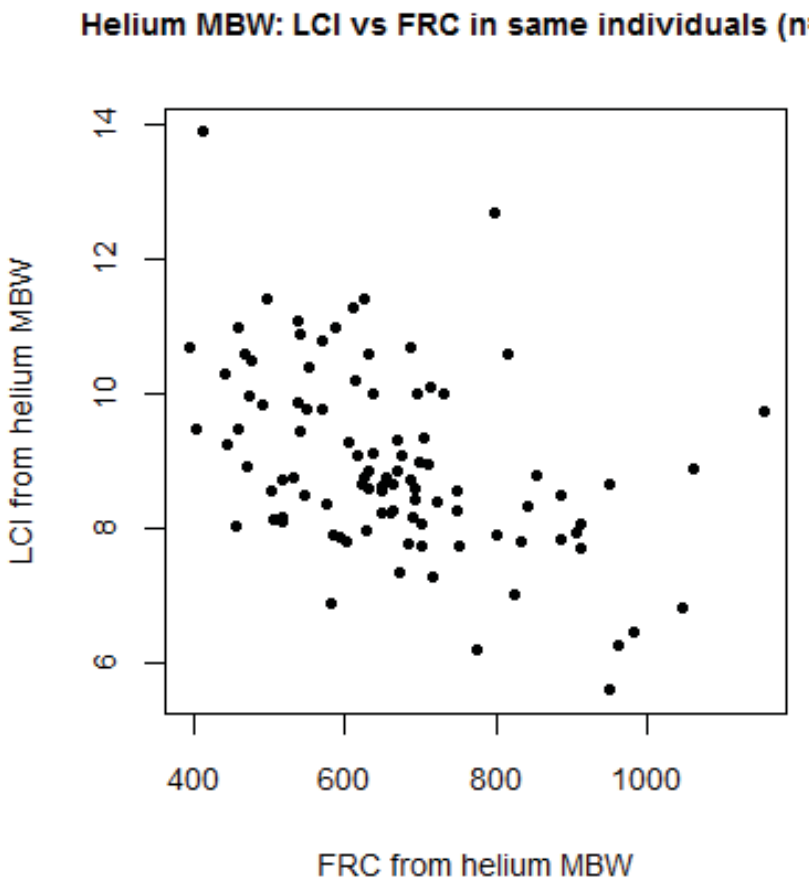
	mean	SD
reference values according to Hibbert	91	17
reference values according to Rosenthal	94	18

Online Supplement Table 3.7.4
Intra-subject coefficient of variation (CV) of functional residual capacity (FRC) from helium MBW at visit 2 (FRC_{MBW}, n=77) and FRC from plethysmography at visit 2 (FRC_{pleth}, n=100)

		median	IQR
CV FRC_{MBW}	%	8.0	(5.5 to 12.9)
CV FRC_{pleth}	%	6.0	(2.8 to 8.7)



Online Supplement Figure 3.7.1:
Agreement of FRC$_{pleth}$ & FRC$_{MBW}$
Bland Altman Plot [Bland and Altman, 1986] showing the absolute (upper figure) and relative (lower figure) difference of functional residual capacity (FRC) measured by plethysmography (FRC$_{pleth}$) and by helium multiple-breath washout (FRC$_{MBW}$) at the age of 6 years, versus the mean of both. Dashed lines represent upper and lower limit of agreement, dotted lines represent confidence interval of mean difference.



Online Supplement Figure 3.7.2.:
Association of FRC$_{MBW}$ and LCI
Functional residual capacity from helium multiple-breath washout (FRC$_{MBW}$) plotted vs. lung clearance index (LCI) of the same individuals (n=99)

Discussion of physiological and technical explanations for the findings

We expected a relative difference between FRC from helium MBW and plethysmography at visit 2 of up to 20%, as described in previous studies [Pillow et al., 2004, Owens et al., 2011, Jensen et al., 2013]. Surprisingly, the observed difference was even higher.

To the best of our knowledge, paediatric reference values for helium MBW were not available. A previous study in children from 4 to 17 years of age reports LCI values from helium MBW; these were higher compared to published reference values using different tracers. FRC values were not reported in this study and main stream molar mass signal was used instead of side stream signal [Vermeulen et al., 2013]. A study in intubated rabbits showed lung volumes measured by helium MBW and chest computer tomography to be in the same range, but volumes were very small and cannot be compared to this paediatric population. Exact values were not given for LCI or FRC; figures indicated that LCI decreased with increasing lung volumes [Albu et al., 2014].

Published FRC values derived from helium dilution technique were lower than FRC values measured by plethysmography [Stocks and Quanjer, 1995]. As FRC_{pleth} measurements were within the expected range [Hibbert et al., 1989, Rosenthal et al., 1993] for children of this age group, the large relative difference was unlikely to be caused by an incorrect overestimation of FRC_{pleth}. Several reasons were likely to contribute to the large difference between the two techniques: Plethysmography detected trapped gas in addition to the lung volume that participated in ventilation. Physiologic variability of FRC and differences in measurement techniques were likely to contribute as well.

Intra-subject-variability, i.e. CV, was slightly higher in MBW compared to plethysmography, but this does not explain the large relative difference between the techniques either. Measurement error was evaluated for the MBW setup, it was within $\pm$10% approximately for FRC values of 600 ml and more [Schmidt et al., 2015b]. For plethysmography, measurement error was unknown. Bland-Altman plot showed a systematic underestimation of FRC in helium MBW. It is possible that specific normative values are needed for different techniques.

LCI was not independent of FRC (see Online Supplement Figure 3.7.2); and this indicated that our results were not reliable. In a cross-sectional paediatric study, upper limit of normal for LCI was constant beyond 6 years of age [Lum et al., 2013] and we expected LCI to be independent of FRC.

Only MBW files without apparent leak were included. From our experience, a flow baseline correction below 1 ml/kg body weight per MBW file is acceptable [Fuchs et al., 2011a]. We cannot exclude that identification of leakage by the software was always

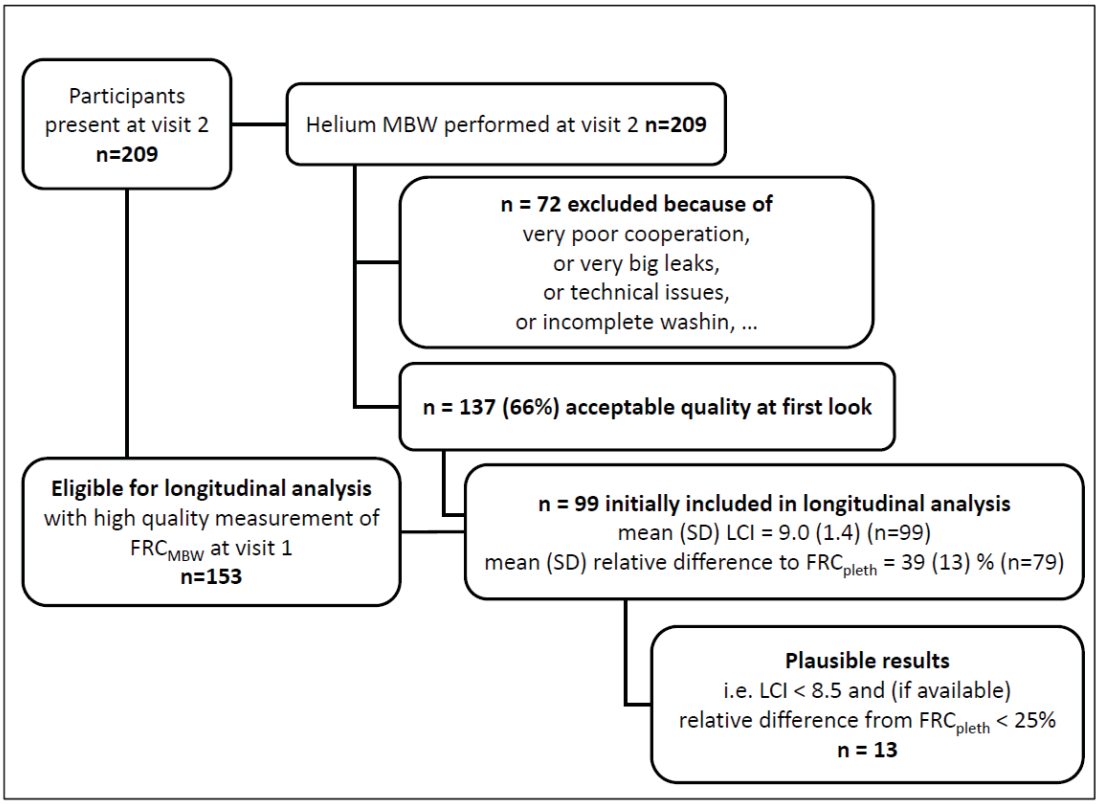
accurate as we had no insights in the details of the software. Leaks can occur at several locations within the setup, and the magnitude can vary within the breath cycle. It was challenging to identify leaks, especially as the software only allowed visual control. The changing tracer concentration was not displayed during measurements. A list of limitations of the helium MBW setup can be found in Online Supplement Table 3.7.5.

Online Supplement Table 3.7.5
Limitations of helium multiple-breath washout

Limitation	Implication for measurement	Problems we observed
low molar mass of helium	very volatile tracer	leaks: might be undetected, might vary over the breath cycle, and might be localised in varying places within the setup
tracer concentration was measured indirectly via end-inspiratory molar mass	several factors had an impact on side stream molar mass: main factors were tracer & CO_2	changes in CO_2 level within the measurement (e.g. hyperventilation) can interfere with determination of tracer concentration
wash-in might be incomplete	tracer concentration could only be ensured by visual control of the molar mass signal	despite experience of the operator, incomplete wash-in or washout was often invisible while measurement was performed
cooperation was limited in young children	both parts, wash-in and washout, need to be appropriate	cooperation might decrease with longer duration of the measurement, and breathing pattern during washout might be less regular than during wash-in

Implications

Unfortunately, most of the measurements we performed had to be excluded, and the remaining successful measurements were too few for a reliable statistical analysis (see Online Supplement Figure 3.7.3). In conclusion, helium MBW performed insufficiently in this population of young children. Helium is very volatile and thus prone to leaks; these leaks were often invisible during measurement, even for an experienced operator. The combination of limited cooperation in young children and suboptimal operator control caused poor quality in many of the measurements. From our experience, nitrogen MBW is easier to perform, especially by young children, as it is less prone to leaks and test duration is shorter.



Online Supplement Figure 2.3.:
Remaining participants for longitudinal analysis with outcome FRC$_{MBW}$
Longitudinal measurements of functional residual capacity (FRC) in children participating in the BILD study[Fuchs et al., 2012a] high quality measurements of FRC from multiple breath-washout (FRC$_{MBW}$) at visit 1 and visit 2 were available in n=99 children. Mean relative difference between FRC$_{MBW}$ and FRC from plethysmography (FRC$_{pleth}$) at the same visit was 39Mean lung clearance index (LCI) at visit 2 was 9.0. After exclusion of problematic helium MBW measurements, the number of children included was too low for a sound statistical analysis.

4 Can infant lung function predict respiratory morbidity during the 1rst year of life in preterm infants?

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4.1 ABSTRACT

Compared with term-born infants, preterm infants have increased respiratory morbidity in the first year of life. We investigated whether lung function tests performed near term predict subsequent respiratory morbidity during the first year of life and compared this to standard clinical parameters in preterms.

The prospective birth cohort included randomly selected preterm infants with and without bronchopulmonary dysplasia. Lung function (tidal breathing and multiple-breath washout) was measure at 44 weeks post-menstrual age during natural sleep. We assessed respiratory morbidity (wheeze, hospitalisation, inhalation and home oxygen therapy) after 1 year using a standardised questionnaire. We first assessed the association between lung function and subsequent respiratory morbidity. Secondly, we compared the predictive power of standard clinical predictors with and without lung function data.

In 166 preterm infants, tidal volume, time to peak tidal expiratory flow/expiratory time ratio and respiratory rate were significantly associated with subsequent wheeze. In comparison with standard clinical predictors, lung function did not improve the prediction of later respiratory morbidity in an individual child.

Although associated with later wheeze, noninvasive infant lung function shows large physiological variability and does not add to clinically relevant risk prediction for subsequent respiratory morbidity in an individual preterm.

4.2 INTRODUCTION

Respiratory morbidity is a common consequence of prematurity. Compared with term-born infants, preterm infants are more likely to have lung function alterations, and to develop respiratory symptoms in early infancy, childhood and adulthood [Baraldi et al., 1997, Latzin et al., 2009a, Vrijlandt et al., 2013, Narang et al., 2008, Doyle et al., 2006]. Several studies identified various perinatal factors leading to impaired lung function (e.g. post menstrual age (PMA) at birth or days on supplemental oxygen) and/or respiratory symptoms in later life (e.g. perinatal adaptation or ventilator support) [Latzin et al., 2009a, Doyle et al., 2006, Schmalisch et al., 2005, Pramana et al., 2011]. Although infants with bronchopulmonary dysplasia (BPD) [Ehrenkranz et al., 2005] are at higher risk of pulmonary morbidity, neither standard perinatal clinical risk predictors nor BPD severity classification can reliably predict subsequent respiratory disease burden in the first year of life for an individual child [Pramana et al., 2011, Lamarche-Vadel et al., 2004].

For clinical management, we need early functional biomarkers to identify infants with particular risks of subsequent respiratory morbidity. Infant lung function has been proposed as such a biomarker predicting respiratory morbidity in early life [Vrijlandt and Duiverman, 2010]. So far, few studies have investigated the association between early lung function and clinical outcome in the first years of life. Most of these studies included babies born at or near term [Gabriele et al., 2012, Giffin et al., 1994, Greenough et al., 1998, Latzin et al., 2006, van Putte-Katier et al., 2012]. For use in daily clinical routine, lung function measurements should not require sedation, and be minimally invasive and easily applicable at bedside, and normative values should be available. We developed such a lung function test compatible with the latest European Respiratory Society (ERS)/American Thoracic Society (ATS) consensus statement in our cohort of healthy infants [Frey et al., 2000a, Fuchs et al., 2012a, Fuchs et al., 2011a].

The aims of the present study were to investigate in infants born prematurely: first, to determine whether noninvasive lung function measured near term (44 weeks PMA) is associated with subsequent respiratory morbidity during the first year of life (pathophysiological association); and second, to assess the usefulness of early lung function tests to

predict later respiratory morbidity in the individual (clinical usefulness). In particular, we investigated whether postnatal lung function adds to the predictive power for subsequent symptoms in addition to standard perinatal predictors and risk scores.

4.3 METHODS

Study design

We prospectively recruited preterm infants during postnatal hospitalisation and performed lung function measurements at the age of 44 weeks PMA [Latzin et al., 2009b]. After 1 year of age, we sent standardised questionnaires to the parents to record respiratory symptoms during the first year of life [Pramana et al., 2011]. The Ethics Committee of the Canton of Bern (Bern, Switzerland) approved the study protocol and parents agreed, with written consent, to participate in the study.

Subjects

Infants born prematurely (<37 weeks PMA) were recruited at the University Childrens Hospital of Bern (Bern, Switzerland) between 1999 and 2010, excluding infants with congenital diaphragmatic hernia. Data on perinatal course were recorded using the neonatology database (NEODAT 4.10, Tubingen, Germany). We additionally assessed commonly used clinical scores such as the clinical risk index for babies (CRIB) [The international neonatal network, 1993] and the ATS JobeBancalari BPD severity classification [Ehrenkranz et al., 2005].

Lung function measurement

At the age of 44 weeks PMA, we performed lung function tests in unsedated infants during non-rapid eye movement sleep (by monitoring behaviour and online flowvolume curves). First, we measured tidal breathing for 10 min, assessing tidal volume (Vt), respiratory rate, minute ventilation (V'E), tidal expiratory flows at 25% (TEF_{25}) and 50% (TEF_{50}) of the remaining Vt, peak flow, and the time to peak tidal expiratory flow (tPTEF)/expiratory time (tE) ratio, the latter describing the shape of the tidal breathing

flowvolume loop. Second, we applied triplicate multiple-breath washout (MBW) measurements (each lasting 3–4 min) using 4% sulfur hexafluoride to estimate functional residual capacity (FRC) and ventilation heterogeneity, assessing lung clearance index (LCI), and first and second moment ratios. LCI is defined as the number of lung volume turnovers (in terms of FRC) required to reduce the sulfur hexafluoride to 1/40th of the starting concentration. It is calculated as cumulative expired volume/FRC. During the measurements, we aimed for a transcutaneous oxygen saturation $>$92%. When infants required supplemental oxygen, we started the analysis from the first constant breaths. Measurements were not undertaken in children whose oxygen saturation fell below 92% while breathing room air. We performed the measurements and the analysis according to the ERS/ATS consensus, using an ultrasonic flow meter device (Exhalyzer D; Eco Medics AG, Duernten, Switzerland), as previously reported [Frey et al., 2000a]. The equipment's dynamic dead space is estimated to be far below the recommended 2 ml/kg^{-1}.

Outcomes and questionnaire

This preterm cohort (recruitment period 1999-2007) has been described in detail in two previous papers [Latzin et al., 2009b, Pramana et al., 2011]. Since then, recruitment has been ongoing. The present study included infants recruited from January 1999 to December 31 2010. Follow-up questionnaires for the additional participants were distributed in 2011.

The questionnaire contained questions modified from that of the International Study on Asthma and Allergies in Childhood (http://isaac.auckland.ac.nz) (online supplement material). We defined four binary outcomes (yes/no): wheeze, inhalation therapy with β-agonists for $>$4 weeks, re-hospitalisation due to respiratory disease (excluding admissions for diagnostic purposes), and home oxygen therapy after hospital discharge. These outcomes can be reproducibly assessed, as previously reported in a subpopulation of this cohort and in other studies [Pramana et al., 2011, Peacock et al., 2013, Strippoli et al., 2007]. The returned questionnaires underwent quality control performed by study nurses and medical doctors. We further validated the answers for re-hospitalisation and home oxygen therapy using medical histories from hospital records. As four out of 45 re-hospitalisations were related to check-ups rather than symptoms, these were recoded.

Statistical analysis

Pathophysiological association

We investigated the association between lung function parameters and subsequent respiratory morbidity separately for each of the four outcomes, using multivariable logistic regression. Adverse perinatal course and pre-existing risk factors may cause respiratory morbidity through an independent pathway not involving lung function. Therefore, to avoid confounding in the association between lung function and respiratory symptoms, we adjusted the models for: maternal atopy (defined as history of hay fever, asthma or eczema); sex; mode of delivery; PMA at birth; Apgar score at 5 min; duration of mechanical ventilation (days of invasive ventilation (intubation) plus days of noninvasive ventilation (continuous positive airway pressure)); weight gain as a standard deviation score (SDS) using the 1990 British Growth Reference from birth until the lung function test date; and PMA, weight in SDS and length in centimetres at the lung function test date. To reduce the risk of collinearity and Type I error, we chose the previously mentioned clinical parameters prior to statistical analysis, guided by clinical evidence [Lamarche-Vadel et al., 2004] and experience from our prospective healthy term cohort from the same region [Pramana et al., 2011, Latzin et al., 2007a]. We describe sensitivity analyses in the online supplementary material.

Clinical relevance

We investigated whether the performed lung function test improved the prediction of respiratory symptoms compared to the standard clinical predictors or scores alone. We carried out logistic regressions using common clinical scores (CRIB and BPD classification), and standard predictors (maternal atopy, sex, PMA at birth, mechanical ventilation and weight gain). We then included lung function parameters in each of the models and compared the area under the receiver operator characteristic curve (AUC) to assess discrimination and the Brier score to assess the overall predictive performance of the models. The Brier score [Pescatore et al., 2014] is the averaged squared difference between the predicted and observed outcomes: the lower the score, the more accurate the prediction, so that a perfect predictor would have a score of 0, a perfect mispredictor a score of 1 and

a predictor acting as a fence-sitter a score of 0.25. In addition, we calculated the p-values of likelihood ratio tests (LRTs) comparing models with and without lung function data included.

We performed the analysis using Stata version 11.2 for Windows (Stata Corporation, College Station, TX, USA).

4.4 RESULTS

Study population

We performed lung function measurements in 236 preterm born infants between 1999 and 2010. 166 (70%) caregivers returned the follow-up questionnaires (online supplementary fig. S1). In the analysis, we used data of 163 subjects with valid tidal measurement and 142 subjects with valid MBW according to ERS/ATS quality criteria [Robinson et al., 2013] (tables 1 and 2, and online supplementary tables S1 and S2).

The 70 caregivers who did not complete questionnaires had infants with a more severe perinatal course: they had lower PMA at birth (mean 27.9 versus 29.2 weeks), lower Apgar score at 5 min (mean 7 versus 7.6), longer mechanical ventilation (mean 39 versus 25 days) and more severe BPD. There were no significant differences in weight (in SDS), length, CRIB score, delivery mode and most lung function parameters (table S3). The cohort differed from the total population of preterms hospitalised at the Childrens Hospital of Bern in that the clinical conditions of the preterms without BPD are somewhat poorer (table S4).

Table 4.1.
Demographics, clinical, anamnestic and lung function data of the cohort infants

	Value	Intrasubject SD (CV %)
Subjects n (%)	166 (100)	
Clinical anamnestic parameters		
Maternal atopy n (%)	25 (15)	
Tobacco exposure during pregnancy[#] n (%)	15 (9)	
Clinical perinatal parameters		
Females n (%)	69 (42)	
Post-menstrual age at birth weeks	29±3 (23–36)	
Vaginal delivery[¶] n (%)	40 (24)	
Weight at birth g	2000±515 (420–2980)	
Length at birth cm	38±4 (27–50)	
Apgar score at 5 min[¶]	7.6±2 (2–10)	
CRIB score[+]	3.9±4 (0–16)	
Chorioamnionitis[§] n (%)	52 (34)	
Malformations[ƒ] n (%)	34 (20)	
Patent ductus arteriosus n (%)	70 (42)	
Atrial septal defect n (%)	6 (4)	
Clinical post-natal parameters		
Mechanical ventilation days	25±21 (0–80)	
Oxygen therapy days	50±52 (0–385)	
Breastfeeding[+] n (%)	112 (74)	
Weight at study date SDS	-1.07±1 (-4.7–3.9)	
Weight gain SDS	-0.3±1 (-4.1–4.7)	
Length at study date cm	52±3 (39–63)	
Post-natal age at study date days	108±26 (37–198)	
Lung function parameters		
Tidal measurements n	163	
V_T/weight mL·kg^{-1}	7.4±1.6 (4.7–19)	1.1 (4)
Respiratory rate breaths per min	51±12 (29–94)	4.1 (8)
t_{PTEF}/t_E %	28±9 (11–61)	5.9 (22)
Minute ventilation mL·min^{-1}	1415±337 (678–2388)	99 (7)
TEF_{50} mL·s^{-1}	57±15 (22–108)	5.8 (10)
TEF_{25} mL·s^{-1}	43±11 (20–76)	5.1 (12)
Peak tidal expiratory flow mL·s^{-1}	66±18 (26–134)	5.8 (8.6)
MBW measurements n	142	
FRC/weight mL·kg^{-1}	23±5 (13–35)	
LCI	6.9±0.7 (5.2–9.4)	
Moment ratio 1	2.1±0.25 (1.6–3)	
Moment ratio 2	9.1±2.7 (4.8–19.7)	

Data are presented as mean±SD (range) unless otherwise stated. Descriptive statistics of clinical and anamnestic data assessed in the neonatal period and lung function parameters assessed at 44 weeks of post-menstrual age. CV: coefficient of variation; CRIB: clinical risk index for babies; SDS: standard deviation score; V_T: tidal volume; t_{PTEF}: time to peak tidal expiratory flow; t_E: expiratory time; TEF_{50}: tidal expiratory flow at 50% of the remaining V_T; TEF_{25}: tidal expiratory flow at 25% of the remaining V_T; MBW: multiple-breath washout; FRC: functional residual capacity at airway opening; LCI: lung clearance index. [#]: data missing for two subjects; [¶]: data missing for one subject; [+]: data missing for 15 subjects; [§]: data missing for 17 subjects; [ƒ]: including dysmorphic syndrome (n=3 including subtelomere 18q deletion and trisomy 21), mega cisterna magna (n=1), plagiocephaly (n=2),partial craniosynostosis (n=1), macrocephaly (n=1), pre-auricular tag and fistula (n=2), cleft palate (n=2), subglottic stenosis (n=1), laryngomalacia (n=2), lung hypoplasia (n=1), ventricular septal defect (n=3), accessory left ventricular chordae tendineae (n=1), Wolf–Parkinson–White syndrome (n=1), kidney anomalies (n=2), vesicoureteral reflux (n=2), hydrocoele (n=2), cryptorchidism (n=2), hypospadias (n=3), hernia inguinalis (n=2) and talipes varus (n=1).

Table 4.2.
Outcomes: respiratory symptoms

Subjects n	166
Wheeze	76 (46)
Inhalation therapy	63 (38)
Re-hospitalisation	41 (25)
Home oxygen therapy	11 (6)

Data are presented as n (%) unless otherwise stated. Symptoms during the first year of life assessed through questionnaire.

Pathophysiological association

Infant lung function was weakly associated with subsequent wheeze and inhalation therapy (table 3). For example, Vt was positively associated with wheeze (adjusted OR (aOR) 1.40 (95%CI 1.04–1.90) per 5 ml increase in Vt) and respiratory rate was negatively associated with wheeze (aOR 0.69 (95%CI 0.50-0.96) per 10 breath per min increase) (figs 1 and 2). A higher tPTEF/tE ratio was negatively associated with several outcomes, most strongly with inhalation therapy (aOR 0.56 (95%CI 0.35-0.89) per 10% increase) and oxygen therapy (aOR 0.27 (95%CI 0.07-1.07) per 10% increase). V'E, FRC, LCI, moment ratios and tidal flows (TEF_{50}, TEF_{25} and peak flow) were not associated with subsequent symptoms (see table 3 for results for all lung function variables except flows). All approaches of the sensitivity analyses confirmed the main results (online supplementary table S5). For instance, the aOR per 10% increase in tPTEF/tE for inhalation therapy was 0.65 (95%CI 0.43-0.97) in the unadjusted model, 0.56 (95%CI 0.35–0.89) in the main model and 0.54 (95%CI 0.29-1.03) when further adjusted. Six infants required oxygen therapy at the time of lung function measurements; none was ventilated. The sensitivity analysis adjusted for additional oxygen showed comparable results. The stratification based on oxygen therapy (yes/no) at 36 weeks PMA (i.e. no or mild BPD versus moderate or severe BPD) also confirmed the main results (data not shown).

Table 4.3.
Associations between lung function at 44 weeks of post-menstrual age and subsequent symptoms during infancy

	Wheeze	Inhalation therapy	Re-hospitalisation	Home oxygen therapy
Respiratory rate per 10 breaths per min	0.69 (0.5–0.96)*	0.89 (0.66–1.21)	1.00 (0.72–1.40)	1.82 (0.93–3.5)
Tidal volume per 5 mL	1.40 (1.04–1.9)*	1.2 (0.89–1.6)	0.85 (0.58–1.26)	0.30 (0.08–1.2)
Minute ventilation per 5 mL·min^{-1}	1.00 (0.99–1.01)	1.00 (0.99–1.01)	1.00 (0.98–1.01)	1.00 (0.99–1.02)
t_{PTEF}/t_E per 10%	0.96 (0.64–1.43)	0.56 (0.35–0.89)*	0.72 (0.43–1.18)	0.27 (0.07–1.07)
FRC per 5 mL	0.93 (0.83–1.05)	0.96 (0.85–1.08)	0.89 (0.77–1.02)	0.77 (0.57–1.04)
LCI	0.71 (0.42–1.2)	1.15 (0.66–1.99)	0.52 (0.27–1.0)	1.6 (0.49–5.3)
Moment ratio 1	0.26 (0.06–1.19)	1.17 (0.26–5.38)	0.15 (0.02–0.96)*	9.88 (0.27–365)
Moment ratio 2	0.9 (0.8–1.02)	1.02 (0.88–1.18)	0.84 (0.71–1)	1.4 (0.97–2.02)

Data are presented as adjusted OR (95%CI) for symptoms per increase in lung function parameter by the increment indicated. The units were arbitrarily chosen for better readability. The multivariable regressions were adjusted for maternal atopy, sex, mode of delivery, post-menstrual age at birth, Apgar score at 5 min, duration of mechanical ventilation, weight gain from birth until the lung function test date as a standard deviation score (SDS), and post-menstrual age, weight as a SDS and length in centimetres on the lung function test date. tPTEF: time to peak tidal expiratory flow; tE: expiratory time; FRC: functional residual capacity at airway opening; LCI: lung clearance index. *: $p<0.05$.

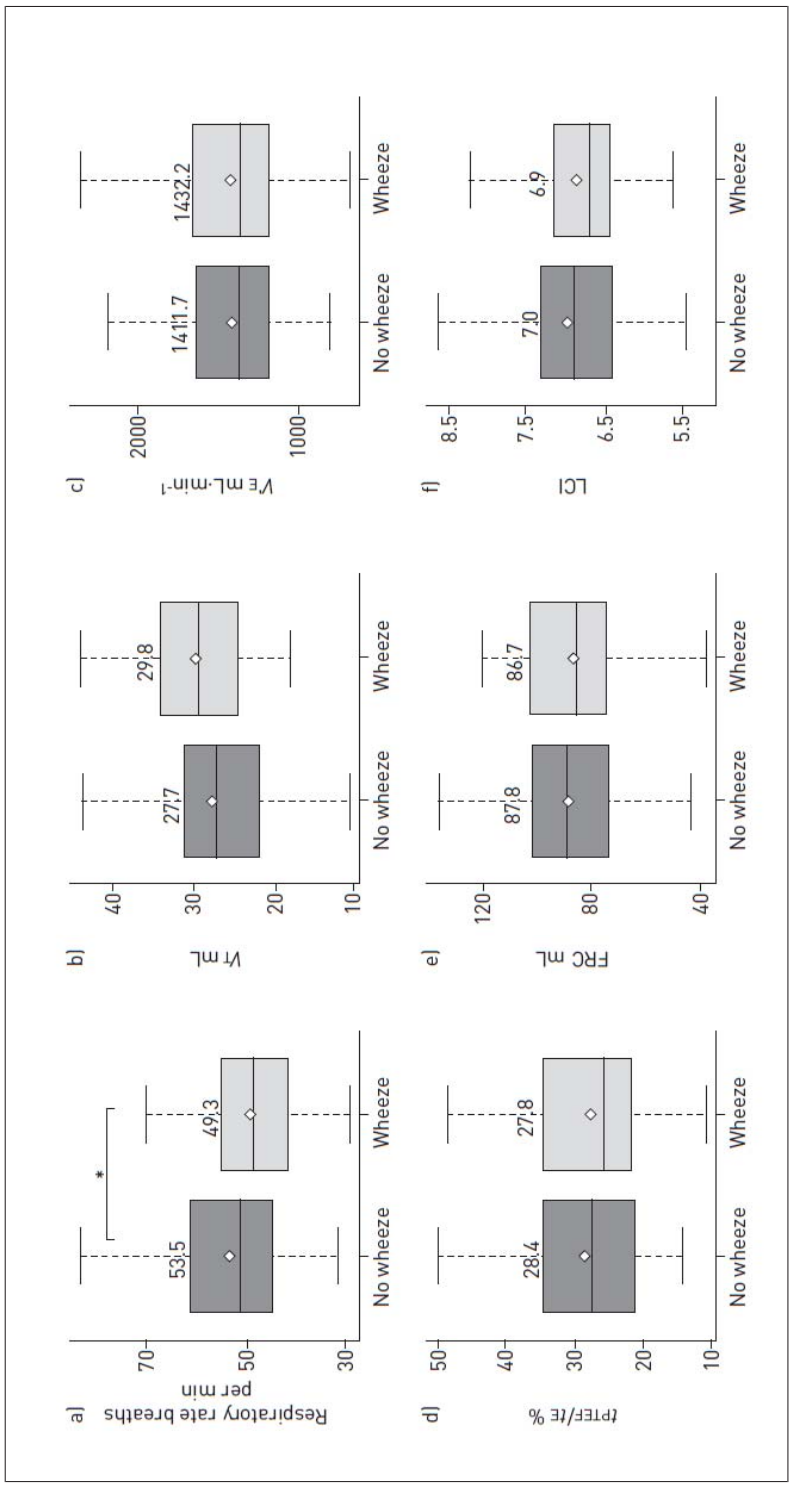


Figure 4.1.
Box plots of a) respiratory rate, b) tidal volume (Vt), c) minute ventilation (V'E), d) time to peak tidal expiratory flow (tPETF)/expiratory time (tE) ratio, e) functional residual capacity (FRC) and f) lung clearance index (LCI), measured at 44 weeks of post-menstrual age, stratified by the outcome wheeze. Numbers and diamonds indicate the mean value of the lung function parameter within the groups, horizontal lines represent the median, boxes represent the interquartile range, and whiskers represent the range. We performed t-tests to compare the mean values of each lung function parameter in the infants who developed subsequent wheeze and in the group who did not develop wheeze. We found a difference in respiratory rate of 4.2 breaths per min between the two groups. *: p,0.05.

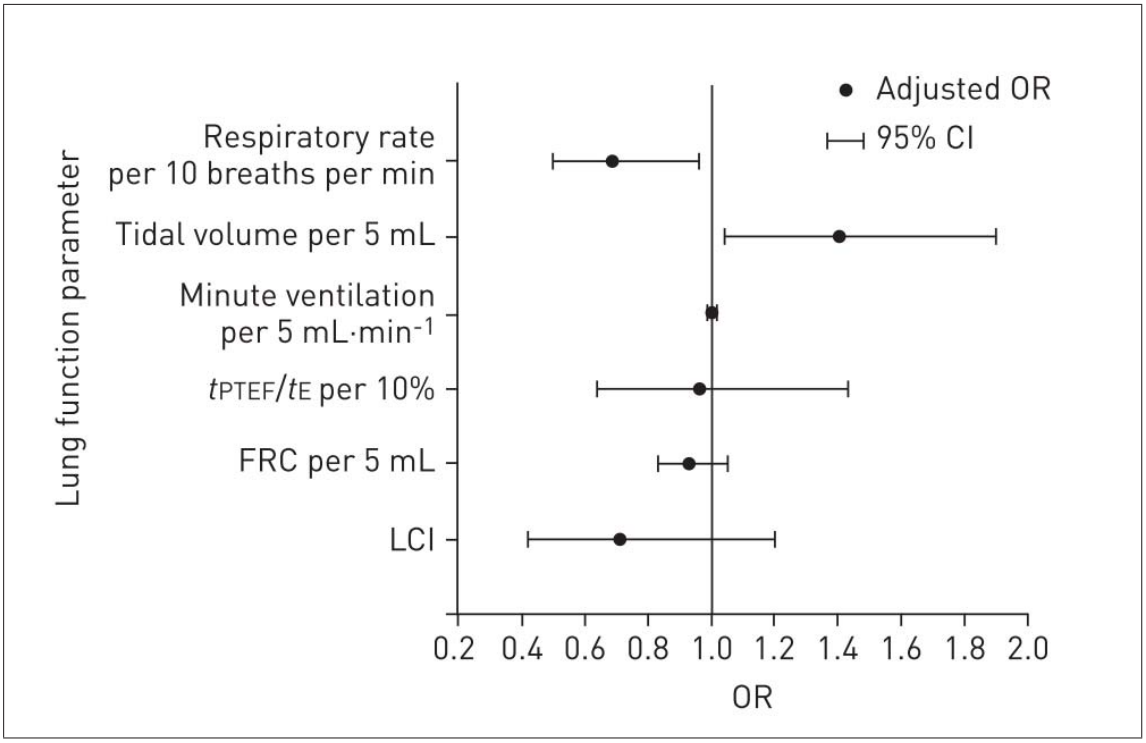


FIGURE 4.2:
Association between the lung function parameters assessed at 44 weeks of post-menstrual age (PMA) and subsequent wheeze during infancy. The models were adjusted for maternal atopy, sex, mode of delivery, PMA at birth, Apgar score at 5 min, duration of mechanical ventilation, weight gain as a standard deviation score (SDS) from birth until the lung function test date, and PMA, weight as SDS and length in centimetres at the lung function test date. Adjusted odds ratio and 95% confidence intervals for developing subsequent wheeze are shown, per increment of the lung function parameter. tPTEF: time to peak tidal expiratory flow; tE: expiratory time; FRC: functional residual capacity; LCI: lung clearance index.

Clinical relevance

The prediction model including common clinical scores only (BPD severity and CRIB score) had the lowest performance, with an AUC of 0.54 and a Brier score of 0.25. The model with the combination of standard clinical predictors had a better performance, with an AUC of 0.67 and a Brier score of 0.23. Adding lung function parameters to

each of these three models did not improve the performance (table 4 and fig. 3). For example, adding Vt, respiratory rate and tPTEF/tE to a combination of standard clinical predictors (sex, PMA, mechanical ventilation, weight gain and maternal atopy), the AUC increased from 0.68 to 0.71 and the Brier score improved from 0.23 to 0.22. The p-value of the LRT was 0.12.

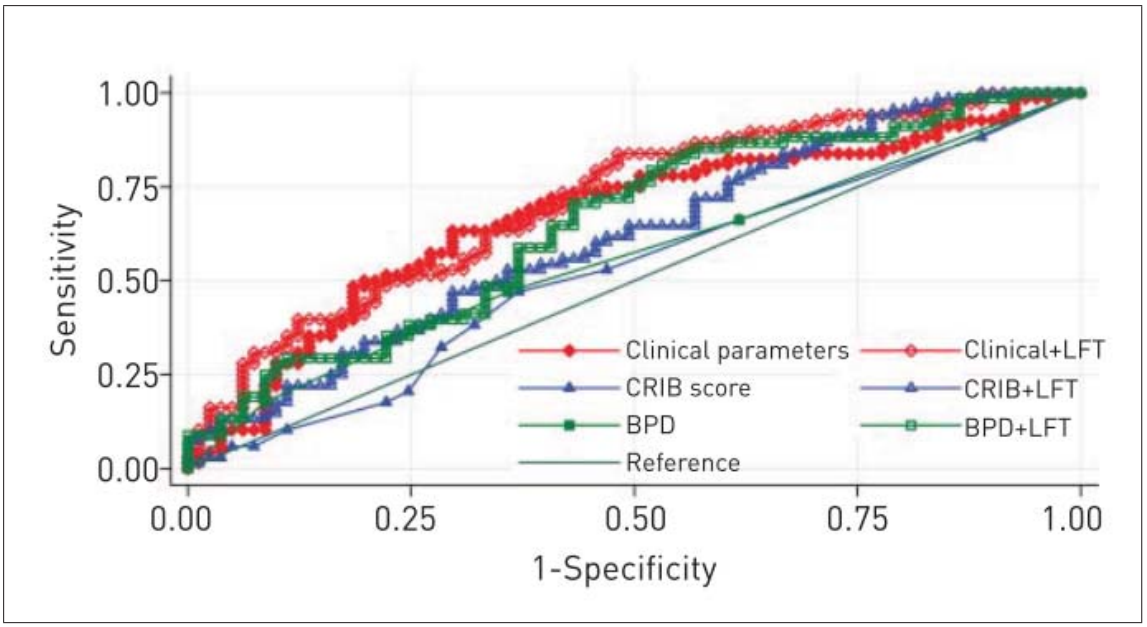


FIGURE 4.3:
Receiver operating characteristic curves comparing bronchopulmonary dysplasia (BPD) classification, clinical risk index for babies (CRIB) score, clinical standard predictors alone and with the lung function parameters (minute ventilation, respiratory rate, time to peak tidal expiratory flow/expiratory time ratio) for prediction of subsequent wheeze during the first year of life. Clinical predictors: sex, post-menstrual age, maternal atopy, weight gain from birth until the time of lung function test and days of mechanical ventilation. LFT: lung function test.

Table 4.4.
Additional value of adding data from lung function tests, compared to a model including standard clinical predictors only, for the prediction of wheeze during the first year of life

	Model with clinical predictors		Models with clinical predictors and lung function test		
	AUC	Brier	LRT p-value	AUC	Brier
BPD classification (four classes)	0.54	0.25	0.15	0.63	0.24
CRIB score (16 points)	0.52	0.25	0.08	0.62	0.23
Clinical standard predictors#	0.68	0.23	0.12	0.71	0.22

Prediction of wheeze using bronchopulmonary dysplasia (BPD) classification, clinical risk index for babies (CRIB) score, clinical standard predictors alone and with the lung function parameters associated with symptoms in the first analysis (minute ventilation, respiratory rate and time to peak tidal expiratory flow/expiratory time ratio). Area under the receiver operating characteristic curve (AUC) and Brier score of the logistic regression models including clinical parameters and with addition of lung function parameters combined are shown. The models with lung function parameters were adjusted for age and weight as a standard deviation score at the time of the lung function test. We compared the models using the likelihood ratio test (LRT). The LRT is used to compare the fits of two similar (nested) models. A p-value <0.05 suggests that one model fits the data significantly better than the other. The Brier score measures the total difference between the event (wheeze) and the prediction probability of that event as an average squared difference. As a benchmark, a perfect predictor would have a Brier score of 0, a perfect mispredictor would have a Brier score of 1 and a fence-sitter (prediction of every wheezer as a "50/50" chance) would have a Brier score of 0.25. #: sex, post-menstrual age, maternal atopy, weight gain from birth until the time of lung function test and days of mechanical ventilation.

4.5 DISCUSSION

Summary

With the present study, we demonstrate weak correlations of tidal breathing parameters with subsequent respiratory symptoms. These findings give interesting insight into adaptive mechanisms in preterm infants but do not help to predict respiratory outcomes in these children.

Pathophysiological association

From a physiological point of view, we observed an interesting breathing pattern among the infants developing subsequent wheeze. This group has a lower respiratory rate and a higher Vt. We hypothesise that this might be caused by adaptive mechanisms only seen in unsedated infants. Greater dynamic dead space and airways more susceptible to collapse may be compensated by higher Vt (lung volume recruitment), leading to lower respiratory rates, in order to maintain constant V'E. An increase in dynamically controlled dead space could lead to increased ventilation heterogeneity, i.e. increased LCI, as reported previously [Latzin et al., 2009b, Hülskamp et al., 2009]. Additional data suggest an immature hypercapnic ventilatory response in the first weeks of life with limited change in respiratory rate but substantial increase of Vt [Putnam et al., 2005]. Whether this immature response still influences the breathing pattern at 44 weeks PMA in premature infants remains unclear, as it has only been studied in the first few weeks of life. Another explanation for higher Vt and lower respiratory rates could be a higher time constant in these infants that is already present near term. This hypothesis is supported by the work of van Putte-Katier et al. [van Putte-Katier et al., 2012], who demonstrated an increased risk of wheeze in the first year of life in term-born infants with higher time constant at the age of 2 months, and by a recent publication by Drysdale et al. [Drysdale et al., 2011], who reported that prematurely born infants admitted to hospital for lower respiratory tract infection in the first year of life had higher resistance of the respiratory system at 36 weeks PMA. To further elucidate the mechanical changes in this group of premature infants, future studies need to explore airway mechanics, flow limitation and adaptive mechanisms in more detail using additional lung function techniques.

An as yet unexplored but potentially highly relevant explanation is that preterm infants have a ventilation perfusion mismatch and a rarefication of the acinar structure. Modelling studies of pulmonary carbon dioxide washout have shown that a potential simplification of acinar structure may affect carbon dioxide clearance mostly at low Vt independently of the respiratory rate [Neufeld et al., 1992]. Thus, in order to maintain V'E and effective carbon dioxide elimination, preterms may predominantly increase Vt and even decrease respiratory rate.

Clinical relevance

Lung function measured at 44 weeks PMA in premature infants is weakly related to respiratory symptoms in the first year of life. The differences in Vt, respiratory rate and tPTEF/tE we found at the group level (between those infants who subsequently developed wheeze or needed inhalation therapy versus those who did not) had poor predictive value for later respiratory symptoms at the individual level. From a clinical point of view, the lung function parameters measured near term in our study do not yet help in predicting later respiratory symptoms in the first year of life.

Predicting respiratory outcome in preterms: comparison with previous literature

Multiple studies aimed to predict respiratory outcome after preterm birth using different clinical factors, such as chorioamnionitis or BPD. Even prematurity without BPD is associated with a higher prevalence of respiratory symptoms in preschool children and schoolchildren [Vrijlandt et al., 2013, Narang et al., 2008, Lamarche-Vadel et al., 2004, Palta et al., 2001, Vrijlandt et al., 2007]. Additionally, preterms can exhibit impaired lung function from preschool age to adulthood [Baraldi et al., 1997, Doyle et al., 2006]. In contrast to our study, these studies used lung function as an outcome but not as a predictor.

Other studies included term-born infants and assessed whether infant lung function predicts short-term outcomes [Greenough et al., 1998, van Putte-Katier et al., 2012, Kavvadia et al., 2000]. Greenough et al. [Greenough et al., 1998] investigated whether lung function testing in term-born infants shortly after birth was predictive of respiratory out-

come in the first year of life. They found that higher airway resistance and higher FRC after birth related to symptoms in the first year of life, but the positive predictive value was weak. Drysdale et al. [Drysdale et al., 2011] studied whether preterm infants developing viral lower respiratory tract infection in the first year of life had poorer lung function at 36 weeks PMA than infants without infection. They found no difference in respiratory system resistance, compliance or FRC, except in children who were hospitalised, whose resistance was significantly higher. It is important to note that measurements of lung function at 36 weeks PMA are technically demanding, show high variability and represent different aspects of lung mechanics to measurements performed in later phases of lung development after term. In contrast to our study, Drysdale et al. [Drysdale et al., 2011] used a combined outcome of cough, wheeze and/or shortness of breath, and they used different lung function techniques, which makes comparison with our data difficult. Consistent with our data, van Putte-Katier et al. [van Putte-Katier et al., 2012] showed in termborn infants that a higher respiratory system compliance before the age of 2 months was associated with a decreased risk of cough and wheeze during the first year of life, whereas higher resistance and time constant were associated with an increased risk of cough and wheeze. Kavvadia et al. [Kavvadia et al., 2000] investigated the predictive value of very early (day 2 of life) lung function measurements and found that FRC $<$19 ml/kg^{-1} body weight was independently predictive of oxygen dependency beyond day 28 or 36 weeks PMA in very preterm infants. In summary, there is little evidence to support regular clinical use of current lung function techniques to estimate respiratory disease outcomes in premature infants.

Strengths and limitations

We investigated the clinical value of early lung function measurements to predict respiratory morbidity during the first year of life in preterm infants. To our knowledge, together with the study by Drysdale et al. [Drysdale et al., 2011], this is the only large study in preterm infants. Ideal for prediction would be a lung function parameter with low variability, such as maximal flow at FRC; however, these techniques are more invasive as they require sedation for squeeze manoeuvres and are less applicable in sick infants, as a bedside application or for widespread clinical use. We used a noninvasive

technique not interfering with potential physiological adaptations of underlying structural and/or functional alterations, being aware of the potentially larger intersubject variability. At 44 weeks PMA, high-quality, standardised lung function measurements are easier to perform, compared with measurements at 36 weeks PMA [Fuchs et al., 2011a]. Nevertheless, normal physiological variability is still rather high, making infant lung function in an individual difficult to interpret.

Retrospective questionnaires always carry the risk of reporting bias. The dropout rate of 30% in the first year of life is comparable to that reported in other studies [Vrijlandt et al., 2013]. However, it entails the risk of selection bias. In fact, the final cohort contained fewer subjects with severe BPD. As the aim of this study was to investigate an association between lung function and symptoms, and not to obtain precise estimates of prevalence or incidence of symptoms, this should not have greatly affected our findings. This is further supported by the fact that results in the subgroup of children with more severe BPD were comparable to those from the whole group. Moreover, the preterms with BPD had a comparable clinical condition to those in the whole population of preterms. We did not include more specific lung function measures that would require tidal phase III slope analyses (e.g. double-tracer gas phase III slope, and ventilation inhomogeneity of conducting and acinar airways).

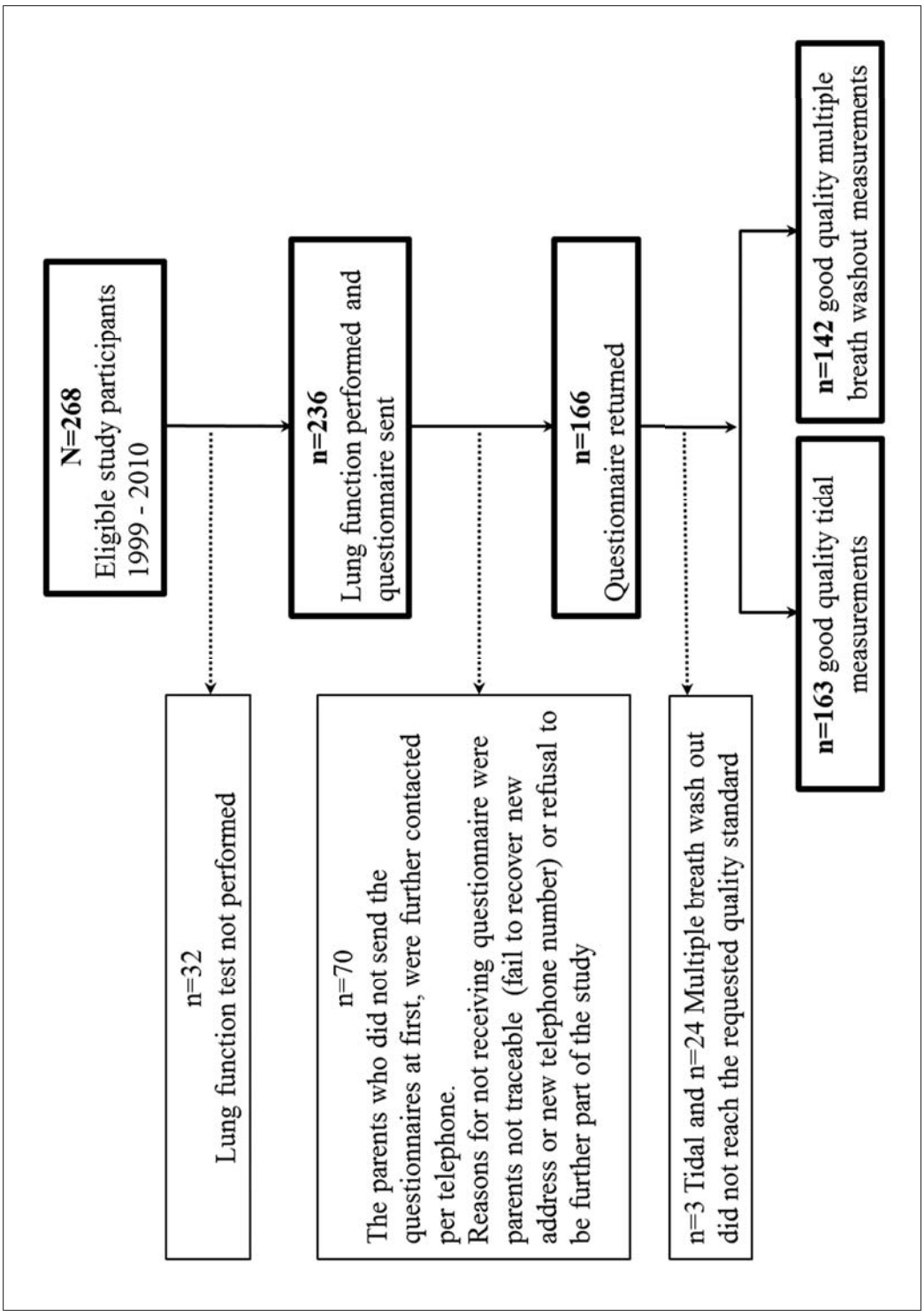
Conclusion

Alterations in tidal breathing pattern measured at 44 weeks were associated with respiratory outcomes in the first year of life in premature infants. For instance, higher Vt and lower respiratory rate were associated with wheeze later in life. This combination potentially reflects an adaptive change in breathing pattern due to underlying structural pathology. However, the clinical value of postnatal lung function testing for the prediction of respiratory morbidity is limited for the individual child: lung function data (tidal breathing and MBW measurement) did not improve prediction of morbidity over and above standard clinical parameters, which are easier to assess. This could partly be due to the high variability of parameters measured during natural sleep. Whether this is different for more invasive lung function tests, such as raised volume rapid thoracic compression, needs to be examined; however, their invasive nature and the need for sedation

preclude clinical application in routine settings.
In summary, we do not recommend routine tidal lung function testing as a clinical tool to predict subsequent symptoms in the first year of life, but we strongly recommend its use in future studies as an important means for understanding respiratory pathophysiology.

Acknowledgements
We thank Olaf Ahrens and Roland Gerull (Division of Neonatology, Department of Paediatrics, University Childrens Hospital Bern, Bern, Switzerland) who provided us with neonatal data; Gisela Wirz, Monika Graf, Sandra Lüscher and Barbara Hofer (study nurses; UKBB, Basel, Switzerland), who were responsible for data management and who were largely responsible for performing lung function measurements; and Janet Maccora (UKBB), who provided English language editorial assistance.

4.6 Can infant lung function predict respiratory morbidity during the 1rst year of life in preterm infants? SUPPLEMENTAL ONLINE FIGURE



Supplemental online figure

5 Functional evidence for continued alveolarization in former preterm children at school age?

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5.1 SUMMARY

Prematurity is the most common disruptor of lung development. The aim of our study was to examine the function of the more vulnerable peripheral airways in former preterm children by multiple-breath washout (MBW) measurements. Eighty-six school-aged children, born between 24 and 35 weeks of gestation and 49 term-born children performed nitrogen MBW. Lung clearance index (LCI), and slope III derived Scond and Sacin were assessed as markers for global, convection-dependent and diffusion-convection dependent ventilation inhomogeneity, respectively. We analyzed data of 77 former preterm (mean age 9.5 years; range 7.2 – 12.8) and 46 term-born children (mean age 9.9 years; range 6.0 - 15.9). LCI and Sacin did not differ between preterm and term-born children. Scond was significantly elevated in preterm compared to term-born participants (mean difference 1.74 z-scores, 95%CI 1.17 - 2.30, $p < 0.001$), with 54% of former preterm children showing elevated Scond. In multivariable regression analysis Scond was significantly related only to gestational age (R^2 0.37). Normal Sacin provides evidence for functionally normal alveolar compartment, while elevated Scond indicates impaired function of more proximal conducting airways. Together, our findings support the concept of continued alveolarization albeit with dysanaptic lung growth in former preterm children.

5.2 INTRODUCTION

On a global scale, the proportion of preterm births has steadily increased over the last decades [Blencowe et al., 2012]. Despite remarkable advances in neonatal and perinatal care [Field et al., 2008], the incidence of bronchopulmonary dysplasia (BPD) remains high affecting up to 40% of the extremely premature infants who survive [Stoll et al., 2010, Jobe, 2012]. Since its first description almost fifty years ago [Northway et al., 1967] advances in the management of respiratory distress syndrome (prenatal steroids, administration of surfactant, minimising of oxygen toxicity and barotrauma) have led to changes in the underlying pathological features of BPD. Thus, the contemporary form of the disease is characterized by impaired alveolar and vascular development which results in abnormal septation, fewer and larger alveoli, and smaller vascular bed, but with only minimal peribronchial fibrosis and airway-muscle hypertrophy [Baraldi and Filippone, 2007, Jobe and Bancalari, 2001, Berkelhamer et al., 2013]. Despite the apparently less severe lung pathology in infants born extremely premature in the post-surfactant era, long-term respiratory impairment still exists. Several studies have shown evidence of airway obstruction [Fawke et al., 2010, Lum et al., 2011, Hacking et al., 2013, Kaplan et al., 2012, Joshi et al., 2013] and air trapping [Lum et al., 2011, Kaplan et al., 2012, Joshi et al., 2013] at school age in such populations. However, only few studies have specifically examined ventilation inhomogeneity (VI) as biomarker of peripheral lung function, and this with inconclusive results. Two studies in preterm infants showed contradictory results, one with elevated VI [Hülskamp et al., 2009] and the other with no sign for VI [Latzin et al., 2009b]. At school age only one study examined VI in former preterm children showing mildly elevated VI in 58% of them [Lum et al., 2011]. Currently, BPD affects mainly premature infants born during the early saccular period of lung development. Thus, because the subsequent alveolar period of lung development is disrupted [Weibel, 2013, Burri, 2006], long-term adverse effects on lung function are not unexpected. The aim of our study was therefore, to examine the function of the most vulnerable peripheral lung regions in former preterm children at school age using multiple-breath washout (MBW) measurements - a well-established technique for the assessment of small airway function [Verbanck et al., 2003, Horsley et al., 2008b, Thompson et al., 2014, Stanojevic

et al., 2015]. In addition to the lung clearance index (LCI) and moment ratios which were used as measures of global VI, we particularly focused on specific parameters of regional VI, namely Sacin for the alveolar lung regions and Scond for the conducting airways. We hypothesized that due to the premature disruption of normal lung development, former preterm children may present an abnormal ventilation distribution particularly in alveolar lung regions.

5.3 METHODS

Study Population

Between February 2012 and June 2014, we prospectively recruited former preterm and term-born children aged 6 to 16 years at the University Childrens Hospital of Bern. Preterm-born participants ($<$37 weeks of gestation) were recruited from a previously described study [Pramana et al., 2011] and the outpatient clinics. Preterm children with congenital bronchopulmonary malformations, or thoracic-abdominal surgery were excluded. According to their gestational age, preterm children were classified into extremely low gestational age neonates (ELGAN) $<$28 weeks of gestation) or no-ELGAN ($\geq$28 weeks of gestation). Term-born controls with birth weight appropriate for gestational age (i.e. $>$2500 g in all cases) were recruited from local schools and playgroups. Term-born children with asthma, or other chronic respiratory diseases were excluded. Exclusion criteria for both preterm and term-born participants were respiratory infections within the last 3 weeks. The study was approved by the Ethics Committee of the Canton Bern, Switzerland. The children's assent was obtained and parents or caregivers provided written informed consent.

Assessment of respiratory symptoms and atopic sensitization in former preterm participants

In preterm children symptoms related to asthma and allergy were assessed by an adapted International Study on Asthma and Allergies in Childhood (ISAAC) questionnaire filled out by the parents [Asher et al., 1995]. For later subgroup analysis, we focused on four binary outcomes (yes/no): Wheeze over the past 12 months, cough without cold, shortness of breath due to cough or asthma, and doctor diagnosed asthma. Current asthma was defined as wheeze or use of asthma medication in the past 12 months or doctor-diagnosed asthma. Atopic sensitization was assessed by positive skin prick testing on at least one common inhalant allergen (mixed trees, mixed grasses, cat, dog, house dust mite and Aspergillus fumigatus). We further measured the fraction of exhaled nitric (FeNO) oxide by the single-breath method using a commercially available analyser (CLD 77 AM; Eco Medics AG). After inhaling NO-free air to TLC, children were exhaling against an expira-

tory resistance with a constant flow of 50ml*s^{-1} for at least 6 seconds. To prove validity of our findings irrespective of other possible changes in lung development, we performed sensitivity analysis comparing three subgroups of preterm children to term-born controls: (i) Absence of respiratory symptoms (absent if all four above mentioned survey questions were negative), (ii) no current asthma, and (iii) absence of atopic sensitization (negative prick testing and FeNO $<$20 ppb; [Dweik et al., 2011]).

Nitrogen multiple-breath washout

All children performed nitrogen MBW (N_2MBW) according to current consensus [Robinson et al., 2013] using a described setup [Singer et al., 2012a, Singer et al., 2013] (Exhalyzer D, Eco Medics AG, Duernten, Switzerland). The application of 100% O_2 washes out the lung resident N_2. The N_2 signal is measured indirectly from O_2, CO_2 and the estimated Argon fraction. Children performed N_2MBW while sitting upright, wearing a nose clip and tidally breathing through a snorkel mouth piece. At least two successful N_2MBW runs were obtained. N_2MBW runs were accepted if functional residual capacity (FRC) varied $<$10% for 2 and $<$25% for 3 trials [Robinson et al., 2013]. Measurements with leaks were excluded. All data were recorded and analyzed using Spiroware 3.1.6 (Eco Medics AG). Outcomes were calculated as currently recommended [Robinson et al., 2013], LCI from cumulative expired volume divided by FRC, resulting in lung turn over units. We used LCI and moment ratios (M1/M0, M2/M0) as a measure of global VI. As specific markers for peripheral lung ventilation we analyzed slopes of alveolar phase III (SIII) of the N_2 expirogram. The first SIII value is thought to reflect VI within diffusion-convection-dependent acinar airways (Sacin), while the subsequent evolution of SIII values from lung turn over 1.5 to 6.0 is thought to reflect VI within convection-dependent conducting airways (Scond) [Robinson et al., 2013]. Scond was calculated according to a validated automated algorithm with SIII determination between 65 to 95% of expired volume and exclusion of breaths deviating $>$25% from median tidal volume with additional visual control of breath quality [Bigler et al., 2015]. Breaths without a clear linear phase III portion of the expirogram, or irregular expiration and oscillation were excluded. SIII values were normalised for mean gas concentration over their calculation interval and corrected for tidal volume [Aurora et al., 2005b]. If insufficient

SIII values were left, i.e. if more than 33% of all SIII values were de-selected, Scond was excluded [Robinson et al., 2013]. We additionally assessed minute ventilation, and airway dead space (VD) using the Fowler's method on the CO_2 expirogram of washout breaths [Fowler, 1948].

Spirometry

Spirometry was performed only in former preterm children after washout measurements using the MasterLab setup (Jaeger, Wurzburg, Germany) and according to standard guidelines [Miller et al., 2005]. Forced expiratory volume in 1 second (FEV_1) and forced expiratory flow between 25-75% of maximal expired volume (FEF_{25-75}) were expressed as z-scores [Stanojevic et al., 2009]. Spirometry was considered to be normal if both FEV_1 and FEF_{25-75} were $>$-1.64 z-scores [Quanjer et al., 2012].

Data analyses

Sample size calculation was based on a baseline mean Scond of 0.01 (SD ± 0.01) from 20 healthy school-aged children measured with the same setup [Bigler et al., 2015]. A sample size of 22 children in each group would provide 90% power to detect differences in washout parameter of one population SD between index and healthy control group, at a two sided 5% significance level. Continuous variables were compared with Student's t test after verification for normal distribution. Categorical variables were compared with chi-square test. Upper limit of normal (ULN) for lung function parameters was defined as mean + 1.64*SD [Quanjer et al., 2012] of a healthy reference population for spirometry [Stanojevic et al., 2009] and from our term-born controls for washout parameters. We assessed the relationships between washout parameters and perinatal factors and other lung function parameters in univariable and multivariable regression analysis. The multivariable model was adjusted for body length, sex and gestational age. All analyses were done using StataTM (Stata Statistical Software: Release 11. College Station, TX: StataCorp LP).

5.4 RESULTS

Study population

A total of 86 former preterm and 49 term-born children were enrolled. At least 2 valid N_2MBW measurements were obtained in 77 (90%) preterm and 46 (94%) term-born participants. Reasons for exclusion were leaks, irregular breathing pattern and FRC variability exceeding 10% for 2 valid trials or 25% for 3 valid trials, respectively. The mean age of the 77 (42 boys) former preterm was 9.5 years (range 7.2 – 12.8) and of the 46 (21 boys) term-born children 9.9 years (range 6.0 – 15.9). Excluded children did not differ from those included in terms of age, sex, weight, length or gestational age (data not shown). Preterm and term-born children showed similar characteristics at inclusion (table 1). Preterm participants had a mean gestational age of 28.7 weeks (range 23.9 – 34.7), and mean birth weight of 1139 g (range 420 – 2980). 75% of them developed BPD, and 21% fulfilled the criteria for current asthma at school age. The characteristics of the preterm participants in relation to their gestational age (ELGAN no-ELGAN) are presented in table 2.

Table 5.1.
Population characteristics at inclusion

	Control	**Preterm**	**p-value*** Control vs Preterm
n (boys)	46 (21)	77 (42)	0.34
Age (years)	9.9 ± 3.6	9.5 ± 1.5	0.37
Height (cm)	138.9 ± 19.2	134.8 ± 9.3	0.12
Weight (kg)	34.1 ± 12.8	30.1 ± 7.6	0.06
BMI ($kg.m^{-2}$)	17.0 ± 2.3	16.7 ± 2.6	0.52

Data are mean ± SD.
*Student's t test or chi-square test, as appropriate.

Table 5.2.
Characteristics of former preterm children, according to extremely low gestational age

Preterm children	< 28 weeks of gestation	≥ 28 weeks of gestation	p-value* ELGAN vs *no*-ELGAN
n (boys)	38 (21)	39 (21)	0.90
Perinatal characteristics			
Gestational age (weeks)	26.7 ± 1.1	30.7 ± 1.9	< 0.001
Birth weight (kg)	0.87 ± 0.22	1.39 ± 0.54	< 0.001
ELBW (%)	28 (74%)	8 (21%)	< 0.001
Antenatal steroids (%)	33 (87%)	32 (82%)	0.56
Surfactant (%)	17 (45%)	14 (36%)	0.43
Respiratory support (days)	45 [30 – 54]	8 [7 – 23]	< 0.001#
Intubation (days)	1 [0 – 4]	0 [0 – 3]	0.11#
Supplementary O_2 (days)	72 [54 – 86]	32 [4 – 47]	< 0.001#
BPD (%)	37 (97%)	21 (54%)	< 0.001
Chorioamnionitis (%)	21 (55%)	10 (26%)	0.007
Maternal smoking during pregnancy	4 (11%)	2 (5%)	0.38
Characteristics at school age			
Age (years)	9.8 ± 1.8	9.2 ± 1.2	0.11
Height (cm)	136.1 ± 10.4	133.6 ± 8.1	0.24
Weight (kg)	31.1 ± 8.6	30.1 ± 6.5	0.56
BMI ($kg.m^{-2}$)	16.6 ± 2.7	16.7 ± 2.5	0.78
Positive skin prick testing	12 (32%)	7 (18%)	0.17

Data are given as mean ± SD or median [interquartile range].
∗: Student's t test, non-parametric Wilcoxon ranksum test (#), or chi-square test as appropriate comparing extremely low gestational age neonates (ELGAN, <28 weeks of gestation) to neonates ≥ 28 weeks of gestation (no-ELGAN).
ELBW: Extremely low birth weight (<1.0 kg), Respiratory support: Days of invasive and non-invasive ventilation, BPD: Bronchopulmonary dysplasia, Positive skin prick testing: Positive on at least one of the tested common inhalant allergens. Missing data in the ELGAN subgroup for prick test (n = 1) and for chorioamnionitis (n = 1), in the no-ELGAN subgroup for chorioamnionitis (n = 1).

Nitrogen multiple-breath washout

Fourteen preterm and 8 term-born children were additionally excluded from Scond analysis, and 7 preterm and 9 term-born from Sacin analysis due to insufficient SIII values left after breath quality control as per current consensus [Robinson et al., 2013]. Excluded children for invalid Scond and Sacin did not differ from those included in terms of age, sex, weight, length or gestational age (data not shown). LCI and M2/M0 showed no significant difference between preterm and term-born children (table 3, figure 1). The moment ratio M1/M0 was significantly higher in preterm children (mean difference 0.49 z-scores, 95%CI 0.05 - 0.92, p = 0.028) (table 3), with 11 (14%) out of 77 preterm children showing M1/M0 values above the ULN. While Sacin did not differ between preterm and term-born participants (figure 2), Scond was significantly higher in former preterm compared to term-born children (mean difference 1.74 z-scores, 95%CI 1.17 - 2.30, p <0.001) (figure 3). Thirty-four out of 63 (54%) preterm children had Scond values >ULN. Scond remained significantly elevated (p <0.001) in subgroups of preterm children without (i) respiratory symptoms, (ii) current asthma, (iii) atopic sensitization, or without any of those three criteria (data not shown). Also in preterm with normal spirometry, Scond was significantly elevated. Results remained similar for raw Scond and Sacin values without tidal volume correction (data not shown). Within preterm children, Scond showed no significant difference between ELGAN and no-ELGAN (table 3), while it was slightly elevated in preterm children with BPD compared to preterm without BPD (mean difference 0.008, 95%CI 0.001 - 0.015, p = 0.026). However after adjustment for gestational age, the difference between BPD and no-BPD disappeared. FRC, minute

ventilation and VD, all corrected for body weight, showed neither a difference between preterm and term-born children, nor between ELGAN and no-ELGAN (table 3).

Spirometry

We found some evidence for airway obstruction: FEV_1 was mean -0.60 z-score (SD ± 1.11) in former preterm children (table 3), thereof 18% (14/77) had decreased values ($<$-1.64 z-score) [Stanojevic et al., 2009]. FEF_{25-75} was mean -0.97 z-score (SD ± 1.20) (table 3), thereof 30% (23/77) with decreased values. There was no significant difference in FEV_1 and FEF_{25-75} between ELGAN and no-ELGAN (table 3), nor between preterm with and without BPD.

Table 5.3.
Lung function values in former preterm and in term-born children.

	Control	Preterm			Mean difference (95% CI)	Mean difference (95% CI)
		All	< 28 weeks of gestation	≥ 28 weeks of gestation	(Preterm – Control)	(ELGAN – *no*-ELGAN)
# Analyzed N_2MBW	n = 46	n = 77	n = 38	n = 39		
# Analyzed Scond	n = 38	n = 63	n = 31	n = 32		
# Analyzed Sacin	n = 37	n = 70	n = 36	n = 34		
LCI	7.04 ± 0.52	7.20 ± 0.64	7.09 ± 0.59	7.30 ± 0.68	0.16 (-0.06 – 0.39)	-0.21 (-0.50 – 0.08)
Moment ratio (M_1/M_0)	1.57 ± 0.10	1.62 ± 0.13	1.60 ± 0.12	1.64 ± 0.13	0.05 (0.01 – 0.09)*	-0.05 (-0.10 – 0.01)
Moment ratio (M_2/M_0)	4.96 ± 0.69	5.27 ± 0.93	5.10 ± 0.80	5.43 ± 1.03	0.309 (-0.005 – 0.623)	-0.33 (-0.75 – 0.09)
Scond	0.017 ± 0.008	0.031 ± 0.012	0.033 ± 0.011	0.029 ± 0.014	0.014 (0.009 – 0.018)**	0.003 (-0.003 – 0.010)
Sacin	0.067 ± 0.029	0.069 ± 0.034	0.070 ± 0.038	0.068 ± 0.030	0.001 (-0.012 – 0.014)	0.002 (-0.014 – 0.018)
FRC (ml/kg)	44.9 ± 8.1	46.2 ± 11.2	48.1 ± 10.4	44.4 ± 11.8	1.3 (-2.5 – 0.5)	3.7 (-1.3 – 8.8)
Minute ventilation (ml/kg)	254.4 ± 92.8	263.6 ± 80.0	263.5 ± 79.8	263.8 ± 81.2	9.2 (-22.2 – 40.6)	-0.3 (-36.9 – 36.3)
V_D (ml/kg)	3.41 ± 0.70	3.37 ± 0.64	3.35 ± 0.59	3.39 ± 0.69	-0.04 (-0.28 – 0.20)	-0.04 (-0.33 – 0.25)
FEV_1 z-score	-	-0.60 ± 1.11	-0.72 ± 1.10	-0.49 ± 1.12	-	-0.23 (-0.73 – 0.27)
FVC z-score	-	-0.52 ± 0.98	-0.62 ± 1.00	-0.43 ± 0.96	-	-0.19 (-0.64 – 0.26)
FEV_1/FVC z-score	-	-0.08 ± 1.34	-0.12 ± 1.35	-0.04 ± 1.35	-	-0.08 (-0.70 – 0.53)
FEF_{25-75} z-score	-	-0.97 ± 1.20	-1.06 ± 1.18	-0.88 ± 1.22	-	-0.18 (-0.73 – 0.37)
FeNO (ppb)	-	13.5 ± 19.2	13.9 ± 18.6	13.1 ± 20.1	-	0.8 (-8.1 – 9.6)
FeNO ≥ 20 ppb	-	9 (12%)	4 (11%)	5 (13%)	-	-

Data are given as mean ± SD and compared by Student's t test. *: p <0.05; **: p <0.001.
ELGAN: extremely low gestational age neonates, N_2MBW: nitrogen multiple-breath washout, LCI: lung clearance index, M1/M0 and M2/M0: moment ratios, FRC: functional residual capacity (from nitrogen multiple-breath washout), VD: airway deadspace, FEV_1: forced expiratory volume in 1 second, FVC: forced vital capacity, FEF_{25-75}: forced expiratory flow between 25-75% expired maximal volume, FeNO: fraction of exhaled nitric oxide in parts per billion (ppb). Missing data for FeNO measurement (n = 1) in the No-ELGAN subgroup.

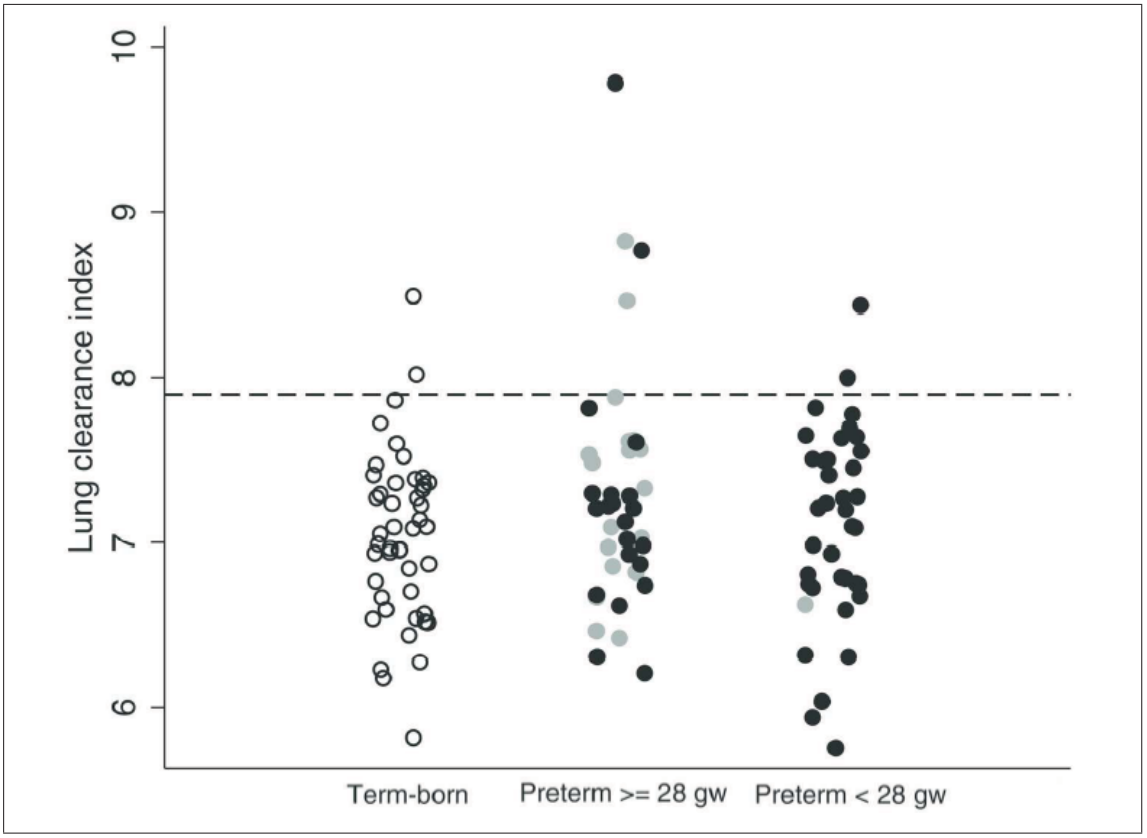

Figure 5.1.
Lung clearance index in former preterm and term-born children.
Lung clearance index (LCI) of nitrogen multiple-breath washout measurement in 46 term-born children, in 39 preterm children born $\geq$ 28 weeks of gestation and in 38 born $<$28 weeks of gestation. Black circles represent preterm children with bronchopulmonary dysplasia (BPD), grey circles without BPD. The dashed line represents the upper limit of normal (ULN). Of the 5 preterm children with LCI $>$ULN, 4 had current asthma, respiratory symptoms, and/or abnormal spirometry.

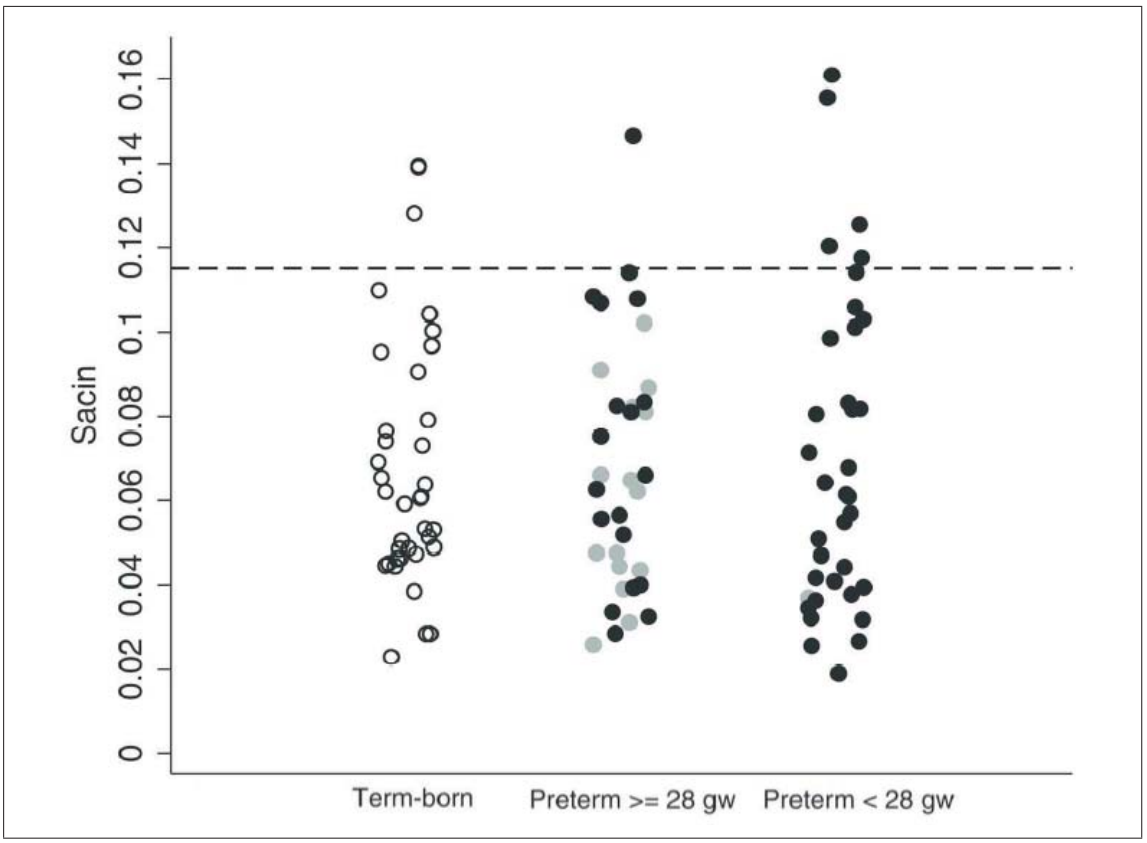

Figure 5.2.
Sacin in former preterm and term-born children.
Sacin of nitrogen multiple-breath washout measurement in 37 term-born children, in 34 preterm children born $\geq$ 28 weeks of gestation and in 36 born $<$28 weeks of gestation. Black circles represent preterm children with bronchopulmonary dysplasia (BPD), grey circles without BPD. The dashed line represents the upper limit of normal.

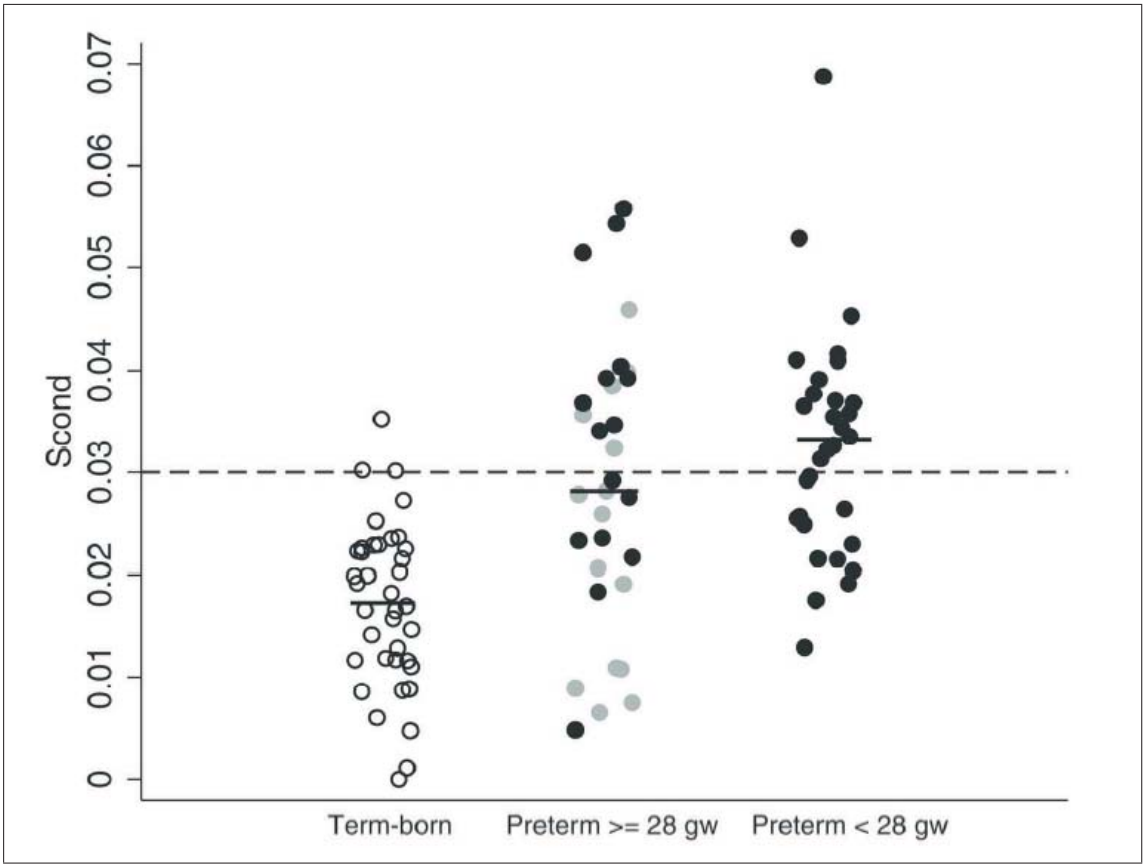

Figure 5.3.
Scond in former preterm and term-born children.
Scond of nitrogen multiple-breath washout measurement in 38 term-born children, in 32 preterm children born $\geq$ 28 weeks of gestation and in 31 born $<$28 weeks of gestation with mean Scond values per group. Black circles represent preterm children with bronchopulmonary dysplasia (BPD), grey circles without BPD. The dashed line represents the upper limit of normal.

Factors associated with elevated Scond

Regression analysis in the whole study population showed that from all significantly associated perinatal factors with Scond in the univariable model (gestational age, BPD, days of supplementary O_2, respiratory support, intubation, surfactant, antenatal steroids) only gestational age remained significantly associated in the multivariable model after adjustment for gestational age, body length and sex (r^2 37%) (table 4). When stratifying for ELGAN within preterm children, the relationship between Scond and gestational age remained significant within the ELGAN group (-0.004, 95%CI -0.008 - -0.001, $p = 0.010$), while it was not significant in the no-ELGAN group. For Sacin and spirometry (FEV_1 and FEF_{25-75}) no perinatal factor was significantly associated (data not shown). Including lung function parameters as exposure variables (Sacin, LCI, VD/kg, FRC/kg, minute ventilation/kg, FEV_1, FEF_{25-75}), only minute ventilation per body weight showed a significant positive association with elevated Scond in the uni- and multivariable model (data not shown).

Table 5.4.
Perinatal factors associated with Scond.

	Univariable model			Multivariable model*		
Scond	β	95% CI	p-value	β	95% CI	p-value
Gestational age (w)	-0.001	-0.002 - -0.001	< 0.001	-0.001	-0.002 - -0.001	< 0.001
No-ELGAN / ELGAN	0.010	0.005 – 0.015	< 0.001	0.010	0.005 – 0.015	< 0.001
BPD absent / present	0.013	0.009 – 0.018	< 0.001	0.005	-0.003 – 0.013	0.26
Supplementary O_2 (d)	0.0001	0.0001 – 0.0002	< 0.001	0.0001	0.0001 – 0.0001	0.072
Respiratory support (d)	0.0003	0.0002 – 0.0004	< 0.001	-0.0001	-0.0002 – 0.0001	0.50
Intubation (d)	0.0003	0.0001 – 0.0007	0.049	0.0001	-0.0003 – 0.0003	0.89
Surfactant no / yes	0.012	0.006 – 0.017	< 0.001	0.003	-0.003 – 0.009	0.30
Antenatal steroids no / yes	0.010	0.005 – 0.015	< 0.001	-0.005	-0.012 – 0.002	0.18

*Adjusted for gestational age, body length and sex.
ELGAN: extremely low gestational age neonates; BPD: bronchopulmonary dysplasia. Univariable and multivariable regression models for Scond. The only remaining significantly associated perinatal factor with Scond in the multivariable model was gestational age (r^2 37%), and ELGAN (r^2 20%).

5.5 DISCUSSION

This study is the first to specifically examine lung function of peripheral airways in former preterm children at school age using the MBW technique. Lung clearance index reflecting global VI did not differ between term-born and former preterm participants, including ELGAN and those with BPD. In contrast to our hypothesis, we found no evidence of diffusion dependent VI at the acinar level, reflected by similar Sacin values between our study groups. However, Scond was elevated in more than half of preterm participants, indicating VI in the more proximal convection-dependent airways. Gestational age was the strongest predictor of Scond in our cohort, independent of other confounders, but in a rather weak association These findings provide evidence for functionally normal alveolar compartment in former preterm children at school age, albeit with disturbed airway function at a more proximal level, in the small convection-dependent airways.

Previous reports on lung function of former preterm children have provided evidence of airway obstruction [Fawke et al., 2010, Lum et al., 2011, Hacking et al., 2013, Kaplan et al., 2012, Joshi et al., 2013], air trapping [Lum et al., 2011, Kaplan et al., 2012, Joshi et al., 2013], and reduced diffusing capacity for carbon monoxide [Lum et al., 2011, Kaplan et al., 2012, Joshi et al., 2013]. However, only one study has assessed global ventilation distribution in school-aged children born extremely preterm [Lum et al., 2011]. Although the investigators reported that 58% of those children had mildly elevated LCI, spirometry performed better in discriminating prematurity or BPD. More specific markers of peripheral VI, such as Sacin and Scond were not assessed. In contrast to Lum et al. [Lum et al., 2011], we found no relevant difference in LCI between former preterm and term-born children, while only 6% of our prematurely-born participants had elevated LCI. This difference may be attributed to the inclusion of children born solely $<$26 weeks of gestation in their study with a high asthma prevalence of 55% [Lum et al., 2011].

As specific regional ventilation parameters, Scond and Sacin have shown to reflect different disease-characteristic pathogenetic mechanisms, such as in cystic fibrosis [Horsley et al., 2008b], asthma [Verbanck et al., 2003], and bronchiolitis obliterans [Thompson et al., 2014]. Our results showed a distinguished pattern of regional VI in former preterm

children: We found normal Sacin values indicating normal ventilation distribution at the acinar level. Thus, our study provides evidence for a functionally normal alveolar compartment in former preterm children. On the other hand, Scond was consistently elevated in more than half of preterm participants without asthma symptoms or other lung function abnormalities, indicating impaired ventilation distribution in the more proximal conducting airways. Multivariable regression analysis confirmed the association between Scond and prematurity, showing that gestational age could explain 37% of the variability in Scond independent of other confounders. Lack of association in the multivariable model between other perinatal factors such as BPD, days of supplementary O_2 and intubation with Scond or spirometry after adjusting for gestational age underlines the pathophysiology of new BPD [Proietti et al., 2014]: functional changes are not primarily caused by the therapy (barotrauma, O_2 toxicity) but rather by prematurity per se. However it remains difficult to disentangle the exact influence of the different interrelating factors, mostly because gestational age and BPD showed strongly collinear effects within our observational study. There was no clear difference for Scond between ELGAN and no-ELGAN within preterm children. Further the association between Scond and gestational age in weeks was strongest within the ELGAN group. Thus, the pathophysiology of the premature lung is complex with many factors contributing and interacting. Whether the association of minute ventilation with Scond indicates an adaptive mechanism upon increased Scond, or a primary regulatory response cannot be answered by our data [Latzin et al., 2009b]. The mechanism for this pattern of ventilation inhomogeneity, could be explained by the fact that these children were born during the late canalicular – early saccular period of lung development, when the peripheral conducting airways are already developed and, thus vulnerable while the alveolar regions develop later and continue to grow after birth [Herring et al., 2014]. Thus, our findings indicate a mismatch in the growth of peripheral conducting airways and lung parenchyma in children born preterm, supporting the concept of a dysanaptic lung growth [Green et al., 1974, Merkus et al., 1993], related to prematurity. Overall there was no evidence for increased global VI in preterm children. While LCI and M2/M0 showed no difference between preterm and term-born children, M1/M0 was elevated in 11 children. Since the weight given to the latter part of the washout increases from M1/M0 to M2/M0 one could postulate a more

proximal involvement of peripheral airways.

Recent advances in imaging techniques [Narayanan et al., 2012] and stereological counting methods [Herring et al., 2014] have provided new evidence for continued alveolarization beyond infancy up to young adulthood in the healthy lung. Of particular interest, a recent study using helium magnetic resonance showed evidence of continued alveolarization at school age both in healthy adolescent [Narayanan et al., 2012] and former preterm children [Narayanan et al., 2013], half of the latter were diagnosed with BPD. Given that the main pathophysiological feature of the new BPD was described as arrest of alveolar development [Baraldi and Filippone, 2007], these findings suggest that the time-window in which alveolarization and potential lung repair occur may be much wider than previously thought. It is unclear whether the morphological term of continued alveolarization can be used in an equivalent and sensible way to describe lung function. Nevertheless, our study provides evidence for a functionally intact continued alveolarization in former preterm, and is thus in line with the structural findings. However our cross-sectional data cannot answer the question of the exact underlying mechanism or structural correlate of this complex process.

The main limitation of our study is the model based interpretation of Sacin and Scond and thus the lack of proof for its association with specific lung morphology. Therefore, the interpretation of our results strongly depends on the assumption that Scond and Sacin are a valid proxy for the regional ventilation distribution in convection- and diffusion-convection-dependent lung zones, respectively [Verbanck et al., 2003, Horsley et al., 2008a, Thompson et al., 2014]. A further limitation is the lack of spirometry in term-born controls and thus the limited comparison to reference values only [Stanojevic et al., 2009]. The lack of precise birth weight records in term-born controls hampered adjustment for birth weight in the regression models. However in analysis limited to preterm children, birth weight showed no significant association to Scond. Concerning the group of preterms we mainly included very preterm born children. This might represent a selection bias towards children with higher frequency of respiratory sequelae. Age distribution in term-born and preterm children was not equal with a greater proportion of older children among the term-born. However, average age was similar and since results

remained stable after adjustment for age, the influence seems negligible. Finally, although present the effect size of gestational age on Scond was relatively weak. The rather small sample size of the subgroups of our study has to be kept in mind when interpreting the associations between perinatal factors and convection-dependent VI. Some of the extremely preterm born participants also had normal Scond values despite low gestational age. Presumably, there are additional factors (e.g., genetic, epigenetic, environmental exposures, etc.) that may influence the susceptibility of the immature lung and determine the long-term outcome of pulmonary injury.

In conclusion, our study provides first evidence for functionally normal alveolar compartment in former preterm children at school age, but impaired ventilation inhomogeneity of conducting airways a pattern supporting a prematurity-related dysanaptic lung growth. Future studies need to elaborate the exact relationship between peripheral lung function and the underlying structural deficit, and thus help to advance prophylactic and therapeutic strategies in prematurely-born infants.

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6 Excessive increase in lung volume during early development in infants recovering from prematurity

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Manuscript ready for submission

Scientific knowledge on the subject

Premature birth disturbs early lung growth, and phenotypes of respiratory diseases in preterm children and adults are likely to differ from those in term-born individuals. During childhood, tracking of lung function parameters was shown for larger airways, and several studies have suggested potential for catch-up growth after prematurity, but longitudinal data on lung volumes is lacking.

What this study adds to the field

From infancy to early childhood, we observed very weak tracking of functional residual capacity in preterm children. Maturity at birth was negatively associated with lung volume increase, which might be explained by catch-up growth of lung parenchyma in children born with immature lungs. In comparison to term-born children, lung volume relative to body length and trapped gas were higher in preterm children at follow-up, which leads us to conclude that compensatory lung growth after prematurity might exceed normal growth.

6.1 ABSTRACT

Rationale & objectives

Prematurity disturbs alveolar growth, but data on lung volumes is sparse. We aimed to assess functional residual capacity (FRC) and potential influencing factors in preterm children from early infancy onwards.

Methods & measurements

FRC was measured in preterm children without sedation in infancy (visit 1) by multiple-breath washout (MBW, FRC_{MBW_V1}) using 4% sulphur hexafluoride, and at school age (visit 2) by nitrogen MBW (FRC_{MBW_V2}) and plethysmography (FRC_{pleth}). We evaluated the association of absolute FRC values at visits 1 and 2, and determinants of lung growth, i.e. increase in standard deviation score (SDS), by linear regression analysis.

Main results

We analysed longitudinal FRC measurements in 80 children (32 females), mean (SD) gestational age at birth and at visit 1 were 29 (3.0) and 45 (2.2) wks., mean (SD) age at visit 2 was 8.7 (1.3) yrs. After adjustment for height and age at visit 2, and for immaturity of the lungs, we found a very weak association between FRC_{MBW_V1} and FRC_{MBW_V2} (beta = 2.6 ml per ml, 95% CI 0.02 to 5.22, R^2 55.7%), with the highest proportion of variance explained by the variables for which we had adjusted. Younger gestational age at birth predicted increase in SDS of FRC_{pleth} (beta = -0.18 after adjustment, 95%CI -0.27 to -0.08, p <0.001). Compared to term-born children, preterm children showed higher SDS for FRC_{pleth}, and more trapped gas at school age.

Conclusions

We observed pulmonary catch-up growth after prematurity in this population, with some evidence pointing to excessive lung volume increase.

6.2 INTRODUCTION

A better understanding of early lung development after preterm birth would allow for the improvement of clinical strategies for both preterm [Islam et al., 2015] and term-born children [Frey et al., 2006]. In preterm children, respiratory diseases beyond the postnatal period, e.g. wheezing disorders in childhood [Greenough, 2013], or chronic obstructive lung disease in adulthood (COPD) [Bush, 2008] are more likely to occur, and disease phenotypes might differ from those in term-born children [Martin and Fanaroff, 2013]. It is well known that prematurity interrupts the growth of airways, alveoli, and pulmonary vessels [Stocks et al., 2013], and sequelae later in life can occur even in children without postnatal respiratory problems [Hoo et al., 2002b]. For clinicians, specific strategies were recommended [Bolton et al., 2015]. Bronchopulmonary dysplasia (BPD) in preterm new-borns, initially described by Northway et al. in 1967 [Northway et al., 1967], is one of the immediate consequences. It was originally characterized by injury of mucosal, alveolar and vascular tissues, and progressive disease stages with prominence of fibroproliferation. Today, the improvement of perinatal management, e.g. antenatal steroids, more gentle ventilation, and artificial surfactant, has changed the clinical picture of BPD. The so-called new BPD is characterized by fewer and larger alveoli, and defined as needing oxygen supplementation at the postnatal age of 28 days, or the corrected age of 36 weeks of gestation [Jobe and Bancalari, 2001]. BPD is inversely associated with gestational age [Costeloe et al., 2012], and different categories of disease severity have been described [Ehrenkranz et al., 2005]. While there is fairly significant evidence in the literature on the impact of prematurity on the functional growth of the airways, literature on the functional growth of the lung volume is lacking. We recently demonstrated a weak tracking of lung volume from infancy to early school age in healthy children [Schmidt et al., 2015a]. In former preterm infants, longitudinal studies regarding airway function showed an association of expiratory flow in infancy and later childhood in individuals after premature birth [Hoo et al., 2002b, Baraldi and Filippone, 2007] with potential for catch-up growth [Narang et al., 2008]. Airways and lung parenchyma can grow disproportionately [Green et al., 1974, Martin et al., 1988]. We hypothesized that lung volume growth in early childhood may exhibit different characteristics compared to

growth of larger airways. The aim of this study was to longitudinally measure lung volume growth from infancy to school age after prematurity, as previously described [Roiha et al., 2007, Latzin et al., 2009b, Pramana et al., 2011, Proietti et al., 2014]. Taking into account known concomitant factors derived from our previous study in term-born children [Schmidt et al., 2015a], we aimed to determine whether there is lung volume tracking and / or functional catch-up growth in preterm children.

6.3 METHODS

Study design and subjects

For this longitudinal observational study, children born <37 weeks of gestation were recruited during postnatal hospitalization, as previously described [Latzin et al., 2009b, Pramana et al., 2011, Proietti et al., 2014]. We measured lung volume, i.e. functional residual capacity (FRC), and ventilation inhomogeneity, i.e. lung clearance index (LCI), repeatedly in 107 children using validated multiple-breath washout (MBW) equipment [Schmidt et al., 2015b, Singer et al., 2012a] and plethysmography [Criée et al., 2011] (see online supplement, OLS). A study in 124 term-born children provided data for comparison [Schmidt et al., 2015a, Fuchs et al., 2012a] (see OLS).

The study was approved by the ethics committee of the Canton of Bern, Switzerland. Informed consent was provided by parents or caregivers. At the second visit, the childs assent was obtained in addition.

Lung function measurements, exposures and health

At the corrected age of 1 month (visit 1), we measured infant lung function without sedation in supine position. We performed tidal breathing tests, and MBW, using a commercially available device (Exhalyzer D, Eco Medics AG, Duernten, Switzerland), and 4% sulphur hexafluoride (SF_6) [Latzin et al., 2007b]. Maximum SF_6 concentration was determined by end-inspiratory molar mass using an improved algorithm [Anagnostopoulou et al., 2015].

At early school age (visit 2), lung function was measured with the awake child sitting upright by nitrogen MBW [Singer et al., 2012a] using the Exhalyzer D, and by spirometry and plethysmography [Criée et al., 2011] using the JAEGER Master-Screen Body (CareFusion, Wuerzburg, Germany).
FRC was measured in triplicates. The mean of high quality FRC measurements was calculated per technique and visit, according to standards; ventilation inhomogeneity was evaluated using the lung clearance index (LCI) [Frey et al., 2000a, Robinson et al., 2013, Wanger et al., 2005]. See OLS for further details .
For definitions of exposures and health status see OLS.

Statistical Methods

Primary outcome was FRC measured by MBW at visit 2 (FRC_{MBW_V2}). We tested the association of FRC measured by MBW at visit 1 (FRCMBW_v1) with FRC_{MBW_V2}. Secondary outcome, allowing comparison with a term-born population, was FRC measured by plethysmography at visit 2 (FRC_{pleth}). For a calculation of relative lung volume increase, gender-specific standard deviation scores (SDS) were generated. Published reference data was available for anthropometric data [World Health Organization, 2006], plethysmography [Rosenthal et al., 1993], and spirometry [Quanjer et al., 2012]. For lung function in infancy, SDS were generated using reference data from our pediatric lung function laboratory [Singer et al., 2012a, Fuchs et al., 2012a]. Relative FRC increase was calculated as the difference between SDS at visit 2 and SDS at visit 1 (ΔSDS).
Hyperinflation at visit 2 was evaluated as (i) trapped gas, calculated as the difference between FRC_{pleth} and FRC_{MBW_V2}, and (ii) residual volume per total lung capacity (RVpTLC).
We tested for dysanapsis by searching for an association of SDS of FRC with expiratory flow.
Associations of variables with the outcome of interest were tested using Pearson correlation coefficient and / or linear regression analysis. After performance of univariable regression, variables with a p-value <0.10 were sorted by physiological plausibility, and variance explained (R^2), and included stepwise in the multivariable model, accounting for potential co-linearity and non-linear associations.

We performed comparisons using paired or unpaired T-tests, or Chi-square tests, as appropriate; a p-value <0.05 was considered significant.
The sample size in the current study is higher than in a longitudinal study on airway function (n=18) [Filippone et al., 2003], but lower than in a study on lung volumes in term-born children (n=124) [Schmidt et al., 2015a]. For tracking analysis, statistical power was 86% to detect a correlation coefficient of 0.35 with a significance level of 0.05 (two-sided).
All analyses were performed in R 3.2.1 (http://www.r-project.org/), and STATA 11 (STATA Corporation, Texas, USA). See OLS for more details.

6.4 RESULTS

Subjects

We recruited 263 preterm children between 1999 and 2010, and performed lung function measurements successfully in 236 children at visit 1 in early infancy. FRC data fulfilling high quality criteria was available in 142 children at visit 1, and 211 families filled in questionnaires regarding exposures and respiratory symptoms during the first year of life. Until December 2014, 107 children attended visit 2 at early school age. Eighty (75%) of them performed FRC measurements at both visits successfully, and were included in this study (see figure 6.1). Their characteristics are shown in table 6.1, together with characteristics of the healthy control group.

Table 6.1
Population (n=80)

Variable	Available in (n)	Preterm Population # (n=80)	Available in (n)	Term-born Population # (n=124)
Neonatal data				
Male gender, n(%)	80	48 (60.0)	124	62 (50.0)
Maternal smoking in pregnancy, n(%)	71	6 (8.5)	124	6(4.8)
Antenatal steroids, n(%)	76	66(86.8)		
Surfactant, n(%)	80	42 (52.5)		
Gestational age at birth, wks	80	29.1 (3.0)	124	39.8 (1.2)
Birth weight, kg	80	1.2 (0.5)	124	3.4 (0.4)
Birth length, cm	78	37.7 (4.6)	124	49.7 (2.0)
BPD moderate or severe, n(%)	80	36 (45.0)		
Oxygen supplementation after birth, d	80	56.0 (60.0)		
Intubation, n(%)	80	50 (62.5)		
CPAP, n(%)	80	75 (93.8)		
At study visit 1				
Age since birth, weeks	80	15.9 (3.7)	124	5.0 (0.6)
Weight, kg	80	4.0 (0.8)	124	4.3 (0.6)
Length, cm	80	52.4 (3.4)	124	54.6 (2.2)
Weight gain per day since birth, g	80	25.4 (5.3)		
Age since date of estimated term delivery, wks	80	1.0 (2.2)	124	4.8 (1.2)
Maternal smoking, n(%)	75	2.0 (2.7)	124	5 (4.0)
Paternal smoking*, n(%)	75	4.0 (5.3)	124	20(16.1)
FRC_{MBW_V1} washin, ml	80	85.2 (20.0)	124	94.9 (15.0)
FRC_{MBW_V1} washout, ml	76	93.0 (23.9)	124	102.7 (17.0)
At study visit 2				
Age, years	80	8.7 (1.3)	124	6.1 (0.2)
Weight, kg	80	28.8 (8.0)	124	22.6 (3.5)
Height, cm	80	131.2 (9.8)	124	117.8 (5.2)
Wheeze ever reported, n(%)	80	25 (31.3)	119	26 (26.3)
Asthma**, n(%)	80	5 (6.3)	121	10 (8.3)
Maternal smoking, n(%)	79	10 (12.7)	121	6 (5.0)
Paternal smoking*, n(%)	78	16 (20.5)	121	15 (12.4)
Parental smoking, n(%)	78	24 (30.8)	121	17 (14.0)
Exposure to tobacco at any point of study, n(%)	73	25 (34.2)	124	31 (25.0)
FRC_{MBW_V2}, ml	71	1287 (291)		
FRC_{pleth}, ml	69	1514 (299)	124	1088.0 (206.0)

Information given in mean (SD) if not otherwise stated

* Defined as smoking of father or other person in the household

** Defined as doctor diagnosed asthma and / or inhalation of bronchodilators and / or steroids

BPD – Bronchopulmonary dysplasia

CPAP – Continuous positive airway pressure

FRC_{MBW_V1} – Functional residual capacity measured by multiple-breath washout at visit 1

FRC_{MBW_V2} – Functional residual capacity measured by multiple-breath washout at visit 2

FRC_{pleth} – Functional residual capacity measured by plethysmography at visit 1

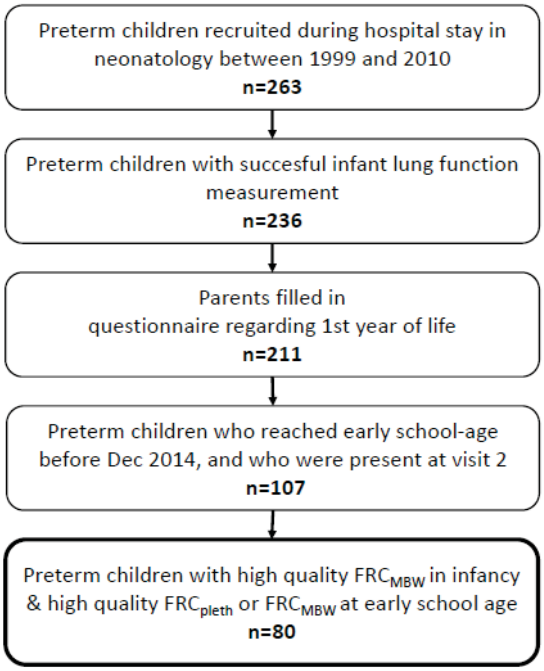


Figure 6.1
Participants
Children born <37 weeks of gestation were recruited between 1999 and 2010. Among 263 participants, 236 performed lung function measurements successfully at visit 1. High quality measurements of functional residual capacity (FRC) were available in 142 children at visit 1, and 211 families filled in questionnaires regarding the first year of life. Until December 2014, 107 children eligible for visit 2 attended, and 80 (75%) of them performed FRC measurements at both visits successfully.

Factors associated with lung volume at school age

Pearson correlation coefficient, and univariable regression analysis showed no association of FRC_{MBW_V1} with FRC_{MBW_V2} at visit 2. A large number of variables showed a positive association with FRC_{MBW_V2}, including lower gestational age at birth, duration of postnatal intubation, days of oxygen supplementation, duration of invasive and non-invasive ventilation, moderate or severe BPD (baseline group no or mild BPD), age since term at visit 1, early weight gain, height and weight increase from visit 1 to visit

2, anthropometric data at visit 2, age at visit 2, and any exposure to parental smoking during the study period.

No association with FRC_{MBW_V2} was found for antenatal steroids, artificial surfactant, maximum inspiratory pressure, duration of continuous positive airway pressure, chorioamnionitis, parental atopy or asthma, weight or length at visit 1, respiratory symptoms in infancy or at follow-up, and male gender.

Many of the variables associated with FRC_{MBW_V2} exhibited co-linearity with the duration of oxygen supplementation, a proxy of lung immaturity at birth. Thus, only the variables height and age at visit 2, and duration of oxygen supplementation, were included in the multivariable model. After adjustment for those variables, we found a very weak association (beta= 2.6 ml per ml, 95% CI 0.02 to 5.22, R^2 55.7%) between FRC_{MBW_V1} and FRC_{MBW_V2}, with the highest proportion of variance explained by anthropometric data at visit 2, and immaturity of the lungs at birth (see table 6.2 and OLS).

Table 6.2
Multivariable regression outcome FRC_{MBW_V2} in ml (n=71)

	Beta (95% CI)	P value
FRC_{MBW_V1} washin (ml)	2.6 (0.02 to 5.22)	0.049
Oxygen supplementation (days)	1.5 (0.69 to 2.27)	< 0.001
Height at visit 2 (cm)	16.8 (8.55 to 25.15)	< 0.001
Age at visit 2 (months)	3.90 (-1.00 to 8.80)	0.117

Adjusted R^2 55.7%

$p < 0.001$

FRC_{MBW_V1} – Functional residual capacity measured by multiple-breath washout at visit 1

FRC_{MBW_V2} – Functional residual capacity measured by multiple-breath washout at visit 2

Factors associated with lung growth

In the univariable regression analysis, relative lung volume increase, i.e. ΔSDS, the difference between FRC SDS at visit 2 and visit 1, was positively associated with younger gestational age at birth, longer duration of oxygen supplementation, severe lung disease, lower SDS for weight and length at visit 1, younger age since term at visit 1, less early

weight gain, lower BMI at visit 2, and with any instance ever, or within 12 months prior to visit 2, of parent reported wheeze.
No association with ΔSDS was found for parental atopy, maximum peak inspiratory pressure during neonatal ventilation, parental smoking, respiratory symptoms during infancy, age or height at visit 2. Male gender was not associated with ΔSDS, as SDS were derived separately for male and females.
Lower gestational age remained in the multivariable model predicting ΔSDS; after adjustment for early weight gain, age since term at visit 1, and BMI at visit 2, beta was -0.18 (95%CI -0.27 to -0.08, p <0.001, see table 6.3 and OLS).

Table 6.3
Multivariable regression outcome relative lung volume increase ΔSDS (n=69)

	Beta (95% CI)	P value
Gestational age at birth	-0.18 (-0.27 to -0.08)	< 0.001

Adjusted R^2 47,4%
p < 0.001

Outcome ΔSDS = (SDS FRC_{pleth} - SDS FRC_{MBW_V1} washin),
result adjusted for weight gain per day before visit 1, age since term at visit 1, and BMI at visit 2.

Comparison with term-born children

We compared FRC SDS at both visits with data from 124 term-born children by unpaired T-test, and found significantly lower SDS for FRC_{MBW_V1} (SDS term-born 0.0 vs SDS preterm -0.6, 95%CI of difference -0.97 to -0.23, p=0.002), but higher SDS for FRC_{pleth} in the preterm group (SDS term-born -0.3 vs SDS preterm 0.6, 95%CI of difference 0.59 to 1.16, p<0.001) (see figure 6.2). The childrens age at visit 1 was comparable. At visit 2, preterm children were older than term-born children, which is why we used height-specific SDS for FRC_{pleth}. FRC_{pleth} relative to height is displayed in figure 6.3.
Within the preterm group, we found elevated LCI values in 30% of preterm children at visit 1, but only in 7% of preterm children at visit 2 (see figure 6.4). A paired t-test showed evidence for a small but significant difference between LCI at visit 1 and 2 (6.9 at

visit 1 vs. 7.2 at visit 2, 95%CI of true difference 0.05 to 0.53, p=0.02). Despite the low percentage of elevated LCI values at visit 2 in preterm children, they showed significantly higher LCI values in comparison to term-born children: mean LCI in term-born children was 7.0, and in 71 preterm children 7.3 (95%CI of difference 0.01 to 0.48, p=0.04). Comparison of SDS for FRC_{MBW_V2} with 22 term-born children showed no significant difference between term-born and preterm children at visit 2 (see OLS).

No difference between term-born and preterm children was found for RVpTLC (see OLS).

Trapped gas in preterm children was significantly higher compared to term-born children (97.4 ml in term-born children vs 232.0 ml in preterm children, 95%CI of difference 64.7 to 204.6 ml, p = <0.001, see figure 6.5).

Evaluation of dysanapsis showed no association of expiratory flow with FRC at either visit in term-born children (n=120), but did show a significant association in preterm children (n=68). Interestingly, this association in preterm children was positive at visit 1 (beta= 0.59, 95% CI 0.36 to 0.82, R^2 25.3%), but negative at visit 2 (beta= -0.30, 95% CI -0.48 to -0.12, R^2 12.6%; see OLS).

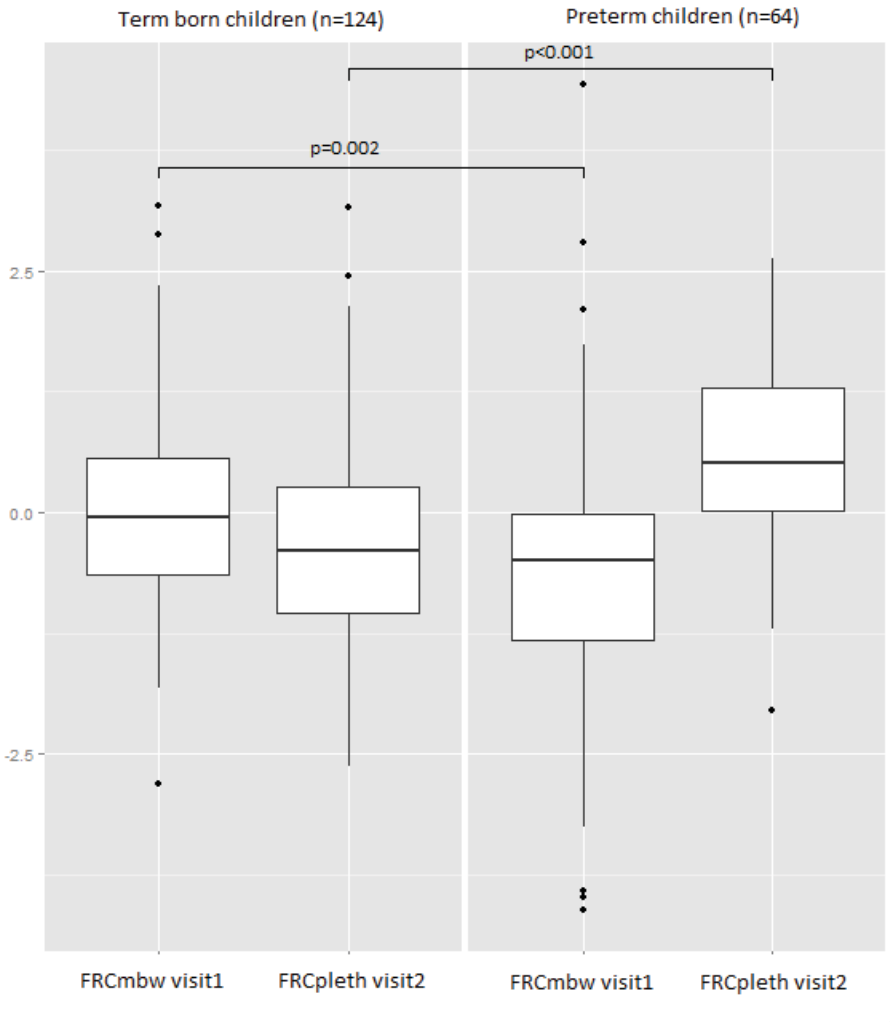


Figure 6.2
FRC SDS over time in term-born and preterm children
In 124 term-born children (left pair of boxes), and in 64 preterm children (right pair of boxes), Standard Deviation Scores (SDS) were generated for both study visits. For visit 1, gender-specific SDS for FRC were generated using mean and SD of FRCMBW of the term-born population. For visit 2, published gender- and height-specific reference values for FRC_{pleth} were used.[Rosenthal et al., 1993] Compared to term-born children, preterm children showed significantly lower FRC SDS at visit 1 (SDS term-born 0.0 vs SDS preterm -0.6, 95%CI of difference -0.97 to -0.23, p=0.002), and significantly higher FRC SDS at visit 2 (SDS term-born -0.3 vs SDS preterm 0.6, 95%CI of difference 0.59 to 1.16, p<0.001)

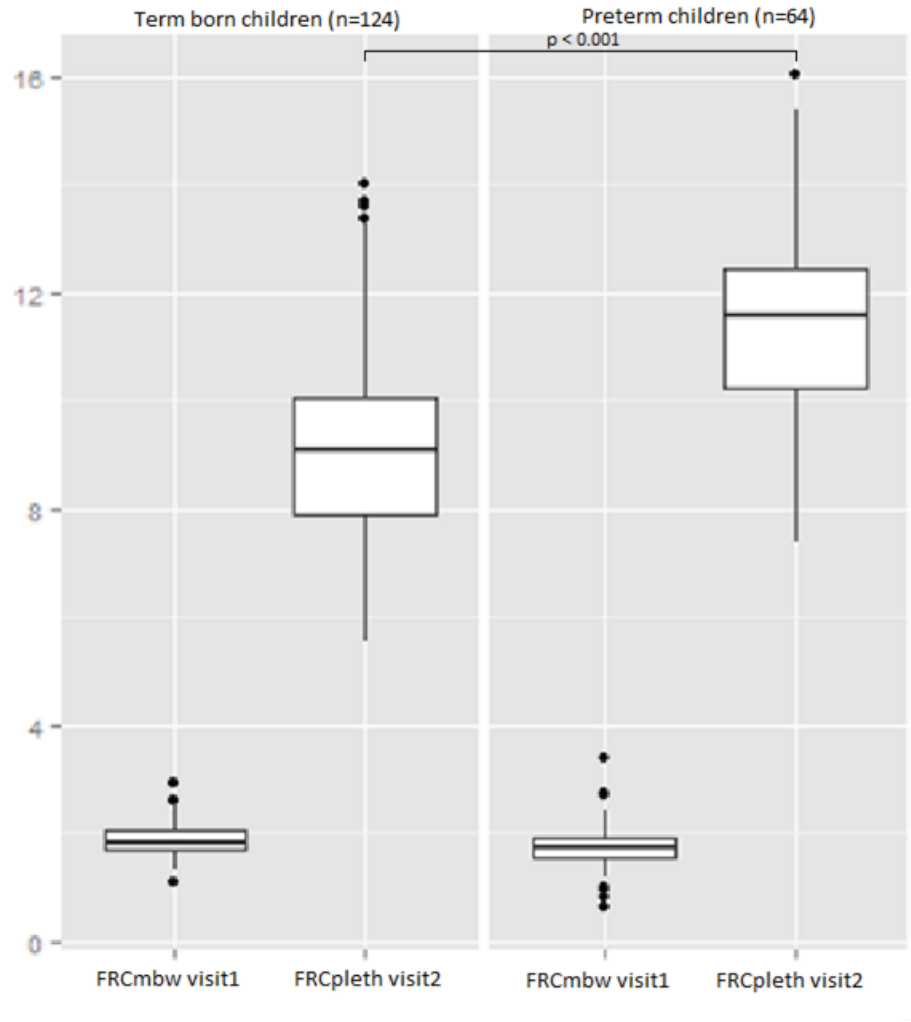


Figure 6.3
FRC relative to height (ml per cm) over time in term-born and preterm children
In 124 term-born children (left pair of boxes), and in 64 preterm children (right pair of boxes), FRC per cm was calculated in order to compare lung volume according to body size. Difference between groups remained statisticall significant even when adjusting for male gender, height in cm at visit 2, height in SDS at visit 2 with and without consideration of gestational age at birth (data not shown, see Online Supplement for SDS of anthropometric data).

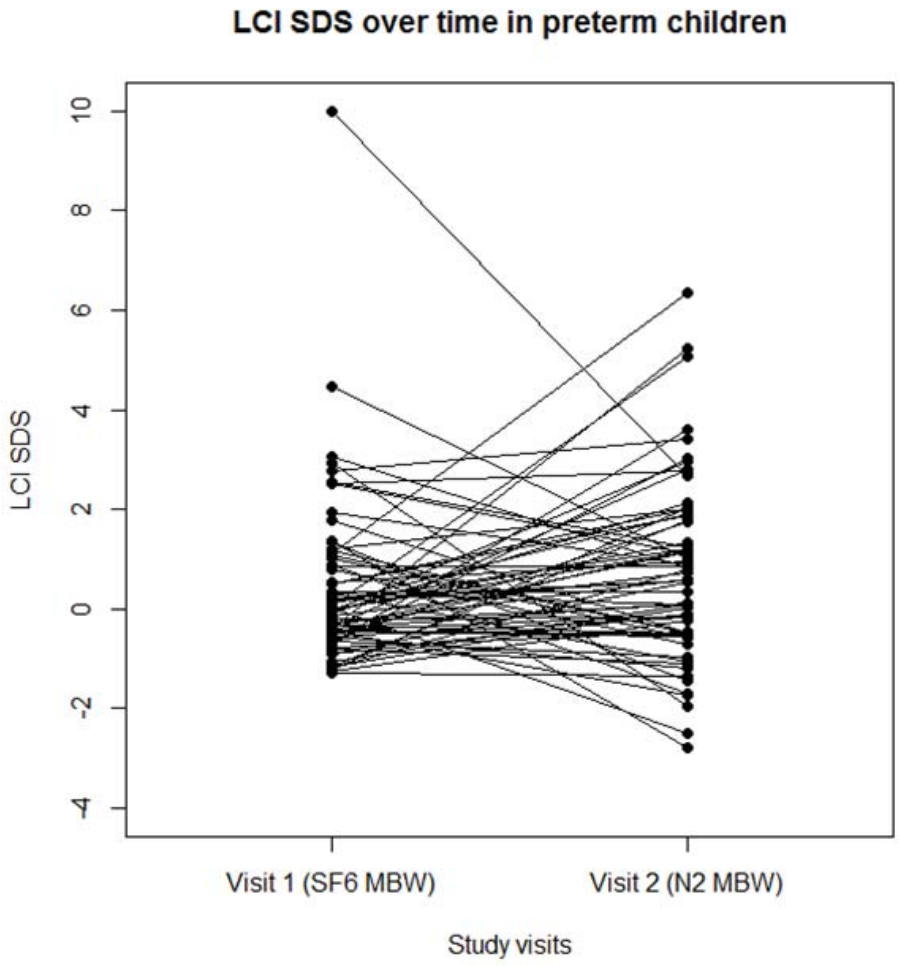


Figure 6.4
LCI over time in preterm children
No tracking of LCI absolute values or LCI SDS was observed (n=71). LCI SDS were derived using gender-specific data from term born children measured at our lung function lab.

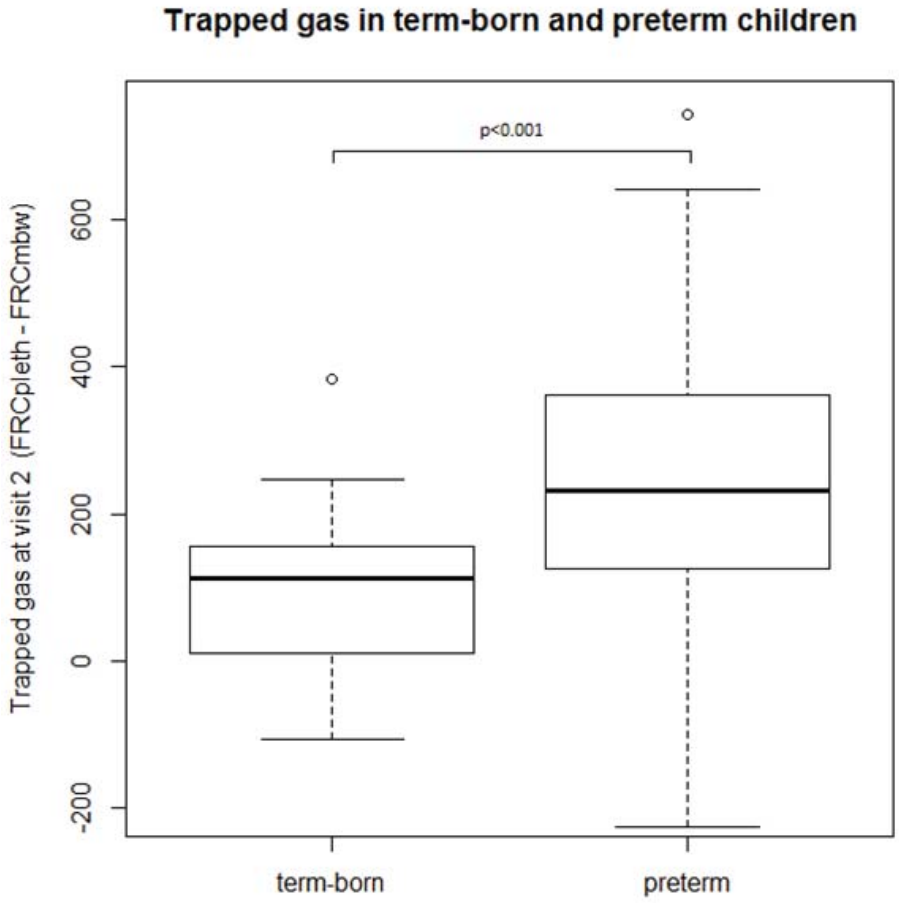


Figure 6.5
Trapped gas in term-born and preterm children
Trapped gas, i.e. the difference between FRC_{pleth} and FRC_{MBW_V2} per child, was calculated for n=22 term-born and n=60 preterm children. Preterm children showed significantly higher trapped gas than term born children for absolute values (97.4 vs 232.0 ml, 95% CI for true difference 64.7 to 204.6 ml, p <0.001), relative to weight (4.4 vs. 8.5 ml/kg body weight, 95%CI for true difference 1.2 to 7.1, p=0.007), and relative to height (0.8 vs 1.8 ml/cm height, 95% CI for true difference 0.4 to 1.5, p=0.001)

6.5 DISCUSSION

In this prospective observational study in preterm children, we found a very weak association of FRC in early childhood with FRC in infancy. Maturity of the lungs at birth was inversely associated with relative lung volume increase. The proportion of children with elevated LCI decreased over time, and FRC and LCI were within normal range for the majority of preterm children at follow-up. Comparisons with term-born children, however, showed larger FRC, stronger FRC increase in relation to body length, and disproportionate growth of large airways and lung parenchyma in preterm children. This additional lung volume growth in preterm infants is likely the expression of a combination of catch-up lung volume growth and hyperinflation, or gas trapping.

Strengths and limitations

To the best of our knowledge, this study is the first to present longitudinal FRC data in preterm children, and one of the strengths was a relatively high number of participants, especially when considering the complexity of measuring FRC. We performed infant MBW without sedation, thus our results reflected the natural breathing pattern of children recovering from prematurity. The opportunity to compare results with data derived from a term-born population increased the value of this analysis. Certainly, some limitations need to be considered when interpreting our findings. Methodological differences between FRC measurements at visit 1 and 2 limited our longitudinal analyses. MBW using SF_6 as a tracer at visit 1 was not entirely comparable to MBW using nitrogen at visit 2. MBW and plethysmography were based on different concepts. It is well known that different techniques and MBW tracers provide different results for FRC. Discrepancies between FRC_{pleth} and FRCMBW were most likely explained by trapped gas, i.e. gas in non-ventilated areas of the lungs. Findings using either FRC_{pleth} or FRCMBW at visit 2 were comparable, which underpinned their physiological validity. Our approach may provide more detailed evidence of the nature of lung volume increase, such as hyperinflation and gas trapping. We challenged our results by comparing them to results when SDS were used instead of absolute values. These sensitivity analyses supported our initial findings.

Statistical power would have been higher if more individuals had been included. However, a very strong tracking would have been detected even in a population of this size.
Because of the availability of published reference data, we focused on group comparisons using SDS from FRC_{pleth}. However, comparisons based on SDS for FRC_{MBW_V2} provided complementary information. Age range at visit 1 was narrow, and SDS could be calculated using data from a term-born group at our pediatric lung function laboratory. At visit 2, age range was broader, and the participants were slightly older than the term-born controls. Regarding FRC_{pleth} SDS results, this small age difference was negligible, as published reference values according to height were available. Regarding FRC_{MBW_V2}, we used data from our laboratory. Longitudinal results from nitrogen MBW in term-born controls were available in 22 children only.
Loss to follow-up and a selection bias, which are common limitations of cohort studies, need to be taken into account. Families with less traumatic experiences during the neonatal period were more likely to participate, especially for follow-up later in childhood, which limits the generalizability of the findings. The majority of parents reported an overall good level of health of their child. Altogether, we were able to include a group with heterogeneous clinical conditions after birth and later in childhood, but our findings might have been more pronounced if attrition rates had been lower.

Comparison with previous findings

To our knowledge, this is the first study reporting lung volume growth in preterm infants. Thus, comparability with previous findings is limited. Data on increasing lung volumes from infancy onwards have only been published up to toddler age, and not beyond. Studies over longer periods reported lung function data from forced expiration, which mainly reflects the growth of airways. Data regarding group comparisons in infancy is available. A negative association of FRC with duration of respiratory assistance was described in infancy, which fits with our finding of stronger lung volume increase in children born at a younger gestational age, and with longer duration of oxygen supplementation. Hülskamp et al. described a negative association of duration of oxygen supplementation with FRC in infancy in a multicentre trial with 219 term-born and preterm infants [Hülskamp et al., 2009]. It is not surprising that alterations in lung function in our study were not necessar-

ily associated with disease severity after birth, as previous findings were heterogeneous. One study in infancy showed FRC abnormalities in preterm infants with severe BPD [Hjalmarson and Sandberg, 2005], others found reduced expiratory flow in the first month of life even in healthy preterm infants [Friedrich et al., 2006].

Schulzke et al. measured FRC repeatedly in 58 preterm infants [Schulzke et al., 2010], and described a negative association of duration of ventilation. Here, FRC increase in infancy was positively associated with gestational age. A study comparing FRC between preterm infants with (n=29) and without BPD (n=26) over approximately 2 years was published by Schmalisch et al. Their main aim was to perform group comparisons over time. FRC in infancy measured by plethysmography and SF_6MBW was equal between the groups when normalized to body weight [Schmalisch et al., 2012]. Our finding of a negative association of FRC increase and gestational age, and of trapped gas at a later period, suggested that catch-up growth happened beyond infancy.

Previous results from this ongoing study were published [Proietti et al., 2014, Riedel et al., 2009, Yammine et al., 2015]. Riedel et al. described a comparison of electrical impedance tomography (EIT) and MBW in infants, and by EIT found more ventilation of the independent areas of the lungs in preterm children than in term-born controls [Riedel et al., 2009]. Proietti et al. investigated whether infant lung function predicts respiratory morbidity in the first year of life in preterm children [Proietti et al., 2014], but no improvement of predictive value was found when infant lung function results were added to established predictors. Interestingly, FRC was not associated with consecutive respiratory symptoms in this analysis either.

Later in childhood, Yammine et al. [Yammine et al., 2015] compared parameters of ventilation heterogeneity in 77 preterm children from this ongoing study to results from 46 term-born children of a similar age range. Indices of ventilation homogeneity at the global and the acinar level were similar in term-born and preterm children. Scond was elevated in $>$50% of preterm participants, indicating ventilation inhomogeneity in the more proximal, convection-dependent airways. Strongest predictor of having an elevated Scond was lower gestational age, which again fits with our finding of the relevance of young gestational age at birth. The finding of a significant difference of LCI between term-born and preterm children in this current analysis needs to be interpreted with caution, as our

comparison was performed with only 22 term-born children for which longitudinal LCI data was available. Despite this weakness, the finding of a smaller percentage of preterm children showing pathologic LCI values at visit 2 than at visit 1 can be interpreted as an indicator of improvement of ventilation on the group level during recovery from prematurity.

Trapped gas is a typical finding in preterm infants [Greenough et al., 2000]. In adolescents and adults after prematurity, evidence for elevated RVpTLC was found by Trachsel et al. in a group of 20 individuals with severe BPD. Participants were born before lung protective ventilation and artificial surfactant were introduced [Trachsel et al., 2012]. Lum et al. described elevated RVpTLC in 11 year-old children from the EPIcure study, in which the children were born <26 weeks of gestation in 1995 [Fawke et al., 2010, Lum et al., 2011]. In our participants, however, we found no evidence for elevated RVpTLC, which might be explained by reduced postnatal damage due to improved ventilation strategies compared to the first study, and by the broader range of gestational age at birth in our population compared to the second study. However, the difference between FRC_{pleth} and FRC_{MBW_V2} at the same visit showed higher values in preterm children compared to term-born controls, suggesting some intra-thoracic gas that does not communicate in ventilation. Information on trapped gas at visit 1 was not available in our population, so we could not exclude the possibility that it was already present in infancy.

There were only few studies investigating disproportionate growth of lungs and airways. One study in healthy children and adolescents reported a weak association of vital capacity (VC) and expiratory flow when 50% of VC was expired [Hopper et al., 1991]. We found no association in the healthy control group, but a change from a positive association in infancy to a negative association later in childhood in the preterm group.

Interpretation and possible mechanisms

The weak tracking, and the higher increase of lung volume in children with less mature lungs at birth, indicated catch-up growth in the investigated population. As alveolar growth was shown even beyond early childhood [Narayanan et al., 2012], normal growth of alveoli and vessels certainly contributed here. However, the complex interaction between alveolar growth, fibrosis, and emphysema is not understood. The negative impact

of fibrosis on lung growth [Shi et al., 2009] may be highly non-linear. Fortunately, lung volumes were within normal range at follow-up. However, compared to term-born children, preterm children showed higher SDS for FRC_{pleth}. This might, in part, be explained by reduced body size after prematurity, as SDS for FRC_{pleth} were height specific. Preterm children had normal SDS for weight and height when using data from a preterm reference population, but reduced SDS when compared to term-born children without consideration of gestational age. Thus, the elevated FRC relative to body size could be explained by reduced body size. Given that we found the difference between FRC_{pleth} and FRCMBW at the same visit to be higher in preterm children than in term-born controls, hyperinflation is also a likely contributor, and could cause excessive lung volume increase. We can only hypothesize about potential mechanisms. As previous studies have described hyperinflation in preterm infants [Greenough, 2013], it is likely that in our participants some hyperinflation was already present infancy. It is possible that preterm children do not achieve the same complex and fully functional lung structure compared to term-born children. The ability to repair the altered lung structure after prematurity might be limited, and despite the presence of growth and repair, small airways might not be fully restored. Over time, this could lead to increasing hyperinflation, and potentially explains the higher incidence of COPD in preterm children later in adulthood.

We were surprised that some of the variables were not associated with either FRC or FRC increase. Perinatal management, i.e. antenatal steroids or artificial surfactant, showed no association with FRC in young childhood, possibly caused by non-random administration. Respiratory symptoms during infancy, or prior to follow-up, showed no association with FRC either, which might be due to a high prevalence of respiratory symptoms in all preterm children, regardless of lung disease severity after birth. Also, as symptoms were reported by parents, their subjective perception of their childs lung and overall health could have led them to report it as better than it actually was. Over time, the association of airway function with lung volume changes from positive to negative in preterm children. This could potentially be explained by the loss of physiological variability and excessive increase of lung volume after prematurity.

Clinical relevance and implications

Specific interventions for preterm children, or subgroups of them, would be highly desirable, but are not within reach. Previous studies have suggested to check for catch-up growth in order to ensure that it occurs in all children affected by prematurity [Greenough, 2013]. In our study, we were finally able to show catch-up growth of lung volume in preterm children, which seemed to be exaggerated. In future studies, we would aim to confirm and understand the mechanisms leading to this early hyperinflation. Such an understanding could enable clinicians to both ensure catch-up growth takes place, and the appropriate extent of said growth.

Conclusion

From infancy to early childhood, we observed very weak tracking of functional residual capacity in preterm children. Maturity at birth was negatively associated with lung volume increase, which might be explained by catch-up growth of lung parenchyma beyond infancy. Compared to term-born children, relative lung volume and trapped gas at follow-up were higher in preterm children; and compensatory lung growth after prematurity might exceed normal growth. The change from a positive to a negative association of lung volume with airway flow indicated a loss of physiological variability of breathing. In conclusion, we observed catch-up growth of lung volume and gas trapping in this group of preterm children, with some evidence pointing to excessive lung growth between infancy and early childhood.

6.6 Excessive increase in lung volume during early development in infants recovering from prematurity

ONLINE SUPPLEMENT - DETAILS ON METHODS AND RESULTS

DETAILS ON METHODS

Study design and participants

Preterm children were recruited for an observational study during postnatal hospitalization at Inselspital Bern, Bern, Switzerland; the study was previously described in detail [Latzin et al., 2009a, Pramana et al., 2011, Proietti et al., 2014]. Briefly, inclusion criteria were birth under 37 weeks of gestation, and the ability of the parents to speak one of the major Swiss languages (German or French). Children with congenital diaphragmatic hernia were excluded.

We measured lung volume and ventilation inhomogeneity in infancy and at early school age, and generated a standard deviation score (SDS) for anthropometric data, functional residual capacity (FRC), and expiratory flow. In a paired design, we tested whether FRC in infancy was associated with FRC at early school age, and which biometric and clinical factors were associated with FRC at early school age and with the change of FRC SDS over time. A group of term-born children, i.e. born between 37 and 42 weeks of gestation, included in a similar study, provided longitudinal lung function data for group comparisons (n=124) [Fuchs et al., 2012a, Schmidt et al., 2015a].

Lung function measurements

All lung function measurements were performed by experienced operators using commercially available equipment and according to standards [Frey et al., 2000a, Robinson et al., 2013, Wanger et al., 2005, Miller et al., 2005].

We measured infant lung function at the corrected age of 44 weeks of gestation (visit 1) in unsedated sleep in supine position. We performed tidal-breath measurements and multiple-breath washout (MBW) using validated equipment [Schmidt et al., 2015b], i.e. the Exhalyzer D (Eco Medics AG, Duernten, Switzerland), and sulphur hexafluoride (SF_6) as an age-appropriate tracer [Latzin et al., 2007b]. Maximum tracer concentration during washin and washout was determined by end-inspiratory molar mass using WBreath 3.28.0 [Anagnostopoulou et al., 2015].

At early school-age (visit 2), we measured lung function with the cooperative, awake child in upright position. Children performed nitrogen MBW [Singer et al., 2012a] using the Exhalyzer D and Spiroware 3.1.6 ext., and after that spirometry, and plethysmogra-

phy [Criée et al., 2011] using the JAEGER Master-Screen Body (CareFusion, Wuerzburg, Germany). For each visit and technique, FRC was measured in triplicates. For MBW, it was calculated as cumulative expired volume (CEV) of tracer divided by the difference between end-tidal tracer gas concentrations at start and end of MBW. For plethysmography it was calculated according to the law of Boyle-Mariotte [Criée et al., 2011]. The mean of high quality FRC measurements was calculated per technique and visit, according to standards [Frey et al., 2000a, Robinson et al., 2013, Wanger et al., 2005, Miller et al., 2005].
Mean lung clearance index (LCI) was calculated from high quality MBW measurements as FRC over CEV until tracer was washed out to 1/40th of the initial concentration [Frey et al., 2000a, Robinson et al., 2013, Wanger et al., 2005, Miller et al., 2005].

Health status and exposures

Gestational age at birth was defined as post-menstrual age at birth in weeks. Severity of bronchopulmonary dysplasia (BPD) was defined according to the Jobe-Bancalari BPD severity classification [Ehrenkranz et al., 2005]. The clinical risk index for babies (CRIB score) was assessed [The international neonatal network, 1993]. Duration of mechanical ventilation in days was defined as duration of invasive and non-invasive ventilation if applicable.
Parental smoking was assessed at several points in time. We generated binary variables for either maternal or paternal smoking (i) during pregnancy, (ii) during the first year of life, or (iii) at early school age; paternal smoking was labelled as positive if the father, or another person in the household, smoked.
Respiratory symptoms during the first year of life were assessed by questionnaire [Pramana et al., 2011].
Parental asthma was defined as either parent reporting doctor-diagnosed asthma. Maternal or paternal atopic disease was defined as mother or father having asthma, atopic dermatitis, or atopic rhinitis.
At the second visit, asthma of the child was defined as either doctor-diagnosed asthma or current inhalation of bronchodilators and / or steroids. Wheeze was assessed based on questions from the ISAAC questionnaire [Asher et al., 1995].

Statistical Methods

Primary outcome was FRC at early school age. We tested for an association between FRC in infancy and FRC at school age using the Pearson correlation coefficient and linear regression analysis.

A standard deviation score (SDS) was generated for anthropometric data, FRC, and expiratory flow. For weight, height, and body mass index (BMI) at both study visits, the zanthro package was used in STATA 11. SDSs were specific for gender and gestational age. [World Health Organization, 2006] In a post-hoc sensitivity analysis, SDSs were generated without consideration of gestational age. Relative increase in body weight or body length was defined as the difference of SDS at visit 2 and SDS at visit 1 (ΔSDS). For FRC and mean expiratory flow at visit 1, SDSs were calculated based on gender-specific mean and standard deviation (SD) within the term-born population (n=124):

SDS = (gender-specific mean within group individual value) / gender-specific SD within group. For FRC_{pleth} at visit 2, SDSs were calculated based on gender- and height-specific reference values [Rosenthal et al., 1993].

For mean expiratory flow at visit 2, SDSs were calculated based on gender-, and age-specific reference data published by the Global Lung Initiative using the software tool for R [Quanjer et al., 2012]. FRC relative to body size was calculated as FRC / body weight (ml/kg) and FRC / height (ml/cm). For FRCMBW at visit 2, gender- and age-specific SDS were calculated from data in healthy children measured at our lung function laboratory. Ventilation inhomogeneity was evaluated using the mean lung clearance index (LCI) from high quality MBW measurements [Frey et al., 2000a, Robinson et al., 2013, Wanger et al., 2005, Miller et al., 2005]. Upper limit of normal for LCI was calculated from values in term-born children measured at our pediatric lung function laboratory, it was 7.67 at visit 1, and 8.06 at visit 2.

Trapped gas was calculated as ml difference between FRC from plethysmography and MBW:

Trapped gas = FRC_{pleth} – FRC_{MBW_V2}.

For spirometric results, SDS were generated using gender- and age-specific reference data [Quanjer et al., 2012].

We performed linear regression analysis. After univariable linear regression, variables associated with the outcome of interest were included stepwise in the multivariable model. For multivariable regression analysis, reasons for exclusion of a variable were co-linearity with a previously included variable and / or increase of R^2 of less than 10%. We tested for non-linear associations by adding quadratic transformations of predictors to the model, and excluded the quadratic term if the likelihood ratio test showed no model improvement.

A group of term-born children included in an observational study provided longitudinal lung volume data for group comparison (n=124 for comparison with FRC_{pleth}, n=22 for comparison with FRC_{MBW_V2}) [Schmidt et al., 2015a].

Group comparisons were performed as follows: For continuous variables, we performed visual control for equally distributed values, and then used the unpaired T-tests, for binary variables we used the Chi-square test. A p-value <0.05 was considered significant.

DETAILS ON RESULTS

Online Supplement Table 6.1:
Characteristics of term-born children from a comparable study, and of preterm children included in the current analysis

	Term-born children (n=124)						Preterm children (n=80)						95% CI of Difference*
	SDS considering gestational age			SDS not considering gestational age			SDS considering gestational age			SDS not considering gestational age			
	Available (n)	mean	SD	Available (n)	mean	SD	Available (n)	mean	SD	Available (n)	mean	SD	
Visit 1													
Weight SDS	124	-0.18	0.92	124	-0.26	0.92	80	-1.03	1.31	56	-3.32	1.12	2.72 – 3.40**
Length SDS	124	0.06	0.92	124	-0.02	1.01	80	-1.23	1.56	50	-3.42	1.12	3.03 – 3.76**
FRC_{MBW} SDS				124	0.00	1.00				76	-0.60	1.43	0.23 – 0.97
LCI SDS				124	0.00	1.00				76	0.34	1.64	-0.76 – 0.07
Visit 2													
Weight SDS	124	0.46	1.00	124	0.45	1.00	80	0.05	1.26	80	-0.09	1.28	0.21 – 0.88
Height SDS	124	0.32	1.04	124	0.32	1.04	80	0.12	1.07	80	-0.09	1.06	0.11 – 0.71
BMI SDS	124	0.37	0.96	124	0.37	0.96	80	-0.02	1.31	80	-0.06	1.30	0.10 – 0.77
FRC_{pleth} SDS				124	-0.29	1.01				69	0.58	0.93	-1.16 – -0.59
FRC_{MBW} SDS				22	0.00	0.98				71	0.18	1.23	-0.70 – 0.33
LCI SDS				22	-0.05	0.95				71	0.71	1.76	-1.34– -0.19

Standard Deviation Scores (SDS) are given with and without consideration of gestational age at birth

* 95% Confidence Interval (95% CI) for difference of mean SDS between term-born and preterm children (unpaired T test comparing SDS without consideration of gestational age)
** For weight and length at visit 1, SDS without consideration of gestational age at birth was out of range in 24 and 30 preterm children respectively, so values are missing, the real difference between the groups is thus even higher

Online Supplement Table 6.2a
Univariable regression analysis
Variables associated with outcome FRC_{MBW_V2} in ml

	Beta	Lower CI	Upper CI	P value	R2
Height at visit 2, cm	22.2	16.6	27.7	< 0.001	46.8
Increase in height from visit 1 to visit 2, cm	19.7	14.2	25.3	< 0.001	41.6
Age at visit 2, months	11.1	7.6	14.7	< 0.001	35.1
Weight at visit 2, kg	18.3	9.7	26.9	< 0.001	19.5
Increase in weight from visit 1 to visit 2, kg	18.4	9.7	27.0	< 0.001	19.5
Increase in height SDS	88.1	42.0	134.3	< 0.001	16.2
LCI visit 1 (multiple-breath washout)	67.9	20.9	114.9	0.005	10.0
Duration of intubation, days	9.0	2.0	16.1	0.012	7.4
Paternal smoking 1st year of life	291.2	-39.7	622.0	0.084	7.3
Oxygen supplementation, days	1.4	0.3	2.5	0.014	7.1
Parental smoking at any point in time during the study	187.3	41.1	333.5	0.013	6.1
Paternal smoking at visit 2*	196.2	31.5	360.9	0.020	5.5
Increase in weight SDS	57.1	3.9	110.2	0.036	4.9
Gestational age at birth, weeks	-22.3	-44.9	0.3	0.053	3.9
CPAP	285.8	-7.7	579.4	0.056	3.8
Duration of mechanical ventilation, days	2.8	-0.1	5.8	0.057	3.8
BPD moderate or severe (compared to no or mild BPD)	116.1	-20.3	252.4	0.094	2.6
Parental smoking at visit 2	133.9	-14.1	281.9	0.076	2.3

* Defined as smoking of father or other person in the household

Increase in SDS was calculated as difference of SDS at visit 2 and SDS at visit 1

BPD – Bronchopulmonary dysplasia

CPAP – Continuous positive airway pressure in neonatal period

Online Supplement Table 6.2b
Univariable regression analysis
Variables not associated with outcome FRC_{MBW_V2} in ml

Variable
Male gender (yes / no)
Parental atopy (yes / no)
Parental asthma (yes / no)
Antenatal steroids (yes / no)
Chorioamnionitis (yes / no)
Surfactant (yes / no)
Intubation (yes / no)
Duration of mechanical ventilation (invasive and/or non-invasive)
Maximum peak inspiratory pressure during neonatal ventilation
Birth weight
Weight gain in infancy from birth to visit 1
Weight at visit 1
Weight SDS at visit 1
Length at visit 1
Length SDS at visit 1
LCI at visit 1
Early weight gain from birth to visit 1
Maternal smoking during pregnancy (yes / no)
Parental smoking during pregnancy (yes / no)
Maternal smoking 1st year of life (yes / no)
Parental smoking 1st year of life (yes / no)
Any respiratory symptoms 1st year of life (yes / no)
Severe respiratory symptoms 1st year of life (yes / no)
Doctor diagnosed asthma at visit 2 (yes / no)
Wheeze within 12 months prior to visit 2 (yes / no)

Online Supplement Table 6.3a
Univariable regression analysis
Variables associated with outcome ΔSDS from visit 1 to visit 2#

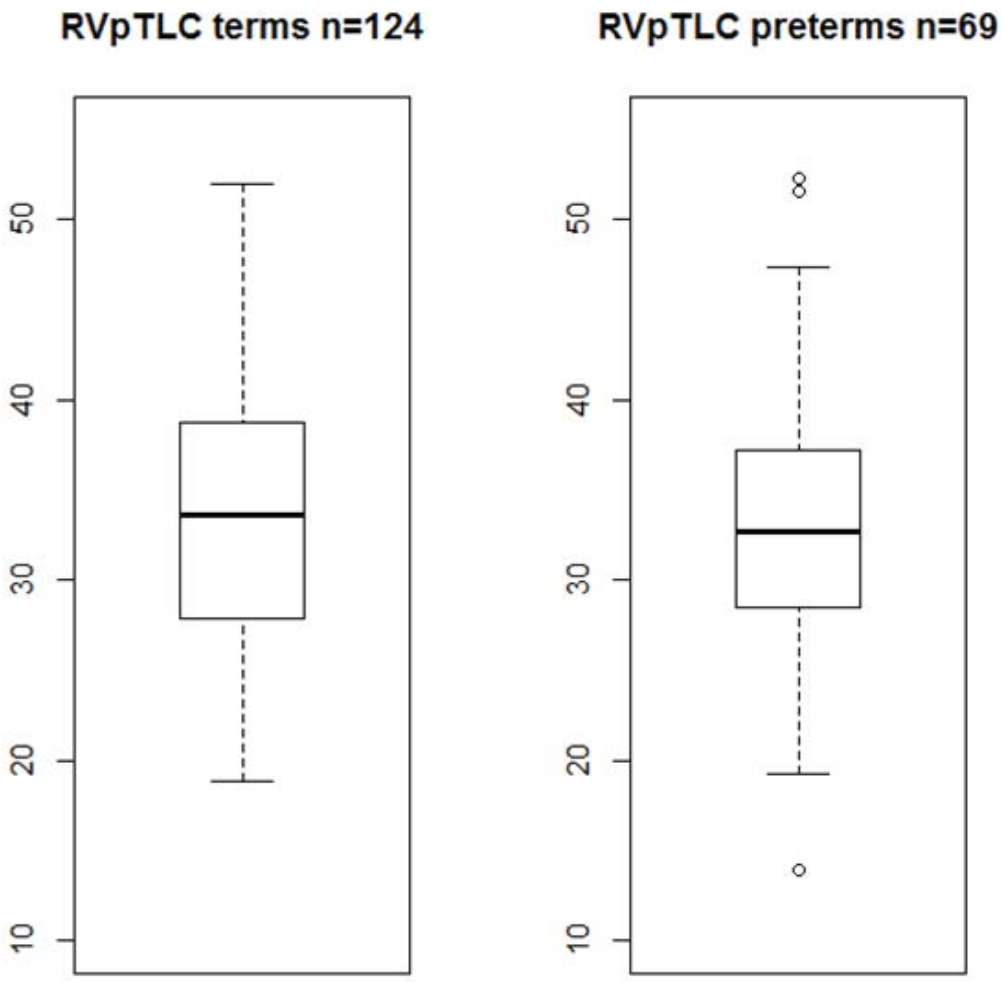
Variable	Beta	Lower CI	Upper CI	P value	R2
Weight at visit 1, g	-0.0009	-0.0013	-0.0005	< 0.001	21.9
Length at visit 1, cm	-0.2116	-0.3071	-0.1161	< 0.001	21.4
Duration of mechanical ventilation, days	0.0325	0.0172	0.0479	< 0.001	19.9
Weight gain in infancy from birth to visit 1, g / day	-0.1297	-0.1925	-0.0668	< 0.001	19.0
Gestational age at birth, weeks	-0.2322	-0.3458	-0.1185	< 0.001	18.7
Weight at birth, g	-0.0013	-0.0020	-0.0006	< 0.001	17.3
Age since term at visit 1, weeks	-0.2872	-0.4480	-0.1263	0.001	14.7
Oxygen supplementation, days	0.0116	0.0045	0.0188	0.002	12.3
Duration of CPAP, days	0.0270	0.0093	0.0448	0.003	10.9
BMI at visit 2, kg/m2	-0.1950	-0.3287	-0.0614	0.005	9.9
Duration of intubation, days	0.0548	0.0165	0.0930	0.006	9.5
CPAP (yes / no)	1.9453	0.5699	3.3206	0.006	9.3
Weight SDS visit 1	-0.3545	-0.6433	-0.0656	0.017	6.8
Length SDS visit 1	-0.2843	-0.5205	-0.0482	0.019	6.6
BPD moderate or severe (compared to no or mild BPD)	0.8813	0.1480	1.6146	0.019	6.5
Wheeze within 12 months prior to visit 2 (yes / no)	1.4232	-0.1292	2.9755	0.072	6.3
CRIB score	0.1105	0.0141	0.2069	0.025	6.2
Surfactant (yes / no)	0.8234	0.0895	1.5573	0.028	5.6
Weight at visit 2, kg	-0.0481	-0.0922	-0.0039	0.033	5.2
Increase in weight from visit 1 to visit 2, kg	-0.0393	-0.0840	0.0053	0.083	3.0
Wheeze ever reported at visit 2 (yes / no)	0.6815	-0.1106	1.4736	0.091	2.8

BMI – Body mass index
BPD – Bronchopulmonary dysplasia
CPAP – Continuous positive airway pressure in neonatal period
CRIB score – Clinical risk index for babies
Outcome ΔSDS = (SDS FRC_{pleth} - SDS $FRC_{MBW\ V1}$ from washin)

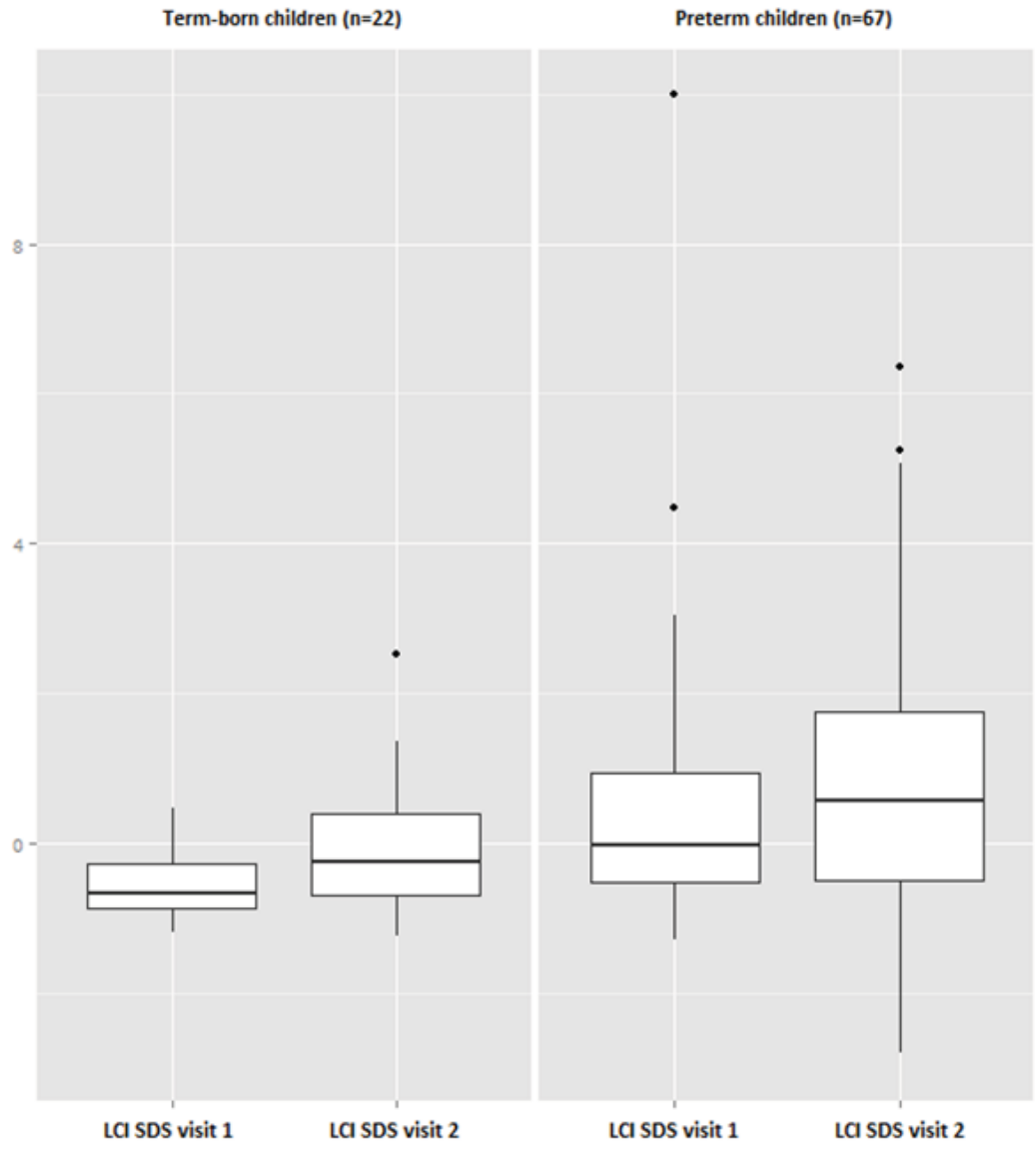
Online Supplement Table 6.3b
Univariable regression analysis
Variables nnot associated with outcome ∆SDS from visit 1 to visit 2#

Variable
Male gender (yes / no)
Parental atopy (yes / no)
Antenatal steroids (yes / no)
Parental smoking during pregnancy (yes / no)
Intubation (yes / no)
Maximum peak inspiratory pressure during neonatal ventilation
Parental smoking 1st year of life (yes / no)
Duration of exclusive breastfeeding
Any respiratory symptoms 1st year of life (yes / no)
Severe respiratory symptoms 1st year of life (yes / no)
Age at visit 2
Height at visit 2
Parental smoking at visit 2 (yes / no)
Doctor diagnosed asthma at visit 2 (yes / no)

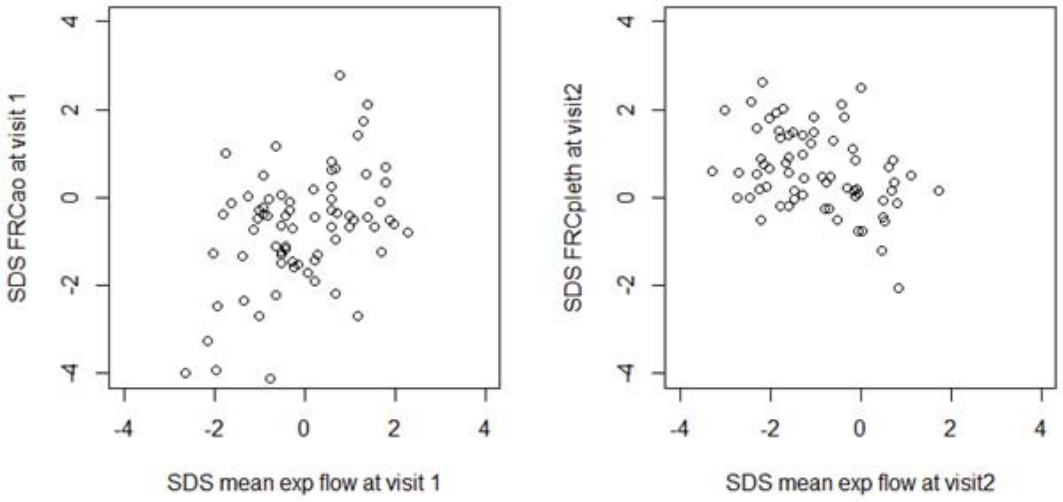
Outcome ΔSDS = (SDS FRC_{pleth} - SDS FRC_{MBW_V1} from washin)



Online Supplement Figure 6.1
RVpTLC in % in term born and preterm children
Residual volume per total lung capacity in term-born (n=124) and preterm children (n=69) at early school-age (visit 2).



Online Supplement Figure 6.2
LCI SDS over time in term-born and preterm children
Standard deviation score (SDS) for lung clearance index (LCI) over time in term-born (n=22) and preterm children (n=67). LCI was measured by SF_6 multiple-breath washout in infancy (visit 1), and by nitrogen multiple-breath washout at early school-age (visit 2).



Online Supplement Figure 6.3
Dysanapsis of lung volume and larger airways in preterm children
In infancy (visit 1), standard deviation score (SDS) for mean expiratory flow was positively associated with functional residual capacity (FRC) measured by SF_6 multiple-breath washout in preterm children (beta=0.59, 95% CI 0.36 to 0.82, p<0.001, R^2 25.3%). In the same group, at early school age (visit 2), SDS for mean expiratory flow was negatively associated with SDS for FRC measured by plethysmography (beta=-0.30, 95% CI -0.48 to -0.12, p=0.002, R^2 12.6%). In the term-born control group, mean expiratory flow and FRC were not associated at either visit (n=124, data not shown).

7 General discussion

This PhD thesis adds new information to the field of early lung development in term-born and preterm children from infancy to early childhood. In the study presented in chapter 2, we showed that our MBW equipment reliably measured lung volumes in models with quasi-physiological conditions, corresponding to the lung sizes of infants and young children. Using both this newly validated and other established equipment, we were able to investigate several aspects of early lung development. Firstly, we investigated early lung growth in term-born children early in childhood, which was presented in chapter 3. Here, we observed a weak tracking of lung volume throughout young childhood, and no association of lung volume and function of larger airways. Secondly, we investigated early lung development in preterm children (i) in infancy, (ii) at school age, (iii) and over time, presented in chapters 4, 5, and 6. In infancy, we tested whether lung function measurements could improve prediction of later respiratory symptoms when compared to established predictive tools, but found no improvement. Thus, infant lung function did not seem to be helpful for immediate clinical decision making. However, in this relatively large population, we observed an altered breathing pattern in those preterm infants with later wheeze, and this gave us reason to speculate on mechanisms for this early respiratory pathophysiology. At early school age, we investigated ventilation homogeneity in preterm children, and found differences in the peripheral lung regions compared to term-born controls. Investigation of lung volumes over time detected excessive pulmonary catch-up growth from infancy to early childhood in preterm children, and reduced physiological variability of breathing pattern. Children born prematurely showed higher lung volume increase when born at younger gestational age. To the best of our knowledge, these are the first studies describing longitudinal FRC data over this long and early period in term-born and preterm children.

7.1 General strengths and limitations

Several strengths of the studies in this work are worth mentioning. The lung models we used had quasi-physiological conditions, and our in-vitro study was the first to challenge the equipment for MBW_{SF6} and MBW_{He} according to current consensus [Robinson et al., 2013]. The studies on early lung growth in term-born and preterm children closed an important gap of knowledge, as data on lung volume over time during this long, early period had not been available until now. In infants after prematurity, it was previously unknown whether or not lung function at the corrected age of 44 weeks of gestation could be helpful for clinical decisions in an individual, and mechanisms of early pathophysiology were not entirely understood. In school children born prematurely, our detailed comparison of peripheral lung ventilation with term-born children of the same age and our investigation of lung volume over time allowed new insights in early lung pathology of the affected children. We added new pieces to the puzzle of early lung development, and answered questions that will allow us to develop new strategies for better overall respiratory health. The term-born and preterm children were unselected, which increases generalisability of our findings. However, some families might have been more motivated to participate, especially at the follow-up visit after several years. Families with a higher interest in the respiratory health of their term-born child, or with less traumatising experiences in the hospital after preterm delivery, might be slightly overrepresented. Also, exposures were not assigned randomly. These were typical issues in observational studies which could not be avoided. However, they needed to be taken into account when interpreting the findings.

The lung function techniques we used were appropriate for the specific age groups. The measurements were performed and analysed in a paediatric lung function laboratory in a team with recognised expertise. We were able to include numerous participants in our studies in spite of the complexity of the measurements, especially of measuring FRC in infants and young children. Inclusion of high quality measurements only limited the sample size.

Methodological differences between the measurement techniques could not be avoided, as the most suitable setups and tracers were age-dependent. Also, vegetative state and

body position of the children differed between the study visits. Our standard of MBW analysis in infancy was recently improved [Anagnostopoulou et al., 2015]. MBW using different tracers was previously shown to require specific reference data. This might have hampered detection of associations of FRC over time. Physical principles of measuring FRC by MBW and plethysmography differed as well, and the poor success rate of helium MBW in term-born children at follow-up was a drawback. Despite our attempts to improve success rate, we had to conclude that helium was not suitable for MBW in the investigated population, and we now perform nitrogen MBW in all children at the follow-up visit. The latter was easier to perform, test duration was shorter and success rate was high. In the current study, longitudinal analyses of FRC in term-born children was limited to FRC_{pleth} as primary outcome, which had the disadvantage of using a different technical principle than FRC_{MBW} measurement in infancy, but the advantage of available height- and gender-specific reference values which could also be compared to preterm children. At any visit, we used the equipment, tracers, and protocols for analyses that were most promising when the study was initiated. During the process, we adapted our procedures based on new knowledge.

The physiological breathing pattern in childhood was variable, and so was FRC, especially as we performed infant lung function tests without sedation. This, combined with some measurement error, probably made detection of associations more difficult than in studies investigating measurements based on forced expiration with less variability. Still, in order to investigate not only larger airways, but lung volumes and ventilation in smaller airways, the tidal breathing lung function tests we performed were valuable tools.

Parameters of peripheral ventilation inhomogeneity, Scond and Sacin, were assumed to represent the convection- and diffusion-convection-dependent lung zones respectively. However, this was a model based interpretation [Verbanck et al., 2003], and proof of association with specific lung morphology was not available.

When comparing findings in the term-born children of the BILD study with those in the preterm population, a few differences between the study protocols and populations had to be considered. Symptoms during the first year of life were assessed prospectively for term-born infants, and retrospectively for preterm infants. Thus, a potential reporting bias in the preterm population needed to be taken into account. This was improved for

the follow-up visit, where questionnaires and interviews for assessment of symptoms were identical in the two populations. At follow-up, success rate for FRC measurements depended on cooperation, and preterm children might be limited in their ability to maintain tight closure around a mouthpiece during measurements with consecutive lower rate of successful trials. We encouraged and helped children as much as possible during measurements, but preterm children with neurological impairment might be under-represented in the group with successful FRC measurements. Age at follow-up was approximately 6 years for term-born children and 9 years for preterm-children. We aimed to overcome this limitation by adjusting for age and body size, by using established reference values whenever these were available, by comparing reference values with and without consideration of gestational age and by challenging our findings in sensitivity analyses using absolute values and values relative to body size.
Statistical power was limited by the sample size. Again, considering the complexity of our measurements, sample size was considerably large. If tracking of FRC had been strong in term-born and preterm children, statistical power in the current sample sizes would have sufficed to detect that.

7.2 Comparison with previous findings and interpretation

Validation of MBW equipment

In the in-vitro lung model trials presented in chapter 2, we were able to show that our equipment reliably measures lung volumes within 10% accuracy over FRC volumes between 80 and 300 ml with the infant setup and between 600 and 1,400 ml with the young child setup. A validation study for an available N_2MBW setup (Exhalyzer D, Eco Medics AG) showed an error within 7% for the small model and within 5% for 98% of the runs in the large lung model. The equipment had several similarities with our setups, e.g. the use of an USFM, but N_2 concentration was determined indirectly via O_2 and CO_2 sensors. Also, CO_2 application to the model differed in this study [Singer et al., 2012a]. Since for infants, SF_6 is currently the recommended tracer [Robinson et al., 2013], the setup we investigated was the more appropriate. For children beyond infancy, both HeMBW and

N_2MBW would be suitable.

Lung growth in term-born children

The study on longitudinal lung volume data presented in chapter 3 showed a weak tracking of FRC in 124 unselected term-born children from infancy to early childhood. The strongest predictors of FRC in early childhood were body size and age at the time of FRC measurement. Male gender and maternal smoking during the first year of life were positively associated with FRC. FRC in infancy alone explained 4% of the variance of FRC_{pleth} in early childhood, the multivariable model explained 22%. As this was the first study on lung volumes over this long and early period, results can only be compared with studies on lung volumes over shorter periods or with studies reporting airway function. A study with a comparable study protocol, i.e. unselected birth cohorts with longitudinal lung function measurements starting very early in childhood, showed a weak tracking of airway function [Stern et al., 2007], with an explained variance of 9 to 14%. Spirometric measurements in infants were performed with sedation, with lower physiological variability than in our measurements without sedation. In preterm children, tracking of airway function was shown [Filippone et al., 2003].

We observed that some investigators used the term tracking for group comparisons, and we suggested the term should be used only when associations of individuals' values over time were evaluated, in order to allow comparability of studies.

Infant lung function in preterm children

In preterm infants, we observed no improvement of prediction of respiratory symptoms in the first year of life when infant lung function was added to established clinical prediction scores. Other investigators observed a weak predictive value of infants lung function parameters, e.g. of higher airway resistance in hospitalised preterm infants [Drysdale et al., 2011], for prediction of symptoms in the first year of life.

We found an altered breathing pattern in infants who developed wheeze, with higher tidal volume and lower respiratory rate. Previous studies in preterm infants showed increased tidal volume [Putnam et al., 2005], and alterations in airway mechanics [van Putte-Katier et al., 2012, Drysdale et al., 2011, Neufeld et al., 1992], and it is possible that thereby

effective CO_2 clearance was maintained despite a ventilation perfusion mismatch and a rarefied acinar structure.

Ventilation in preterm children at school age

In former preterm children at early school age, ventilation on the overall and the acinar level was normal, but function of more proximal conducting airways was impaired in the majority of the investigated population. This confirms what was suggested previously, i.e. that alveolar growth continues after term and preterm birth [Narayanan et al., 2012, Narayanan et al., 2013, Herring et al., 2014]. In a population of 11-year old children born <26 weeks of gestation [Lum et al., 2011], the proportion of children with inhomogeneous ventilation on a global level, i.e. elevated LCI, was higher than in our study, which was most likely explained by the different range of gestational age at birth. The finding of ventilation inhomogeneity on the conductive level, but not on the more peripheral acinar level, was unexpected. We speculated that it was explained by the timing of lung growth disruption: if the peripheral conducting airways were damaged after they had already developed, while the alveolar regions developed later, this could explain why Scond was elevated in the majority of preterm children, but Sacin was not.

Excessive lung growth after prematurity

In our longitudinal analysis of lung volume from infancy to early childhood in preterm children, which was presented in chapter 5, we found only a very weak association of the individuals' FRC values over time. This was in contrast to strong tracking of airway function that was previously described [Filippone et al., 2003]. Relative FRC increase was negatively associated with gestational age, which we interpreted as catch-up growth after preterm birth. When we compared the data in preterm children with a population of term-born children (see chapter 2), we found some differences between the groups with regards to ventilation heterogeneity, and hyperinflation. In the investigated population, trapped gas was higher than in a group of term-born children. Even though we cannot exclude that trapped gas was already present in infancy, our findings and what was described by other investigators gave us reason to speculate that catch-up growth after premature birth happened beyond infancy, and that it was overshooting. In the preterm

population, FRC was within normal range at early school age. However, FRC SDS was higher in preterm children compared to the term-born population. This might be explained in part by reduced body size in the preterm group, but the difference remained significant even after adjustment. Because of that, and because of the higher values regarding trapped gas, we interpreted our observations as excessive pulmonary catch-up growth after premature birth.

7.3 Clinical and public health relevance

Our observations gave new insights into early lung development in term-born and preterm children. Even though our findings do not have immediate consequences in clinical management, they were encouraging, and our understanding of lung growth in early childhood was advanced. This new basic knowledge will facilitate the design of further studies and it will be beneficial for communication with affected families.

Using validated MBW equipment, we were able to show the natural course of early lung growth, with anthropometric values being the strongest predictors of lung volume at follow-up. Tracking was weak, and measurements of lung volume and larger airway function were not associated in term-born children. In preterm children, we observed no improvement of prediction of respiratory morbidity by adding infant lung function to established prediction tools, but we did note interesting alterations of breathing pattern in children who developed wheeze in the first year of life. Thus, infant lung function in preterm children did not seem to be advantageous for clinical decision making, but it was beneficial for understanding early pathophysiology. At school age, differences in ventilation homogeneity between term-born and preterm children were subtle, and this was an encouraging observation. It confirmed previous observations of ongoing alveolar growth during childhood. The finding of pulmonary catch-up growth after prematurity, which we described in a longitudinal data set for the first time, was encouraging. We expected to find fewer sequelae after prematurity compared to populations born in earlier periods. However, recovery from interrupted lung growth seemed even better than expected.

The incidence of preterm birth will not drop in the future, and survival even after very

premature birth will be improving, so clinicians in neonatology and paediatric respiratory medicine will continue to see children suffering from pulmonary impairment after prematurity, and many of these patients will require respiratory treatment in adulthood. Our goal would clearly be to ensure catch-up growth in these children, and to minimise early hyperinflation. Due to non-random administration of potentially beneficial treatments we were not able to identify specific strategies for the future. New studies will be necessary to answer the open questions.

7.4 Outlook

In order to bring beneficial strategies within reach, the next scientific steps would be (a) to confirm our findings and combine them with information on morphology or function, e.g. by combining measurements of small airway function and hyperinflation or with functional measurements like diffusion of carbon monoxide, or with modern imaging techniques like magnetic resonance imaging (MRI). The latter can display lung tissue in increasing quality without exposing the children to radiation, and combined longitudinal investigations of small airway function and structural lung growth would be of great interest in healthy children as well as in those with chronic lung diseases. Histology might add valuable information, but acquisition of samples is invasive. Further scientific steps would be (b) to continue follow-up of the investigated populations into adolescence and adulthood to see whether hyperinflation increases in preterm children, and whether it predicts later respiratory symptoms; (c) to observe the effect of potentially harmful substances, i.e. outdoor air pollution and active smoking, and to compare susceptibility between term-born and preterm-children; (d) to investigate gene-environment interactions, and (e) to compare clinical management after preterm birth during childhood with regards to later lung volume as well as the function of larger and smaller airways, e.g. in a randomised controlled trial (RCT). RCTs are certainly limited by ethical considerations, resources and number of potential participants. To increase sample size, (f) collaborations for meta-analyses are an interesting approach, which at the moment predominantly have an observational design using classic lung function tests with a very broad availability. Last but not least,

(g) an improvement of the already available new, non-invasive lung function tests would help us to increase the quality of information that we can gain. At the same time, we should aim for tests to be short and easy to perform with children.
Many of these steps are currently being undertaken in our group (see Appendix [Singer et al., 2014b, Anagnostopoulou et al., 2015, Fuchs et al., 2015]), in collaborations (see Appendix [Sonnenschein-van der Voort et al., 2014]), and by other research groups. In the coming years this will increase our knowledge of potentially harmful exposures and of best clinical management after preterm birth, and we will aim for the best possible short-term and long-term outcomes of respiratory health in all children.

7.5 Conclusions

We can conclude from our in-vitro study that, in a lung model, the investigated setups for multiple-breath washout were valid and reliable for measuring lung volumes in infancy and early childhood.
Tracking of lung volume in term-born children early in childhood was weak, and lung volume was not associated with function of larger airways. The weakness of tracking was partially explained by the high physiological variability of functional residual capacity and multiple influencing factors.
In preterm infants, lung function at the corrected age of 44 weeks of gestation did not improve prediction of respiratory symptoms compared with standard clinical parameters. Preterm infants with wheeze during infancy showed a different breathing pattern compared to non-wheezers, possibly because of an adaptation to their altered lung structure.
In preterm children at school age, ventilation homogeneity measured by multiple-breath washout differed from term-born children in the peripheral, conducting airways. Results for ventilation on the overall and the acinar level showed no significant differences, which suggested continued alveolar growth after preterm birth.
Tracking of lung volume in preterm children early in childhood was very weak. Stronger increase of lung volume when born at younger gestational age suggested catch-up growth,

while subtle differences to term born children regarding ventilation homogeneity and air trapping pointed towards incomplete recovery from interrupted structural growth due to prematurity.

Altogether, tracking was weak in both term-born and preterm children, which confirms that early childhood is a window of vulnerability, but also of opportunity for lung development. Lung volume in early childhood was not largely determined by lung volume in infancy, and this stressed the importance of protection from potentially harmful substances throughout childhood. However, efforts to prolong intra-uterine development as much as possible must still be regarded as beneficial: in our studies, respiratory health and growth of peripheral lung structure beyond infancy did not seem to be determined by harmful or preventive therapeutic interventions, but by prematurity per se.

8 References

[Albu et al., 2014] Albu, G., Petak, F., Zand, T., Hallback, M., Wallin, M., and Habre, W. (2014). Lung volume assessments in normal and surfactant depleted lungs: agreement between bedside techniques and ct imaging. *BMC Anesthesiol*, 14:64.

[Anagnostopoulou et al., 2015] Anagnostopoulou, P., Yammine, S., Schmidt, A., Korten, I., Kieninger, E., Mack, I., Trachsel, D., Hafen, G., Moeller, A., Casaulta, C., and Latzin, P. (2015). False normal lung clearance index in infants with cystic fibrosis due to software algorithms. *Pediatr Pulmonol*, 50(10):970–7.

[Asher et al., 1995] Asher, M., Keil, U., Anderson, H., Beasley, R., Crane, J., Martinez, F., Mitchell, E., Pearce, N., Sibbald, B., a.W. Stewart, Strachan, D., Weiland, S., and Williams, H. (1995). International study of asthma and allergies in childhood (ISAAC): rationale and methods. *European Respiratory Journal*, 8(3):483–491.

[Aurora et al., 2005a] Aurora, P., Bush, A., Gustafsson, P., Oliver, C., Wallis, C., Price, J., Stroobant, J., Carr, S., and Stocks, J. (2005a). Multiple-breath washout as a marker of lung disease in preschool children with cystic fibrosis. *Am J Respir Crit Care Med*, 171(3):249–56.

[Aurora et al., 2005b] Aurora, P., Kozlowska, W., and Stocks, J. (2005b). Gas mixing efficiency from birth to adulthood measured by multiple-breath washout. *Respir Physiol Neurobiol*, 148(1-2):125–39.

[Aurora et al., 2011] Aurora, P., Stanojevic, S., Wade, A., Oliver, C., Kozlowska, W., Lum, S., Bush, A., Price, J., Carr, S. B., Shankar, A., and Stocks, J. (2011). Lung clearance index at 4 years predicts subsequent lung function in children with cystic fibrosis. *American journal of respiratory and critical care medicine*, 183(6):752–8.

[Baker and Alvira, 2014] Baker, C. D. and Alvira, C. M. (2014). Disrupted lung development and bronchopulmonary dysplasia: opportunities for lung repair and regeneration. *Current opinion in pediatrics*, 26(3):306–14.

[Baraldi and Filippone, 2007] Baraldi, E. and Filippone, M. (2007). Chronic Lung Disease after Premature Birth. *The New England journal of medicine*, 357:1946–1955.

[Baraldi et al., 1997] Baraldi, E., Filippone, M., Trevisanuto, D., Zanardo, V., and Zacchello, F. (1997). Pulmonary function until two years of life in infants with bronchopulmonary dysplasia. *American Journal of Respiratory and Critical Care Medicine*, 155(1):149–155.

[Barker et al., 1991] Barker, D. J., Godfrey, K. M., Fall, C., Osmond, C., Winter, P. D., and Shaheen, S. O. (1991). Relation of birth weight and childhood respiratory infection to adult lung function and death from chronic obstructive airways disease. *BMJ*, 303(6804):671–5.

[Beck et al., 2010] Beck, S., Wojdyla, D., Say, L., Betran, A. P., Merialdi, M., Requejo, J. H., Rubens, C., Menon, R., and Van Look, P. F. (2010). The worldwide incidence of preterm birth: a systematic review of maternal mortality and morbidity. *Bull World Health Organ*, 88(1):31–8.

[Been et al., 2014] Been, J. V., Nurmatov, U. B., Cox, B., Nawrot, T. S., van Schayck, C. P., and Sheikh, A. (2014). Effect of smoke-free legislation on perinatal and child health: a systematic review and meta-analysis. *Lancet*, 383(9928):1549–60.

[Belgrave et al., 2014] Belgrave, D. C. M., Buchan, I., Bishop, C., Lowe, L., Simpson, A., and Custovic, A. (2014). Trajectories of lung function during childhood. *American journal of respiratory and critical care medicine*, 189(9):1101–9.

[Berkelhamer et al., 2013] Berkelhamer, S. K., Mestan, K. K., and Steinhorn, R. H. (2013). Pulmonary hypertension in bronchopulmonary dysplasia. *Semin Perinatol*, 37(2):124–31.

[Bigler et al., 2015] Bigler, A., Yammine, S., Singer, F., Riedel, T., and Latzin, P. (2015). Feasibility of automated slope iii and scond analysis in children. *Pediatr Pulmonol*, 50(8):805–13.

[Bland and Altman, 1986] Bland, J. M. and Altman, D. G. (1986). Statistical methods for assessing agreement between two methods of clinical measurement. *Lancet*, 1(8476):307–10.

[Blencowe et al., 2012] Blencowe, H., Cousens, S., Oestergaard, M. Z., Chou, D., Moller, A. B., Narwal, R., Adler, A., Vera Garcia, C., Rohde, S., Say, L., and Lawn, J. E. (2012). National, regional, and worldwide estimates of preterm birth rates in the year 2010 with time trends since 1990 for selected countries: a systematic analysis and implications. *Lancet*, 379(9832):2162–72.

[Bolton et al., 2015] Bolton, C. E., Bush, A., Hurst, J. R., Kotecha, S., and McGarvey, L. (2015). Lung consequences in adults born prematurely. *Thorax*, 70:574–580.

[Brunner et al., 1985] Brunner, J. X., Wolff, G., Cumming, G., and Langenstein, H. (1985). Accurate measurement of n2 volumes during n2 washout requires dynamic adjustment of delay time. *J Appl Physiol*, 59(3):1008–12.

[Burri, 2006] Burri, P. H. (2006). Structural aspects of postnatal lung development - alveolar formation and growth. *Biol Neonate*, 89(4):313–22.

[Bush, 2008] Bush, A. (2008). COPD: a pediatric disease. *COPD*, 5(1):53–67.

[Costeloe et al., 2012] Costeloe, K. L., Hennessy, E. M., Haider, S., Stacey, F., Marlow, N., and Draper, E. S. (2012). Short term outcomes after extreme preterm birth in england: comparison of two birth cohorts in 1995 and 2006 (the epicure studies). *BMJ*, 345:e7976.

[Criée et al., 2011] Criée, C. P., Sorichter, S., Smith, H. J., Kardos, P., Merget, R., Heise, D., Berdel, D., Köhler, D., Magnussen, H., Marek, W., Mitfessel, H., Rasche, K., Rolke, M., Worth, H., and Jörres, R. a. (2011). Body plethysmography–its principles and clinical use. *Respiratory medicine*, 105(7):959–71.

[Darling et al., 1940] Darling, R. C., Cournand, A., Mansfield, J. S., and Richards, D. W. (1940). Studies on the intrapulmonary mixture of gases. i. nitrogen elimination from blood and body tissues during high oxygen breathing. *J Clin Invest*, 19(4):591–7.

[Doyle et al., 2006] Doyle, L. W., Faber, B., Callanan, C., Freezer, N., Ford, G. W., and Davis, N. M. (2006). Bronchopulmonary dysplasia in very low birth weight subjects and lung function in late adolescence. *Pediatrics*, 118(1):108–13.

[Drysdale et al., 2011] Drysdale, S. B., Wilson, T., Alcazar, M., Broughton, S., Zuckerman, M., Smith, M., Rafferty, G. F., Johnston, S. L., and Greenough, A. (2011). Lung function prior to viral lower respiratory tract infections in prematurely born infants. *Thorax*, 66(6):468–473.

[Dweik et al., 2011] Dweik, R. A., Boggs, P. B., Erzurum, S. C., Irvin, C. G., Leigh, M. W., Lundberg, J. O., Olin, A. C., Plummer, A. L., and Taylor, D. R. (2011). An official ats clinical practice guideline: interpretation of exhaled nitric oxide levels (feno) for clinical applications. *Am J Respir Crit Care Med*, 184(5):602–15.

[Ehrenkranz et al., 2005] Ehrenkranz, R. A., Walsh, M. C., Vohr, B. R., Jobe, A. H., Wright, L. L., Fanaroff, A. A., Wrage, L. A., and Poole, K. (2005). Validation of the national institutes of health consensus definition of bronchopulmonary dysplasia. *Pediatrics*, 116(6):1353–60.

[Fawke et al., 2010] Fawke, J., Lum, S., Kirkby, J., Hennessy, E., Marlow, N., Rowell, V., Thomas, S., and Stocks, J. (2010). Lung function and respiratory symptoms at 11 years in children born extremely preterm: The EPICure study. *American Journal of Respiratory and Critical Care Medicine*, 182(2):237–245.

[Field et al., 2008] Field, D. J., Dorling, J. S., Manktelow, B. N., and Draper, E. S. (2008). Survival of extremely premature babies in a geographically defined population: prospective cohort study of 1994-9 compared with 2000-5. *BMJ*, 336(7655):1221–3.

[Filippone et al., 2003] Filippone, M., Sartor, M., Zacchello, F., and Baraldi, E. (2003). Flow limitation in infants with bronchopulmonary dysplasia and respiratory function at school age. *Lancet*, 361(9359):753–4.

[Fowler, 1948] Fowler, W. S. (1948). Lung function studies; the respiratory dead space. *Am J Physiol*, 154(3):405–16.

[Frey, 2001] Frey, U. (2001). Clinical applications of infant lung function testing : does it contribute to clinical decision making ? *Paediatric respiratory reviews*, 2:126–130.

[Frey et al., 2006] Frey, U., Maksym, G., Silverman, M., and Suki, B. (2006). New approaches to the understanding of complex chronic lung diseases. *European Respiratory Monograph*, (37):345–360.

[Frey et al., 2000a] Frey, U., Stocks, J., Coates, A., Sly, P., Bates, J., and ATS ERS Task Force, . (2000a). Specifications for equipment used for infant pulmonary function testing. *European Respiratory Journal*, 16:731–740.

[Frey et al., 2000b] Frey, U., Stocks, J., Sly, P., Bates, J., and Force, A. E. T. (2000b). Specifications for signal processing and data handling used for infant pulmonary function testing. *European Respiratory Journal*, 16:1016–1022.

[Frey and Suki, 2008] Frey, U. and Suki, B. (2008). Complexity of chronic asthma and chronic obstructive pulmonary disease : implications for risk assessment , and disease progression and control. *Lancet*, 372:1088 – 1099.

[Friedrich et al., 2006] Friedrich, L., Stein, R. T., Pitrez, P. M. C., Corso, A. L., and Jones, M. H. (2006). Reduced lung function in healthy preterm infants in the first months of life. *American journal of respiratory and critical care medicine*, 173(4):442–447.

[Fuchs et al., 2015] Fuchs, O., Gorlanova, O., Latzin, P., Schmidt, A., Schieck, M., Toncheva, A. A., Michel, S., Gaertner, V. D., Kabesch, M., and Frey, U. (2015). 6q12 and 11p14 variants are associated with postnatal exhaled nitric oxide and respiratory symptoms. *Journal of Allergy and Clinical Immunology*, submitted.

[Fuchs et al., 2012a] Fuchs, O., Latzin, P., Kuehni, C. E., and Frey, U. (2012a). Cohort profile: the Bern infant lung development cohort. *International journal of epidemiology*, 41(2):366–76.

[Fuchs et al., 2011a] Fuchs, O., Latzin, P., Thamrin, C., Stern, G., Frischknecht, P., Singer, F., Kieninger, E., Proietti, E., Riedel, T., and Frey, U. (2011a). Normative

data for lung function and exhaled nitric oxide in unsedated healthy infants. *The European respiratory journal*, 37(5):1208–16.

[Fuchs et al., 2011b] Fuchs, S., Buess, C., and Gappa, M. (2011b). In vitro validation of nitrogen multiple breath washout using ultrasonic equipment. *Eur Respir J*, 38: Suppl. 55:355s.

[Fuchs et al., 2006] Fuchs, S. I., Buess, C., Lum, S., Kozlowska, W., Stocks, J., and Gappa, M. (2006). Multiple breath washout with a sidestream ultrasonic flow sensor and mass spectrometry: a comparative study. *Pediatr Pulmonol*, 41(12):1218–25. Fuchs, Susanne I Buess, Christian Lum, Sooky Kozlowska, Wanda Stocks, Janet Gappa, Monika Pediatr Pulmonol. 2006 Dec;41(12):1218-25.

[Fuchs et al., 2009] Fuchs, S. I., Eder, J., Ellemunter, H., and Gappa, M. (2009). Lung clearance index: normal values, repeatability, and reproducibility in healthy children and adolescents. *Pediatr Pulmonol*, 44(12):1180–5.

[Fuchs et al., 2012b] Fuchs, S. I., Ellemunter, H., Eder, J., Mellies, U., Grosse-Onnebrink, J., Tummler, B., Staab, D., Jobst, A., Griese, M., Ripper, J., Rietschel, E., Zeidler, S., Ahrens, F., and Gappa, M. (2012b). Feasibility and variability of measuring the lung clearance index in a multi-center setting. *Pediatr Pulmonol*, 47(7):649–57.

[Gabriele et al., 2012] Gabriele, C., Jaddoe, V. W., van Mastrigt, E., Arends, L. R., Hofman, A., Moll, H. A., and de Jongste, J. C. (2012). Exhaled nitric oxide and the risk of wheezing in infancy: the generation r study. *Eur Respir J*, 39(3):567–72.

[Giffin et al., 1994] Giffin, F., Greenough, A., and Yuksel, B. (1994). Relationship between lung function results in the first year of life and respiratory morbidity in early childhood in patients born prematurely. *Pediatr Pulmonol*, 18(5):290–4.

[Gonem et al., 2012] Gonem, S., Singer, F., Siddiqui, S., and Gustafsson, P. (2012). Validation of a multiple breath inert gas washout technique using a lung phantom. *Eur Respir J*, 40: Suppl. 56:694s.

[Green et al., 1974] Green, M., Mead, J., and Turner, J. M. (1974). Variability of maximum expiratory flow-volume curves. *Journal of Applied Physiology*, 37(2).

[Greenough, 2013] Greenough, A. (2013). Long-term respiratory consequences of premature birth at less than 32 weeks of gestation. *Early human development*, 89 Suppl 2:S25–7.

[Greenough et al., 2000] Greenough, A., Dimitriou, G., Johnson, A. H., Calvert, S., Peacock, J., and Karani, J. (2000). The chest radiograph appearances of very premature infants at 36 weeks post-conceptional age. *Br J Radiol*, 73(868):366–9.

[Greenough et al., 1998] Greenough, A., Naik, S., Itakura, Y., Yuksel, B., Cheeseman, P., and Nicolaides, K. H. (1998). Perinatal lung function measurements and prediction of respiratory problems in infancy. *Physiol Meas*, 19(3):421–6.

[Gustafsson, 2005] Gustafsson, P. M. (2005). Inert gas washout in preschool children. *Paediatr Respir Rev*, 6(4):239–45. Gustafsson, Per M England Paediatr Respir Rev. 2005 Dec;6(4):239-45. Epub 2005 Nov 9.

[Gustafsson et al., 2008] Gustafsson, P. M., De Jong, P. A., Tiddens, H. A., and Lindblad, A. (2008). Multiple-breath inert gas washout and spirometry versus structural lung disease in cystic fibrosis. *Thorax*, 63(2):129–34.

[Hacking et al., 2013] Hacking, D. F., Gibson, A. M., Robertson, C., and Doyle, L. W. (2013). Respiratory function at age 8-9 after extremely low birthweight or preterm birth in victoria in 1997. *Pediatr Pulmonol*, 48(5):449–55.

[Henderson, 2014] Henderson, a. J. (2014). The child is father of the man: the importance of early life influences on lung development. *Thorax*.

[Herring et al., 2014] Herring, M. J., Putney, L. F., Wyatt, G., Finkbeiner, W. E., and Hyde, D. M. (2014). Growth of alveoli during postnatal development in humans based on stereological estimation. *Am J Physiol Lung Cell Mol Physiol*, 307(4):L338–44.

[Hibbert et al., 1990] Hibbert, M., Hudson, I., Lanigan, A., Landai, L., and Phelan, P. (1990). Tracking of Lung Function in Healthy Children and Adolescents. *Pediatric pulmonology*, 8:172–177.

[Hibbert et al., 1989] Hibbert, M., Lannigan, A., Landau, L., and Phelan, P. (1989). Lung Function Values From a Longitudinal Study of Healthy Children and Adolescents. *Pediatric Pulmonology*, 7:101–109.

[Hjalmarson and Sandberg, 2005] Hjalmarson, O. and Sandberg, K. L. (2005). Lung function at term reflects severity of bronchopulmonary dysplasia. *Journal of Pediatrics*, 146(1):86–90.

[Hoo et al., 2002a] Hoo, A. F., Dezateux, C., Hanrahan, J. P., Cole, T. J., Tepper, R. S., and Stocks, J. (2002a). Sex-specific prediction equations for vmax(frc) in infancy: a multicenter collaborative study. *Am J Respir Crit Care Med*, 165(8):1084–92.

[Hoo et al., 2002b] Hoo, A.-F., Dezateux, C., Henschen, M., Costeloe, K., and Stocks, J. (2002b). Development of airway function in infancy after preterm delivery. *The Journal of pediatrics*, 141(5):652–8.

[Hopper et al., 1991] Hopper, J. L., Hibbert, M. E., Macaskill, G. T., Phelan, P. D., and Landau, L. I. (1991). Longitudinal analysis of lung function growth in healthy children and adolescents. *Journal of applied physiology (Bethesda, Md. : 1985)*, 70(2):770–7.

[Horsley et al., 2008a] Horsley, a. R., Gustafsson, P. M., Macleod, K. a., Saunders, C., Greening, a. P., Porteous, D. J., Davies, J. C., Cunningham, S., Alton, E. W. F. W., and Innes, J. a. (2008a). Lung clearance index is a sensitive, repeatable and practical measure of airways disease in adults with cystic fibrosis. *Thorax*, 63(2):135–40.

[Horsley et al., 2008b] Horsley, A. R., Macleod, K. A., Robson, A. G., Lenney, J., Bell, N. J., Cunningham, S., Greening, A. P., Gustafsson, P. M., and Innes, J. A. (2008b). Effects of cystic fibrosis lung disease on gas mixing indices derived from alveolar slope analysis. *Respir Physiol Neurobiol*, 162(3):197–203.

[Hülskamp et al., 2009] Hülskamp, G., Lum, S., Stocks, J., Wade, A., Hoo, a. F., Costeloe, K., Hawdon, J., Deeptha, K., and Pillow, J. J. (2009). Association of prematurity, lung disease and body size with lung volume and ventilation inhomogeneity in unsedated neonates: a multicentre study. *Thorax*, 64(3):240–245.

[Islam et al., 2015] Islam, J. Y., Keller, R. L., Aschner, J. L., Hartert, T. V., and Moore, P. E. (2015). Understanding the short- and long-term respiratory outcomes of prematurity and bronchopulmonary dysplasia. *Am J Respir Crit Care Med*, 192(2):134–56.

[Jensen et al., 2013] Jensen, R., Stanojevic, S., Gibney, K., Salazar, J. G., Gustafsson, P., Subbarao, P., and Ratjen, F. (2013). Multiple breath nitrogen washout: a feasible alternative to mass spectrometry. *PloS one*, 8(2):e56868.

[Jobe, 2012] Jobe, A. H. (2012). What is bpd in 2012 and what will bpd become? *Early Hum Dev*, 88 Suppl 2:S27–8.

[Jobe and Bancalari, 2001] Jobe, A. H. and Bancalari, E. (2001). Bronchopulmonary Dysplasia. *Am J Resp Crit Care Med*, 163:1723–1729.

[Joshi et al., 2013] Joshi, S., Powell, T., Watkins, W. J., Drayton, M., Williams, E. M., and Kotecha, S. (2013). Exercise-induced bronchoconstriction in school-aged children who had chronic lung disease in infancy. *J Pediatr*, 162(4):813–818 e1.

[Kaplan et al., 2012] Kaplan, E., Bar-Yishay, E., Prais, D., Klinger, G., Mei-Zahav, M., Mussaffi, H., Steuer, G., Hananya, S., Matyashuk, Y., Gabarra, N., Sirota, L., and Blau, H. (2012). Encouraging pulmonary outcome for surviving, neurologically intact, extremely premature infants in the postsurfactant era. *Chest*, 142(3):725–33.

[Kavvadia et al., 2000] Kavvadia, V., Greenough, A., and Dimitriou, G. (2000). Early prediction of chronic oxygen dependency by lung function test results. *Pediatr Pulmonol*, 29(1):19–26.

[Kieninger et al., 2011] Kieninger, E., Singer, F., Fuchs, O., Abbas, C., Frey, U., Regamey, N., Casaulta, C., and Latzin, P. (2011). Long-term course of lung clearance index between infancy and school-age in cystic fibrosis subjects. *Journal of cystic fibrosis*, 10(6):487–90.

[Kinsella et al., 2006] Kinsella, J. P., Greenough, A., and Abman, S. H. (2006). Bronchopulmonary dysplasia. *Lancet*, 367:1421–1431.

[Kotecha et al., 2013] Kotecha, S. J., Edwards, M. O., Watkins, W. J., Henderson, a. J., Paranjothy, S., Dunstan, F. D., and Kotecha, S. (2013). Effect of preterm birth on later FEV1: a systematic review and meta-analysis. *Thorax*, 68(8):760–6.

[Kuehni et al., 2001] Kuehni, C. E., Davis, A., Brooke, A. M., and Silverman, M. (2001). Are all wheezing disorders in very young (preschool) children increasing in prevalence ? *Lancet*, 357:1821–1825.

[Lamarche-Vadel et al., 2004] Lamarche-Vadel, A., Blondel, B., Truffer, P., Burguet, A., Cambonie, G., Selton, D., Arnaud, C., Lardennois, C., du Mazaubrun, C., N'Guyen, S., Mathis, J., Breart, G., and Kaminski, M. (2004). Re-hospitalization in infants younger than 29 weeks' gestation in the epipage cohort. *Acta Paediatr*, 93(10):1340–5.

[Latzin et al., 2011] Latzin, P., Frey, U., Armann, J., Kieninger, E., Fuchs, O., Roosli, M., and Schaub, B. (2011). Exposure to moderate air pollution during late pregnancy and cord blood cytokine secretion in healthy neonates. *PLoS One*, 6(8):e23130.

[Latzin et al., 2007a] Latzin, P., Frey, U., Roiha, H. L., Baldwin, D. N., Regamey, N., Strippoli, M. P. F., Zwahlen, M., and Kuehni, C. E. (2007a). Prospectively assessed incidence, severity, and determinants of respiratory symptoms in the first year of life. *Pediatric pulmonology*, 42(1):41–50.

[Latzin et al., 2006] Latzin, P., Kuehni, C. E., Baldwin, D. N., Roiha, H. L., Casaulta, C., and Frey, U. (2006). Elevated exhaled nitric oxide in newborns of atopic mothers precedes respiratory symptoms. *American journal of respiratory and critical care medicine*, 174(12):1292–8.

[Latzin et al., 2009a] Latzin, P., Röösli, M., Huss, a., Kuehni, C. E., and Frey, U. (2009a). Air pollution during pregnancy and lung function in newborns: a birth cohort study. *The European respiratory journal*, 33(3):594–603.

[Latzin et al., 2009b] Latzin, P., Roth, S., Thamrin, C., Hutten, G. J., Pramana, I., Kuehni, C. E., Casaulta, C., Nelle, M., Riedel, T., and Frey, U. (2009b). Lung volume, breathing pattern and ventilation inhomogeneity in preterm and term infants. *PloS one*, 4(2):e4635.

[Latzin et al., 2007b] Latzin, P., Sauteur, L., Thamrin, C., Schibler, a., Baldwin, D., Hutten, G. J., Kyburz, M., Kraemer, R., Riedel, T., and Frey, U. (2007b). Optimized temperature and deadspace correction improve analysis of multiple breath washout measurements by ultrasonic flowmeter in infants. *Pediatric pulmonology*, 42(10):888–97.

[Lum et al., 2011] Lum, S., Kirkby, J., Welsh, L., Marlow, N., Hennessy, E., and Stocks, J. (2011). Nature and severity of lung function abnormalities in extremely pre-term children at 11 years of age. *European Respiratory Journal*, 37(5):1199–1207.

[Lum et al., 2013] Lum, S., Stocks, J., Stanojevic, S., Wade, A., Robinson, P., Gustafsson, P., Brown, M., Aurora, P., Subbarao, P., Hoo, A.-F., and Sonnappa, S. (2013). Age and height dependence of lung clearance index and functional residual capacity. *The European respiratory journal*, 41(6):1371–7.

[Martin and Fanaroff, 2013] Martin, R. J. and Fanaroff, A. a. (2013). The preterm lung and airway: Past, present, and future. *Pediatrics and Neonatology*, 54(4):228–234.

[Martin et al., 1988] Martin, T. R., Feldman, H. A., Fredberg, J. J., Castile, R. G., Mead, J., and Wohl, M. E. (1988). Relationship between maximal expiratory flows and lung volumes in growing humans. *J Appl Physiol (1985)*, 65(2):822–8.

[McEvoy et al., 2014] McEvoy, C. T., Jain, L., Schmidt, B., Abman, S., Bancalari, E., and Aschner, J. L. (2014). Bronchopulmonary dysplasia: NHLBI Workshop on the Primary Prevention of Chronic Lung Diseases. *Annals of the American Thoracic Society*, 11 Suppl 3(April):S146–53.

[Merkus et al., 1993] Merkus, P. J., Borsboom, G. J., Van Pelt, W., Schrader, P. C., Van Houwelingen, H. C., Kerrebijn, K. F., and Quanjer, P. H. (1993). Growth of airways and air spaces in teenagers is related to sex but not to symptoms. *J Appl Physiol (1985)*, 75(5):2045–53.

[Miller et al., 1995] Miller, J., Law, A. B., Parker, R. A., Sundell, H. W., Lindstrom, D. P., and Cotton, R. B. (1995). Validation of a nitrogen washout system to mea-

sure functional residual capacity in premature infants with hyaline membrane disease. *Pediatr Pulmonol*, 20(6):403–9.

[Miller et al., 2005] Miller, M. R., Hankinson, J., Brusasco, V., Burgos, F., Casaburi, R., Coates, a., Crapo, R., Enright, P., van der Grinten, C. P. M., Gustafsson, P., Jensen, R., Johnson, D. C., MacIntyre, N., McKay, R., Navajas, D., Pedersen, O. F., Pellegrino, R., Viegi, G., and Wanger, J. (2005). Standardisation of spirometry. *The European respiratory journal*, 26(2):319–38.

[Narang et al., 2008] Narang, I., Rosenthal, M., Cremonesini, D., Silverman, M., and Bush, A. (2008). Longitudinal evaluation of airway function 21 years after preterm birth. *Am J Respir Crit Care Med*, 178(1):74–80.

[Narayanan et al., 2013] Narayanan, M., Beardsmore, C. S., Owers-Bradley, J., Dogaru, C. M., Mada, M., Ball, I., Garipov, R. R., Kuehni, C. E., Spycher, B. D., and Silverman, M. (2013). Catch-up alveolarization in ex-preterm children: evidence from (3)he magnetic resonance. *Am J Respir Crit Care Med*, 187(10):1104–9.

[Narayanan et al., 2012] Narayanan, M., Owers-Bradley, J., Beardsmore, C. S., Mada, M., Ball, I., Garipov, R., Panesar, K. S., Kuehni, C. E., Spycher, B. D., Williams, S. E., and Silverman, M. (2012). Alveolarization continues during childhood and adolescence: new evidence from helium-3 magnetic resonance. *American journal of respiratory and critical care medicine*, 185(2):186–91.

[Neufeld et al., 1992] Neufeld, G. R., Schwardt, J. D., Gobran, S. R., Baumgardner, J. E., Schreiner, M. S., Aukburg, S. J., and Scherer, P. W. (1992). Modelling steady state pulmonary elimination of he, sf6 and co2: effect of morphometry. *Respir Physiol*, 88(3):257–75.

[Northway et al., 1967] Northway, W. H., J., Rosan, R. C., and Porter, D. Y. (1967). Pulmonary disease following respirator therapy of hyaline-membrane disease. bronchopulmonary dysplasia. *N Engl J Med*, 276(7):357–68.

[Owens et al., 2011] Owens, C. M., Aurora, P., Stanojevic, S., Bush, A., Wade, A., Oliver, C., Calder, A., Price, J., Carr, S. B., Shankar, A., and Stocks, J. (2011). Lung

clearance index and hrct are complementary markers of lung abnormalities in young children with cf. *Thorax*, 66(6):481–8.

[Palta et al., 2001] Palta, M., Sadek-Badawi, M., Sheehy, M., Albanese, A., Weinstein, M., McGuinness, G., and Peters, M. E. (2001). Respiratory symptoms at age 8 years in a cohort of very low birth weight children. *Am J Epidemiol*, 154(6):521–9.

[Peacock et al., 2013] Peacock, J. L., Lo, J. W., D'Costa, W., Calvert, S., Marlow, N., and Greenough, A. (2013). Respiratory morbidity at follow-up of small-for-gestational-age infants born very prematurely. *Pediatr Res*, 73(4 Pt 1):457–63.

[Pescatore et al., 2014] Pescatore, A. M., Dogaru, C. M., Duembgen, L., Silverman, M., Gaillard, E. a., Spycher, B. D., and Kuehni, C. E. (2014). A simple asthma prediction tool for preschool children with wheeze or cough. *The Journal of allergy and clinical immunology*, 133(1):111–8.e1–13.

[Pillow et al., 2004] Pillow, J. J., Ljungberg, H., Hulskamp, G., and Stocks, J. (2004). Functional residual capacity measurements in healthy infants: ultrasonic flow meter versus a mass spectrometer. *Eur Respir J*, 23(5):763–8.

[Postma et al., 2014] Postma, D. S., Bush, A., and van den Berge, M. (2014). Risk factors and early origins of chronic obstructive pulmonary disease. *Lancet*, 385(9971):899–909.

[Pramana et al., 2011] Pramana, I., Latzin, P., Schlapbach, L., Hafen, G., Kuehni, C. E., Nelle, M., Riedel, T., and Frey, U. (2011). Respiratory symptoms in preterms infants: Burden of disease in the first year of life. *Europena Journal of Medical Research*, 16:223–230.

[Proietti et al., 2014] Proietti, E., Riedel, T., Fuchs, O., Pramana, I., Singer, F., Schmidt, A., Kuehni, C., Latzin, P., and Frey, U. (2014). Can infant lung function predict respiratory morbidity during the first year of life in preterm infants? *The European respiratory journal*, 43(6):1642–51.

[Proquitte et al., 2006] Proquitte, H., Kusztrich, A., Auwarter, V., Pragst, F., Wauer, R. R., and Schmalisch, G. (2006). Functional residual capacity measurement by hep-

tafluoropropane in ventilated newborn lungs: in vitro and in vivo validation. *Crit Care Med*, 34(6):1789–95.

[Putnam et al., 2005] Putnam, R. W., Conrad, S. C., Gdovin, M. J., Erlichman, J. S., and Leiter, J. C. (2005). Neonatal maturation of the hypercapnic ventilatory response and central neural co2 chemosensitivity. *Respir Physiol Neurobiol*, 149(1-3):165–79.

[Quanjer et al., 2012] Quanjer, P. H., Stanojevic, S., Cole, T. J., Baur, X., Hall, G. L., Culver, B. H., Enright, P. L., Hankinson, J. L., Ip, M. S., Zheng, J., Stocks, J., and Initiative, E. R. S. G. L. F. (2012). Multi-ethnic reference values for spirometry for the 3-95-yr age range: the global lung function 2012 equations. *Eur Respir J*, 40(6):1324–43.

[Riedel et al., 2009] Riedel, T., Kyburz, M., Latzin, P., Thamrin, C., and Frey, U. (2009). Regional and overall ventilation inhomogeneities in preterm and term-born infants. *Intensive Care Medicine*, 35(1):144–151.

[Robinson et al., 2009] Robinson, P. D., Goldman, M. D., and Gustafsson, P. M. (2009). Inert gas washout: theoretical background and clinical utility in respiratory disease. *Respiration*, 78(3):339–55.

[Robinson et al., 2013] Robinson, P. D., Latzin, P., Verbanck, S., Hall, G. L., Horsley, A., Gappa, M., Thamrin, C., Arets, H. G., Aurora, P., Fuchs, S. I., King, G. G., Lum, S., Macleod, K., Paiva, M., Pillow, J. J., Ranganathan, S., Ratjen, F., Singer, F., Sonnappa, S., Stocks, J., Subbarao, P., Thompson, B. R., and Gustafsson, P. M. (2013). Consensus statement for inert gas washout measurement using multiple- and single- breath tests. *Eur Respir J*, 41(3):507–22.

[Roiha et al., 2007] Roiha, H. L., Kuehni, C. E., Zanolari, M., Zwahlen, M., Baldwin, D. N., Casaulta, C., Nelle, M., and Frey, U. (2007). Alterations of exhaled nitric oxide in pre-term infants with chronic lung disease. *The European respiratory journal*, 29(2):251–8.

[Rosenfeld et al., 2013] Rosenfeld, M., Allen, J., Arets, B. H. G. M., Aurora, P., Beydon, N., Calogero, C., Castile, R. G., Davis, S. D., Fuchs, S., Gappa, M., Gustaffson, P. M.,

Hall, G. L., Jones, M. H., Kirkby, J. C., Kraemer, R., Lombardi, E., Lum, S., Mayer, O. H., Merkus, P., Nielsen, K. G., Oliver, C., Oostveen, E., Ranganathan, S., Ren, C. L., Robinson, P. D., Seddon, P. C., Sly, P. D., Sockrider, M. M., Sonnappa, S., Stocks, J., Subbarao, P., Tepper, R. S., and Vilozni, D. (2013). An official American Thoracic Society workshop report: Optimal lung function tests for monitoring cystic fibrosis, bronchopulmonary dysplasia, and recurrent wheezing in children less than 6 years of age. *Annals of the American Thoracic Society*, 10(2):1–11.

[Rosenthal et al., 1993] Rosenthal, M., Bain, S., Cramer, D., Helms, P., Denison, D., Bush, A., and Warner, J. (1993). Lung function in white children aged 4 to 19 years: I-Spirometry. *Thorax*, 48:794–802.

[Scalfaro et al., 2000] Scalfaro, P., Cotting, J., and Sly, P. D. (2000). In vitro assessment of an ultrasonic flowmeter for use in ventilated infants. *Eur Respir J*, 15(3):566–9.

[Schibler et al., 2002] Schibler, a., Hall, G., Businger, F., Reinmann, B., Wildhaber, J., Cernelc, M., and Frey, U. (2002). Measurement of lung volume and ventilation distribution with an ultrasonic flow meter in healthy infants. *European Respiratory Journal*, 20(4):912–918.

[Schmalisch et al., 2012] Schmalisch, G., Wilitzki, S., Roehr, C. C., Proquitté, H., and Bührer, C. (2012). Development of lung function in very low birth weight infants with or without bronchopulmonary dysplasia: longitudinal assessment during the first 15 months of corrected age. *BMC pediatrics*, 12(1):37.

[Schmalisch et al., 2005] Schmalisch, G., Wilitzki, S., and Wauer, R. R. (2005). Differences in tidal breathing between infants with chronic lung diseases and healthy controls. *BMC pediatrics*, 5(Cld):36.

[Schmidt et al., 2015a] Schmidt, A., Proietti, E., Fuchs, O., Egger, B., Reppucci, D., Delgado-Eckert, E., Kuehni, C., Frey, U., and Latzin, P. (2015a). Early lung growth in term-born children: Weak tracking of end-expiratory lung volume from infancy to early childhood. ready for submission, see chapter 2.

[Schmidt et al., 2015b] Schmidt, A., Yammine, S., Proietti, E., Frey, U., Latzin, P., Riedel, T., and Singer, F. (2015b). Validation of multiple-breath washout equipment for infants and young children. *Pediatr Pulmonol*, 50(6):607–614.

[Schulzke and Frey, 2013] Schulzke, S. and Frey, U. (2013). Editorial: Consensus statement on inert gas washout measurement: at the threshold of clinical use. *Eur Respir J*, 41:500–502.

[Schulzke et al., 2010] Schulzke, S. M., Hall, G. L., Nathan, E. a., Simmer, K., Nolan, G., and Pillow, J. J. (2010). Lung Volume and Ventilation Inhomogeneity in Preterm Infants at 15-18 Months Corrected Age. *Journal of Pediatrics*, 156(4):542–549.e2.

[Schweizer Bundesamt für Statistik, 2015] Schweizer Bundesamt für Statistik, . (2015). Gesundheit der neugeborenen.

[Shi et al., 2009] Shi, W., Xu, J., and Warburton, D. (2009). Development, repair and fibrosis: what is common and why it matters. *Respirology*, 14(5):656–65.

[Silverman, 1995] Silverman, M. (1995). Asthma and wheezing in young children. *N Engl J Med*, 332(3):181–2.

[Silverman and Kuehni, 2007] Silverman, M. and Kuehni, C. E. (2007). Early lung development and copd. *Lancet*, 370(9589):717–9.

[Singer et al., 2012a] Singer, F., Houltz, B., Latzin, P., Robinson, P., and Gustafsson, P. (2012a). A realistic validation study of a new nitrogen multiple-breath washout system. *PLoS ONE*, 7(4):1–8.

[Singer et al., 2012b] Singer, F., Houltz, B., Robinson, P., Latzin, P., and Gustafsson, P. (2012b). Bench test of a mass spectrometer based multiple-breath washout system using a realistic lung model. *Eur Respir J*, 40: Suppl. 56:841s.

[Singer et al., 2013] Singer, F., Kieninger, E., Abbas, C., Yammine, S., Fuchs, O., Proietti, E., Regamey, N., Casaulta, C., Frey, U., and Latzin, P. (2013). Practicability of nitrogen multiple-breath washout measurements in a pediatric cystic fibrosis outpatient setting. *Pediatric Pulmonology*, 48(8):739–746.

[Singer et al., 2014a] Singer, F., Yammine, S., Schmidt, A., Proietti, E., Kieninger, E., Barben, J., Casaulta, C., Regamey, N., Gustafsson, P., Frey, U., and Latzin, P. (2014a). Ventilatory response to nitrogen multiple-breath washout in infants. *Pediatr Pulmonol*, 49(4):342–7.

[Singer et al., 2014b] Singer, F., Yammine, S., Schmidt, A., Proietti, E., Kieninger, E., Barben, J., Casaulta, C., Regamey, N., Gustafsson, P., Frey, U., and Latzin, P. (2014b). Ventilatory response to nitrogen multiple-breath washout in infants. *Pediatric pulmonology*, 49(4):342–7.

[Sonnenschein-van der Voort et al., 2014] Sonnenschein-van der Voort, A. M. M., Arends, L. R., de Jongste, J. C., Annesi-Maesano, I., Arshad, S. H., Barros, H., Basterrechea, M., Bisgaard, H., Chatzi, L., Corpeleijn, E., Correia, S., Craig, L. C., Devereux, G., Dogaru, C., Dostal, M., Duchen, K., Eggesbø, M., van der Ent, C. K., Fantini, M. P., Forastiere, F., Frey, U., Gehring, U., Gori, D., van der Gugten, A. C., Hanke, W., Henderson, a. J., Heude, B., Iñiguez, C., Inskip, H. M., Keil, T., Kelleher, C. C., Kogevinas, M., Kreiner-Mø ller, E., Kuehni, C. E., Küpers, L. K., Lancz, K., Larsen, P. S., Lau, S., Ludvigsson, J., Mommers, M., Nybo Andersen, A.-M., Palkovicova, L., Pike, K. C., Pizzi, C., Polanska, K., Porta, D., Richiardi, L., Roberts, G., Schmidt, A., Sram, R. J., Sunyer, J., Thijs, C., Torrent, M., Viljoen, K., Wijga, A. H., Vrijheid, M., Jaddoe, V. W. V., and Duijts, L. (2014). Preterm birth, infant weight gain, and childhood asthma risk: a meta-analysis of 147,000 European children. *The Journal of allergy and clinical immunology*, 133(5):1317–29.

[Stanojevic et al., 2015] Stanojevic, S., Jensen, R., Sundaralingam, D., Salazar, J. G., Yammine, S., Singer, F., Latzin, P., Amin, R., Subbarao, P., Gustafsson, P., and Ratjen, F. (2015). Alternative outcomes for the multiple breath washout in children with cf. *J Cyst Fibros*, 14(4):490–6.

[Stanojevic et al., 2009] Stanojevic, S., Wade, A., Cole, T. J., Lum, S., Custovic, A., Silverman, M., Hall, G. L., Welsh, L., Kirkby, J., Nystad, W., Badier, M., Davis, S., Turner, S., Piccioni, P., Vilozni, D., Eigen, H., Vlachos-Mayer, H., Zheng, J., Tomalak, W., Jones, M., Hankinson, J. L., and Stocks, J. (2009). Spirometry centile charts for

young Caucasian children: the Asthma UK Collaborative Initiative. *American journal of respiratory and critical care medicine*, 180(6):547–52.

[Stern et al., 2007] Stern, D. a., Morgan, W. J., Wright, A. L., Guerra, S., and Martinez, F. D. (2007). Poor airway function in early infancy and lung function by age 22 years: a non-selective longitudinal cohort study. *Lancet*, 370(9589):758–64.

[Stern et al., 2013] Stern, G., Latzin, P., Röösli, M., Fuchs, O., Proietti, E., Kuehni, C., and Frey, U. (2013). A prospective study of the impact of air pollution on respiratory symptoms and infections in infants. *American journal of respiratory and critical care medicine*, 187(12):1341–8.

[Stocks et al., 2013] Stocks, J., Hislop, A., and Sonnappa, S. (2013). Early lung development: lifelong effect on respiratory health and disease. *The Lancet. Respiratory medicine*, 1(9):728–42.

[Stocks and Quanjer, 1995] Stocks, J. and Quanjer, P. H. (1995). Reference values for residual volume, functional residual capacity and total lung capacity. ats workshop on lung volume measurements. official statement of the european respiratory society. *Eur Respir J*, 8(3):492–506.

[Stoll et al., 2010] Stoll, B. J., Hansen, N. I., Bell, E. F., Shankaran, S., Laptook, A. R., Walsh, M. C., Hale, E. C., Newman, N. S., Schibler, K., Carlo, W. A., Kennedy, K. A., Poindexter, B. B., Finer, N. N., Ehrenkranz, R. A., Duara, S., Sanchez, P. J., O'Shea, T. M., Goldberg, R. N., Van Meurs, K. P., Faix, R. G., Phelps, D. L., Frantz, I. D., r., Watterberg, K. L., Saha, S., Das, A., and Higgins, R. D. (2010). Neonatal outcomes of extremely preterm infants from the nichd neonatal research network. *Pediatrics*, 126(3):443–56.

[Strippoli et al., 2007] Strippoli, M. P., Silverman, M., Michel, G., and Kuehni, C. E. (2007). A parent-completed respiratory questionnaire for 1-year-old children: repeatability. *Arch Dis Child*, 92(10):861–5.

[Tai et al., 2014] Tai, A., Tran, H., Roberts, M., Clarke, N., Wilson, J., and Robertson, C. F. (2014). The association between childhood asthma and adult chronic obstructive

pulmonary disease. *Thorax*, 69(9):805–10.

[The international neonatal network, 1993] The international neonatal network, . (1993). The crib (clinical risk index for babies) score: a tool for assessing initial neonatal risk and comparing performance of neonatal intensive care units. the international neonatal network. *Lancet*, 342(8865):193–8.

[Thompson et al., 2014] Thompson, B. R., Hodgson, Y. M., Kotsimbos, T., Liakakos, P., Ellis, M. J., Snell, G. I., and Verbanck, S. (2014). Bronchiolitis obliterans syndrome leads to a functional deterioration of the acinus post lung transplant. *Thorax*, 69(5):487–8.

[Trachsel et al., 2012] Trachsel, D., Brutsche, M. H., Hug-Batschelet, H., and Hammer, J. (2012). Progressive static pulmonary hyperinflation in survivors of severe bronchopulmonary dysplasia by mid-adulthood. *Thorax*, 67(8):747–748.

[Turner et al., 2014] Turner, S., Fielding, S., Mullane, D., Cox, D., Goldblatt, J., Landau, L., and le Souef, P. (2014). A longitudinal study of lung function from 1 month to 18 years of age. *Thorax*, pages 1–6.

[Twisk et al., 1998] Twisk, J., Staal, B., Brinkman, M., Kemper, H., and van Mechelen, W. (1998). Tracking of lung function parameters and the longitudinal relationship with lifestyle. *European Respiratory Journal*, 12(3):627–634.

[van Putte-Katier et al., 2012] van Putte-Katier, N., van der Gugten, A. C., Uiterwaal, C. S., de Jong, B. M., Numans, M. E., Kimpen, J. L., Verheij, T. J., and van der Ent, C. K. (2012). Early life lung function and respiratory outcome in the first year of life. *Eur Respir J*, 40(1):198–205.

[Verbanck et al., 2003] Verbanck, S., Schuermans, D., Paiva, M., and Vincken, W. (2003). Nonreversible conductive airway ventilation heterogeneity in mild asthma. *J Appl Physiol (1985)*, 94(4):1380–6.

[Vermeulen et al., 2013] Vermeulen, F., Ophoff, J., Proesmans, M., and De Boeck, K. (2013). Comparison of lung clearance index measured during helium washin and washout in children with cystic fibrosis. *Pediatric Pulmonology*, 48(10):962–969.

[Vollsæ ter et al., 2013] Vollsæ ter, M., Rø ksund, O. D., Eide, G. E., Markestad, T., and Halvorsen, T. (2013). Lung function after preterm birth: development from mid-childhood to adulthood. *Thorax*, 68(8):767–76.

[von Mutius, 2001] von Mutius, E. (2001). Paediatric origins of adult lung disease. *Thorax*, 56(2):153–7.

[Vrijlandt and Duiverman, 2010] Vrijlandt, E. and Duiverman, E. (2010). *Pulmonary function testing in premature infants and infants with bronchopulmonary dysplasia*. in Paediatric Lung Function. Eur Respir Monogr, U. Frey and P. Merkus, Editors. 2010. p. 251-262.

[Vrijlandt et al., 2007] Vrijlandt, E. J., Boezen, H. M., Gerritsen, J., Stremmelaar, E. F., and Duiverman, E. J. (2007). Respiratory health in prematurely born preschool children with and without bronchopulmonary dysplasia. *J Pediatr*, 150(3):256–61.

[Vrijlandt et al., 2013] Vrijlandt, E. J., Kerstjens, J. M., Duiverman, E. J., Bos, A. F., and Reijneveld, S. A. (2013). Moderately preterm children have more respiratory problems during their first 5 years of life than children born full term. *Am J Respir Crit Care Med*, 187(11):1234–40.

[Wang et al., 2013] Wang, H., St. Julien, K. R., Stevenson, D. K., Hoffmann, T. J., Witte, J. S., Lazzeroni, L. C., Krasnow, M. a., Quaintance, C. C., Oehlert, J. W., Jelliffe-Pawlowski, L. L., Gould, J. B., Shaw, G. M., and O'Brodovich, H. M. (2013). A Genome-Wide Association Study (GWAS) for Bronchopulmonary Dysplasia. *Pediatrics*, 132(2):290–297.

[Wanger et al., 2005] Wanger, J., Clausen, J. L., Coates, A., Pedersen, O. F., Brusasco, V., Burgos, F., Casaburi, R., Crapo, R., Enright, P., van der Grinten, C. P., Gustafsson, P., Hankinson, J., Jensen, R., Johnson, D., Macintyre, N., McKay, R., Miller, M. R., Navajas, D., Pellegrino, R., and Viegi, G. (2005). Standardisation of the measurement of lung volumes. *Eur Respir J*, 26(3):511–22.

[Wauer et al., 2003] Wauer, J., Leier, T. U., Henschen, M., Wauer, R. R., and Schmalisch, G. (2003). In vitro validation of an ultrasonic flowmeter in order to

measure the functional residual capacity in newborns. *Physiol Meas*, 24(2):355–65.

[Weibel, 2013] Weibel, E. R. (2013). It takes more than cells to make a good lung. *American journal of respiratory and critical care medicine*, 187(4):342–6.

[World Health Organization, 2006] World Health Organization, . (2006). Who child growth standards: Length/height-for-age, weight-for-age, weight-for-length, weight-for-height and body mass index-for-age: Methods and development.

[World Health Organization, 2015] World Health Organization, . (2015). World health statistics 2015.

[Yammine et al., 2015] Yammine, S., Schmidt, A., Sutter, O., Fouzas, S., F., S., Frey, U., and Latzin, P. (2015). Functional evidence for continued alveolarization in former preterm children at school age? *Eur Respir J*, doi: 10.1183/13993003.00478-2015.

[Yammine et al., 2013] Yammine, S., Singer, F., Abbas, C., Roos, M., and Latzin, P. (2013). Multiple-breath washout measurements can be significantly shortened in children. *Thorax*, 68(6):586–587.

9 Appendix

Joining an internationally connected research group with broad expertise allowed me to contribute to several studies and collaborations in addition to the focus of my thesis. I worked in an interdisciplinary team, and this broadened my scientific experience. We shared our experiences and knowledge in frequent team meetings and ensured maintenance of the data set by mutual support. We constantly challenged our MBW protocols and equipment and were able to improve those even further. Our collaborations gave me insights into the work in a Genetic laboratory, and our contribution to meta analyses showed me the potential of a well-coordinated, collective effort.
We investigated the associations of genetic variants with inflammatory markers in term-born infants, tested improved protocols for measurement and analysis of infant lung function, and contributed to two European meta analyses on asthma development. The manuscripts and abstracts are shown in this appendix.

9.1 Additional manuscript
6q12 and 11p14 variants are associated with postnatal exhaled nitric oxide and respiratory symptoms

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6q12 and 11p14 variants are associated with postnatal exhaled nitric oxide and respiratory symptoms

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Running Head

Genetic variants and exhaled nitric oxide in infants

Key Words

Infants, exhaled nitric oxide, genetics, airway inflammation, genome-wide association study, wheeze, asthma

Article word count: 3,677/3,500 excl. abstract, abbreviations and acknowledgments

Key messages

- Genetic determinants at 6q12 and 11p14 are associated with higher postnatal eNO levels in healthy term-born infants while there were no associations on a genome-wide level with known genetic determinants of eNO in later life.
- These genetic determinants are subsequently associated with less and less extensive respiratory symptoms during the first year of life in our study population.
- Our findings imply potential differences in the relation between eNO metabolism and lung disease in early infancy in comparison to later childhood.

Capsule Summary

The identification of novel genetic determinants of infant eNO, at previously unknown loci 6q12 and 11p14, may implicate that postnatal eNO metabolism in healthy infants prior to first viral infections and sensitization is related to mechanisms other than those associated with asthma, atopy or increased risk thereof later in life.

DEFINITION OF ABBREVIATIONS

ANO3	Human anoctamin 3 gene locus
BILD	Basel/Bern Infant Lung Development (Cohort)
bp	base pair
BPD	Bronchopulmonary dysplasia
cM	CentiMorgan
CEU	Utah residents with ancestry from northern and western Europe
CTCF	11-zinc finger protein or CCCTC- binding factor
DENND1B	DENN/MADD domain containing 1B
ECR	Evolutionary conserved genomic region
EGF(R)	Epithelial growth factor (receptor)
(e)NO	(Exhaled) nitric oxide
ETS	Environmental tobacco smoke
EYS	Human eyes shut homolog gene locus
GxE	Gene-environment interaction
GxG	Gene-gene interaction
GSDMB	Human gasdermin B gene locus
GWAS	Genome-wide association study
HWE	Hardy-Weinberg equilibrium
IBD	Identity-by-descent
IRR	Incidence risk ratio
LD	Linkage disequilibrium
LUZP2	Human leucine zipper protein 2 gene locus
LYRM9	Human LYR motif containing 9 gene locus

MAF	Minor allele frequency
Mb	Megabase
MUC15	Human mucin 15 gene locus
NOS	Human nitric oxide synthase gene locus
OLR	Online repository
OR	Odds ratio
PC(A)	Principal component (analysis)
p_{int}	Interaction p-value
QC	Quality control
QQ	Quantile-quantile plot
QTL	Quantitative trait locus
rs	Reference SNP cluster code (dbSNP)
SD	Standard deviation
SNP	Single-nucleotide polymorphism
TFBS	Transcription factors binding sites
UCB	Umbilical cord blood
VEGF	Vascular endothelial growth factor
V'E	Minute ventilation
V'NO	Nitric oxide output
V_T	Tidal volume
95% CI	95% confidence interval

ABSTRACT

Background: Exhaled nitric oxide (eNO) is a biomarker of airway inflammation and seems to precede respiratory symptoms, such as asthma in childhood. Identifying genetic determinants of postnatal eNO may aid in unraveling the role of eNO in epithelial function or airway inflammation and disease.

Objective: To identify genetic determinants of early postnatal eNO and subsequent respiratory symptoms during the first year of life.

Methods: Within a population-based birth cohort, eNO was measured in healthy term infants aged 5 weeks during quiet tidal breathing in unsedated sleep. We assessed associations of single-nucleotide polymorphisms with eNO in a genome-wide association study, and subsequent symptoms of lower respiratory tract infections during the first year of life; and asked if this was modified by prenatal and early-life environmental factors.

Results: We identified so far unknown determinants of infant eNO: rs208515 ($p=3.3 \times 10^{-8}$) located at 6q12, explaining 10.3% of eNO variance, and furthermore rs1441519 ($p=1.6 \times 10^{-6}$) at 11p14. The 6q12 locus was inversely associated with subsequent respiratory symptoms ($p<0.05$) and time to recovery after first respiratory symptoms during the first year of life ($p<0.05$).

Conclusion: The identification of novel genetic determinants of infant eNO may implicate that postnatal eNO metabolism in healthy infants prior to first viral infections and sensitization is related to mechanisms other than those associated with asthma, atopy or increased risk thereof later in life.

INTRODUCTION

Exhaled nitric oxide (eNO) is an important biomarker of airway inflammation. Low levels of outdoor air pollution and environmental tobacco smoke (ETS) affect eNO levels in newborns.(1, 2) Moreover, eNO seems to also be an early marker for future respiratory morbidity in infants(3) and to precede transient early but not persistent wheeze in infants,(4) and may thus help to differentiate early forms of infant wheeze, at least in individuals at risk. Later, eNO serves as an established indicator for allergic airway inflammation, aiding in discriminating childhood wheeze and asthma phenotypes(5-7) and in identifying exacerbation risk or asthma control.(8, 9)

Interestingly, respiratory epithelial-derived NO is also important for cell signaling. There is an emerging role of NO in airway development and pulmonary angiogenesis both in rodent models(10, 11) and for premature children at risk for bronchopulmonary dysplasia (BPD).(12, 13) In the context of lung epithelial-mesenchymal crosstalk,(14) NO has been shown to modulate recovery from lung injury and to attenuate transition of epithelial cells to myofibroblasts.(15) In rodents, this has important implications for epithelial function, structural maintenance, and lung remodelling in the postnatal phase.(16) In humans however, the role of postnatal eNO as a marker of epithelial function or for future respiratory morbidity irrespectively of underlying risk remains unclear. Thus, before it may be used as a marker for "FeNOtyping",(6) it is important to better understand putative genetic determinants of postnatal eNO.

Several lung cell types are able to produce NO from L-arginine through the action of nitric oxide synthase isoforms (NOSs).(17, 18) Previous work in infants at risk of atopy(19) and following a candidate-gene approach in older children(20-23) has suggested that variants in genes such as *DENN/MADD domain containing 1B* (*DENND1B*) and *NOS2* may affect eNO levels. Only recently, a large genome-wide association study (GWAS) in individuals not at increased risk for atopy or asthma identified the loci *LYR motif containing 9* (*LYRM9*), *NOS2*,

and one near *gasdermin B* (*GSDMB*) at 17q21 to be associated with eNO.(24) All these analyses have been performed in older children where eNO levels may also be affected by a number of other factors, e.g. concomitant disease and environmental effects. Here, we present a GWAS in a unique, unselected population-based cohort of term-born infants not at increased risk of atopy or asthma, the Basel/Bern Infant Lung Development (BILD) cohort.(25) There, eNO levels were measured shortly after birth in order to determine early genetic effects which may influence eNO levels and may precede disease development from early on.

METHODS

A more detailed description of the study population and methods is provided in the online repository (OLR).

Study population, study participants

All data were collected in the ongoing prospective BILD birth cohort of unselected, healthy neonates.(25) The study was approved by all involved Ethics Committees. All caregivers provided written informed consent for this study.

Measurements and quality control of tidal breathing and nitric oxide

Tidal breathing and mixed eNO were measured at five weeks of age as described previously.(25, 26) Outcome parameters were tidal volume (V_T), minute ventilation ($V'E=V_T$ x respiratory rate), eNO, and NO output ($V'NO$=eNO concentration x expiratory flow).(26)

Measurement of respiratory symptoms and time to recovery from first respiratory infection during the first year of life

Respiratory symptom data was collected by weekly telephone interviews.(25) Respiratory symptoms were defined as 'cough, wheezing, difficulty breathing and reduced activity during night and day'. Weeks with symptoms were defined as total number of weeks with respiratory symptoms. Time to recovery was defined as total number of weeks that the infant suffered from first respiratory symptoms during the first 6 and 12 months of life.

Genotyping, genetic data quality control

Whole-genome genotyping (Illumina HumanOmniExpress Bead Chips, Illumina Inc., San Diego, USA) was performed in two separate batches in all n=425 study participants (in 2011 for n=329 and in 2013 for additional n=96 children).We checked for a batch effect due to two genotyping time-points. See Figure 1 for details on post-hoc exclusion of individuals and

quality control (QC) of eNO measurements and genotyping data. Principal component analysis (PCA) was performed in order to obtain eigenvectors as covariates for later association tests. The first principal component (PC1) was subsequently included for adjustment.

Statistical analyses and association testing

Analyses were restricted to additive models. No transformation was necessary for eNO or V'NO values. Associations between SNPs and eNO or V'NO were analyzed by adjusted linear regression. The Bonferroni method was chosen to control for multiple testing.

Associations between SNPs and number of weeks with respiratory symptoms and time to recovery were analyzed by adjusted Poisson and logistic regression. For the latter, a binary variable (resolution of symptoms < 2 week or ≥ 2 weeks) was defined. Results are expressed as incidence risk ratio (IRR) and odds ratio (OR), respectively. Gene-gene (GxG) and gene-environment interaction (GxE) were assessed by ANOVA. The corresponding p-value was labeled p_{int}. A p-value <0.05 was considered significant.

Association computations were done in R version 3.0.2 (www.r-project.org) (27) using the GenABEL package (28) and Stata 12.1 (STATA Corporation, College Station, TX, USA) both final subset of all n=229 individuals that were left for subsequent analyses as well as for each genotyping batch from either 2011 or 2013, separately. Regional plots were generated by LocusZoom (http://csg.sph.umich.edu/locuszoom). Permutation analyses were performed by chromosome using PLINK version 1.07.(29) Linkage disequilibrium (LD) was evaluated by PLINK version 1.07(29) based on 1000 Genomes Project (CEU population) data.(30) Regional LD plots were generated in R 3.0.0. Base pair (bp) positions correspond to genome assembly GRCh37.p13 and gene annotations are according to the NCBI RefSeqGene Project (accessed March 24th 2015).

Post-hoc power estimation

Post-hoc statistical power was estimated with the Genetic Power Calculator (31). With a sample size of n=229, there was more than 90% power to detect a quantitative trait locus (QTL) explaining 10.3% of the trait (eNO) variance together with accurate eNO measurements shortly after birth and therefore little environmental influence thereon.

In-silico functional analyses

Several databases (see OLR) were used to check the relationship of target polymorphisms with published data on expression (eQTL) and methylation QTLs (mQTL). ECR Browser (http://ecrbrowser.dcode.org/) was used to check whether the target polymorphisms are located in evolutionary conserved genomic regions (ECRs). CCCTC- binding factor (CTCF) binding sites and chromatin topological domains were determined using data from CTCFBSDB 2.0 - (http://insulatordb.uthsc.edu/).(32)

RESULTS

Between 1999 and 2013, the study enrolled 425 eligible infants, of whom n=353 presented for lung function measurements at the age of 5 weeks after gestation. After secondary exclusion, technically acceptable data were obtained from n=344 infants. As indicated in Figure 1, n=229 individuals with data for n=567,864 SNPs were then subjected to analyses. Table 1 presents anthropometric and demographic characteristics and distribution of known and possible confounders among all study participants for whom we had complete datasets on eNO measurements and from genotyping after aforementioned QC. Values for eNO and V'NO were highly correlated ($r=0.71$, $p<0.0001$). We did not find any differences in baseline characteristics between infants for whom data were in- or generally or specifically excluded from subsequent analyses (data not shown).

Calculated genomic inflation factors for eNO and V'NO were below 1.006, showing no evidence for population stratification. While we did not find any association of *NOS* gene loci with our outcomes, *LYRM9* and *GSDMB* loci were related to eNO and V'NO with the same effect direction as previously published(24) however not reaching genome-wide significance ($p=0.082$ and $p=0.034$, respectively). After adjustment for confounders and correction for multiple testing, we identified polymorphisms significantly associated with eNO and V'NO. Two SNPs on 6q12 (reference SNP (rs) cluster codes rs208515 and rs208520), were in high LD ($r^2>0.8$) and significantly associated with eNO and V'NO, respectively. The T-allele (risk allele) of rs208515 and the G-allele (risk allele) of rs208520 were associated with increased eNO values (explained variance 10,3% and 10,2%, respectively) as well as increased V'NO values (explained variance 9,9% and 9,9%, respectively). Two variants on 11p14 (rs12223678 and rs1441519) were also in high LD ($r^2>0.8$) and were indicative for associations with eNO and V'NO. Here, the T-alleles (risk alleles) of rs1441519 and rs12223678 were associated with lower eNO values (explained variance 7,9% and 7,3%, respectively) as well as with lower V'NO values (explained variance 9,0% and 8,7%, respectively). Detailed association

data for the total final subset of n=229 individuals are provided in OLR Tables E1A and B, and summary statistics of associated SNPs are listed in OLR Table E2. Figures E1A and B in the OLR display quantile-quantile (QQ) plots and Figures 2A and B Manhattan plots for eNO and V'NO. Figures E2A and B in the OLR as well as OLR Figures E3A and B display regional plots for eNO and V'NO, respectively. The above mentioned top SNPs were also robust with regard to effect direction and of comparable effect size albeit not significant for the smaller batch from 2013 when analyses were performed for each genotyping batch from 2011 or 2013, separately (see OLR Tables E3A and B for associations with eNO). Moreover, they remained also significantly associated with measured outcomes when we performed additional permutation analyses. For the 6q12 top SNPs this was also the case after accounting for multiple testing within the permutation analysis, while for 11p14 hitsa trend was observed (see OLR Table E4 for eNO).

In a previous study, no association was found between postnatal eNO levels and respiratory symptoms during the first year of life for the entire study population but for the subgroup of mothers smoking during pregnancy or who were atopic.(3) We therefore investigated wether the identified genetic determinants were also relevant for n=209/229 children with respiratory symptoms during the first year of life. Table E5A in the OLR displays details on duration of respiratory symptoms in weeks for the study population and across group levels. The T-allele of rs208515 (6q12) was negatively associated with the number of weeks with respiratory symptoms with an IRR of 0.80 (95%CI 0.68-0.95, p=0.009, see Figure 3). This was independent of symptom severity (data not shown) and remained significant also after adjusting for confounders (sex, having older siblings, nursery care, maternal atopy, maternal smoking during pregnancy, V'E, and eNO) with an adjusted IRR (aIRR) of 0.81 (95%CI 0.69-0.95, p=0.011, see OLR Table E5B). The same association with comparable effect size and direction was found for rs208520. In contrast, we did not find any significant associations between 11p14 variants and the number of weeks with respiratory symptoms (see OLR Table

E5B for adjusted and Figure 4 for unadjusted results). Stratifying for maternal atopy or maternal smoking during pregnancy did neither modify the association between SNPs and eNO or V'NO, nor between SNPs and subsequent symptoms for either locus. Furthermore, association of SNPs with assessed outcomes was not dependent of each other, i.e. there was no GxG for variants at 6q12 or 11p14 identified to be associated with eNO and V'NO in infants (data not shown).

Lastly, we analyzed wether the effect of identified genetic determinants was also relevant for the time to recovery from first respiratory infections. There was no effect of genetic determinants on the duration of first respiratory infection in the study population with symptoms across the first 12 months of life (n=207) in either univariable or adjusted models (data not shown). Constricting our analyses to children with symptoms during the first 6 months of life (n=130) however, rs208515 or rs208520 were significantly associated with faster recovery (i.e. less than two weeks) in both univariable models (data not shown) and models adjusted for known and possible confounders (sex, age, having older siblings, nursery care, maternal atopy, maternal smoking during pregnancy, season, being breast-fed at time of first respiratory symptoms, V'E, and eNO) with an aOR=0.34, p=0.008 and an aOR of 0.24, p=0.002, respectively.

As association signals on chromosome 6q12 (rs208515 and rs208520) and chromosome 11p14 (rs12223678 and rs1441519) are located in non-coding regions, we applied *in-silico* and database analyses to search for putative causal SNPs in the respective regions driving the observed signals. The closest RefSeq genes identified on 6q12 were *SLC25A51P1*, *EYS* and *LOC441155* located upstream of rs208515 (446kb, 529kb and 930kb distance respectively), whereas no gene was listed within 1,000kb downstream of rs208515 (see Figure 3A). On 11p14, *LUZP2* was the closest gene upstream of rs12223678 (812kb distance). The closest downstream genes of rs1441519 were *ANO3*, *MUC15* and *SLC5A12* (424kb, 651kb and 759kb distance, respectively, see Figure 3B). Linkage disequilibrium analyses in at least

1,000kb surrounding the associated polymorphisms on 6q12 identified an LD $r^2 \geq 0.8$ for 177 SNPs for rs208515 and 88 SNPs for rs208520. Due to r^2 of 0.82 between rs208515 and rs208520, an overlap in tagged SNPs exists, resulting in 201 unique SNPs in the 6q12 tagging block (OLR Tables 6A and B). None of the tagged SNPs is located in close vicinity to known genes in the 6q12 locus (closest SNP: rs1563929 located 203kb from *SLC25A51P1*). Analysis for 11p14 identified an LD of r^2 0.96 between rs12223678 and rs1441519. Both SNPs are tagging for the identical set of SNPs at $r^2 \geq 0.8$. (n=13 SNPs; OLR Tables 6C and D). None of these 13 SNPs is in close vicinity to genes on the 11p14 locus (closest SNPs: rs11028996 with 802kb distance to upstream *LUZP2* and rs1348169 with 424kb distance to downstream *ANO3*).

All identified SNPs (n=214) were subjected to further evaluation, whether they locate within regulatory sequences such as enhancers/ super-enhancers and insulators, transcription factors binding sites (TFBS) or evolutionary conserved regions (ECRs) determined in the respective regions as depicted in Figure 5A and B. Indeed, we identified 5 associated SNPs on chromosome 6q12 in binding sites for the CCCTC-binding factor (CTCF), which is specifically allocated to insulator regions in humans and was shown to play an important developmental role (OLR Tables E7A and B).(33-35) Three variants (rs9453663, rs9453664, rs12200490) belong to a sequence that allows cell type-specific binding of CTCF in lung fibroblasts. Polymorphisms rs3899423 and rs4618506 locate in regions where CTCF binds in embryonic stem cells and lymphoblastoid cells, respectively. *In-silico* predictions suggested that all 13 SNPs at 11p14 are located in an area that is part of two cell type-specific (human embryonic stem cells and fetal lung myofibroblasts, IMR-90) chromatin structures called topologically associating domains.

DISCUSSION

In this unselected birth cohort,(25) we found two new loci, 6q12 and 11p14, to be associated with NO levels, measured in healthy term-born infants shortly after birth. The 6q12 locus explains a significant proportion of variance at a reasonably high significance level. Furthermore, this locus is also inversely related with the number of weeks with respiratory symptoms and time to recovery after first respiratory symptoms during the first year of life and may thus be of functional relevance.

The most significantly associated SNPs (rs208515 and rs208520) are located at 6q12 downstream the *EYS* gene locus, a member of the human epithelial growth factor (EGF) homolog family. EGF and homologs regulate several signal cascades(36, 37) that participate in functions such as proliferation, differentiation, mitosis, cell survival, and apoptosis on the transcription level.(36) Additional downstream events encompass glucose metabolism and cell migration.(37) The second identified locus at 11p14 (rs12223678 and rs1441519) is situated in vicinity to the human *ANO3/MUC15* gene locus. While *ANO3* belongs to a gene family encoding calcium-activated chloride channels,(38) the MUC15 protein belongs to the mucin family, representing glycoproteins important as mucus constituents as part of the physical barrier of the airway epithelium.(38) Remarkably, it also shares structural motifs with the EGF receptor (EGFR) and has been shown to modulate EGFR-signaling after EGF stimulation.(39)

However, our long-distance LD analyses revealed that no polymorphisms in LD with eNO-associated SNPs are located in close vicinity to so far identified genes in the respective regions. Therefore, a direct impact of eNO-associated polymorphisms on gene function (e.g. amino acid sequence, splicing, promoter activity) in either of the two chromosomal loci could not be established. Unfortunately, existing eQLT datasets are of no use to study gene expression effects in early childhood and thus, further targeted experiments will be needed. Interestingly, the extensive intergenic regions, where identified SNPs are located, are

predicted to harbor regulatory regions. Five of the variants from associated tagging bins at 6q12 are placed in insulator sequences, defined as binding sites for the major human insulator protein CTCF. ChipSeq database derived data revealed that CTCF binds in a cell type-specific manner, and more importantly, in cell types involved in embryotic development and potentially relevant to the course of airway inflammation (lung fibroblasts and lymphoblastoid cells).(33-35) Prediction analysis for the 11p14 variants indicates cell type-specific CTCF-driven formation of topologically associating domains in embryonic stem cells and in fetal lung fibroblasts, potentially orchestrating 11p14 chromatin rearrangements and affecting genes involved in lung tissue development. Thus, allele-specific effects on chromatin insulator function in both 6q12 and 11p14 regions might contribute to the association signals that were observed.

Chromatin insulators regulate the effects of enhancers and silencers on a gene promoter(40, 41) and thus they might induce or suppress transcription activity. Moreover, insulator activity is mainly driven by DNA conformation and the global chromatin architecture mediated by CTCF,(33) which has been identified as a major regulator in the genome.(33, 34) CTCF establishes boundaries between topologically associating domains in a cell type-specific and developmental fashion.(34, 42) It is possible that 6q12 and 11p14 polymorphisms influence CTCF binding and activity and thus gene expression in larger areas in both regions. Recently it was reported that CTCF might play a role in lung diseases such as cystic fibrosis(43, 44) by modulating *CFTR* expression through insulator activity in the *CFTR* locus.(45) Whether and how 6q12 and 11p14 polymorphisms affect CFTR binding, insulator activity and gene expression in the region, remains to be shown.

Importantly, we measured mixed eNO in our study, thus containing eNO of nasal and bronchial origin. There is no data supporting that there is a difference between strictly oral or nasal NO (nNO) in term infants.(26, 46) However, such differences could be demonstrated for premature infants very early in life and before any possible significant bacterial colonization

and pneumatisation of paranasal sinuses, with much higher levels for nNO, predominantly under iNOS influence,(17) than for bronchial eNO.(47, 48) The link to the already mentioned role of NO metabolism in cell signaling, VEGF-mediated airway development,(10-13) lung epithelial-mesenchymal crosstalk,(14) and epithelial function is striking. Similarly to a possible role for endogenous NO in premature lungs, one could speculate that in our study, given that these are healthy term infants for whom there is no data on a relevant share of nNO, elevated eNO as an expression of elevated bronchial epithelial-derived NO might confer a counter regulatory effect. This might happen in the context of EGF metabolism and epithelial function independently from underlying risk factors for subsequent respiratory morbidity. These are functional implications and have to be taken with caution, as we are currently lacking clear functional outcomes such as measurements of gene expression in our study. Moreover, further genes adjacent to the most significant SNPs at 6q12 and 11p14 may importantly also play their roles.

Remarkably, we neither found associations with known genetic determinants of eNO levels in children at risk of developing atopy(19) nor in older children or adults on a genome-wide level.(20, 21, 23, 24, 49) Interestingly, the *ANO3/MUC15* locus at 11p14 has recently been related to atopic eczema in adults ascertained through asthma(38) but the genetic signal in that study is independent from the signal found in our study. Earlier in this study, the role of infant eNO as a marker for later respiratory symptoms was evident for the subgroup of smoking or atopic mothers.(3) This was confirmed by others.(4) We investigated the potential discrepancy to current findings and found several differences. The population included by *Latzin et al.* was smaller and had higher exposure to ETS (14.0% in (3) vs. 8.7%) or air pollution.(3) Furthermore, siblings were strictly excluded in the current study. We reproduced the former findings for the smaller population used by *Latzin et al.*(3) We speculate, that discrepancies could be due to sample size differences or differences in environmental exposure.

This study is to the best of our knowledge the first effort so far to uncover genetic determinants of eNO levels, which were collected in healthy term infants shortly after birth(25) according to latest standards and already published as part of a set of normative data, including extensive QC during data collection, and subsequent analyses.(26) Despite being the first effort so far, we are limited by the number of healthy subjects very early in life included to generate sufficient statistical power to detect all variants with at least modest effects and especially with regard to possible replication in independent populations. To our knowledge no such cohort exists, and a decade was needed to build up the presented study. Still, the analyses within the two different batches may nevertheless serve as discovery and replication samples. Analysing both batches seperately, they display the same effects, with same effect direction and comparable effect sizes while showing different levels of significance given their unequal numbers of individuals. Moreover, with this GWAS we were able to reach genome-wide significant levels with a significant effect size even with the most conservative method to adjust for multiple testing and with significant results for the identified top SNPs also after permutation. Still, additional larger and especially longitudinal studies are needed to characterize functional implications, as well as further loci with only modest effects that remain to be identified for eNO values during later life.

Conclusion

We identified novel genetic determinants of postnatal eNO in healthy infants not at increased risk for atopy or asthma. One identified locus is 6q12, for which rs208515 explained up to 13% of outcome variance with a high level of significance. In addition, suggestive association was detected for SNPs at chromosome 11p14. Given the association with respiratory symptoms, these results may provide new insight into possible biological mechanisms underlying eNO levels, their regulation, and their functional relevance within the spectrum of respiratory disease especially in young children. The identification of these variants may

implicate that postnatal eNO metabolism in healthy infants not at increased risk for atopy is rather related to epithelial function prior to first viral infections and postnatal sensitization.

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REFERENCES

1. Frey U, Kuehni C, Roiha H, Cernelc M, Reinmann B, Wildhaber JH, et al. Maternal atopic disease modifies effects of prenatal risk factors on exhaled nitric oxide in infants. Am J Respir Crit Care Med. 2004;170(3):260-5.
2. Gabriele C, Asgarali R, Jaddoe VW, Hofman A, Moll HA, de Jongste JC. Smoke exposure, airway symptoms and exhaled nitric oxide in infants: the Generation R study. Eur Respir J. 2008;32(2):307-13.
3. Latzin P, Kuehni CE, Baldwin DN, Roiha HL, Casaulta C, Frey U. Elevated exhaled nitric oxide in newborns of atopic mothers precedes respiratory symptoms. Am J Respir Crit Care Med. 2006;174(12):1292-8.
4. Chawes BL, Buchvald F, Bischoff AL, Loland L, Hermansen M, Halkjaer LB, et al. Elevated exhaled nitric oxide in high-risk neonates precedes transient early but not persistent wheeze. Am J Respir Crit Care Med. 2010;182(2):138-42.
5. Pijnenburg MW, De Jongste JC. Exhaled nitric oxide in childhood asthma: a review. Clin Exp Allergy. 2008;38(2):246-59.
6. de Jongste JC. To wheeze or not to wheeze: prospective FENO-typing in early infancy. Am J Respir Crit Care Med. 2006;174(12):1281-2.
7. Singer F, Luchsinger I, Inci D, Knauer N, Latzin P, Wildhaber JH, et al. Exhaled nitric oxide in symptomatic children at preschool age predicts later asthma. Allergy. 2013;68(4):531-8.
8. Pijnenburg MW, Hofhuis W, Hop WC, De Jongste JC. Exhaled nitric oxide predicts asthma relapse in children with clinical asthma remission. Thorax. 2005;60(3):215-8.
9. van der Valk RJ, Baraldi E, Stern G, Frey U, de Jongste JC. Daily exhaled nitric oxide measurements and asthma exacerbations in children. Allergy. 2012;67(2):265-71.
10. Duong-Quy S, Hua-Huy T, Pham H, Tang X, Mercier JC, Baud O, et al. Early inhaled nitric oxide at high dose enhances rat lung development after birth. Nitric oxide : biology and chemistry / official journal of the Nitric Oxide Society. 2014;38:8-16.
11. Young SL, Evans K, Eu JP. Nitric oxide modulates branching morphogenesis in fetal rat lung explants. American journal of physiology Lung cellular and molecular physiology. 2002;282(3):L379-85.
12. Cole FS, Alleyne C, Barks JD, Boyle RJ, Carroll JL, Dokken D, et al. NIH Consensus Development Conference statement: inhaled nitric-oxide therapy for premature infants. Pediatrics. 2011;127(2):363-9.
13. Mercier JC, Olivier P, Loron G, Fontaine R, Maury L, Baud O. Inhaled nitric oxide to prevent bronchopulmonary dysplasia in preterm neonates. Seminars in fetal & neonatal medicine. 2009;14(1):28-34.
14. Knight DA, Holgate ST. The airway epithelium: structural and functional properties in health and disease. Respirology. 2003;8(4):432-46.
15. Vyas-Read S, Shaul PW, Yuhanna IS, Willis BC. Nitric oxide attenuates epithelial-mesenchymal transition in alveolar epithelial cells. American journal of physiology Lung cellular and molecular physiology. 2007;293(1):L212-21.
16. Kanazawa H. Role of vascular endothelial growth factor in the pathogenesis of chronic obstructive pulmonary disease. Medical science monitor : international medical journal of experimental and clinical research. 2007;13(11):RA189-95.

17. Michel T, Feron O. Nitric oxide synthases: which, where, how, and why? The Journal of clinical investigation. 1997;100(9):2146-52.

18. Guo FH, Comhair SA, Zheng S, Dweik RA, Eissa NT, Thomassen MJ, et al. Molecular mechanisms of increased nitric oxide (NO) in asthma: evidence for transcriptional and post-translational regulation of NO synthesis. J Immunol. 2000;164(11):5970-80.

19. Chawes BL, Bischoff AL, Kreiner-Moller E, Buchvald F, Hakonarson H, Bisgaard H. DENND1B gene variants associate with elevated exhaled nitric oxide in healthy high-risk neonates. Pediatr Pulmonol. 2015;50(2):109-17.

20. Dahgam S, Nyberg F, Modig L, Naluai AT, Olin AC. Single nucleotide polymorphisms in the NOS2 and NOS3 genes are associated with exhaled nitric oxide. Journal of medical genetics. 2012;49(3):200-5.

21. Salam MT, Bastain TM, Rappaport EB, Islam T, Berhane K, Gauderman WJ, et al. Genetic variations in nitric oxide synthase and arginase influence exhaled nitric oxide levels in children. Allergy. 2011;66(3):412-9.

22. Salam MT, Byun HM, Lurmann F, Breton CV, Wang X, Eckel SP, et al. Genetic and epigenetic variations in inducible nitric oxide synthase promoter, particulate pollution, and exhaled nitric oxide levels in children. J Allergy Clin Immunol. 2011.

23. Bouzigon E, Monier F, Boussaha M, Le Moual N, Huyvaert H, Matran R, et al. Associations between nitric oxide synthase genes and exhaled NO-related phenotypes according to asthma status. PloS one. 2012;7(5):e36672.

24. van der Valk RJ, Duijts L, Timpson NJ, Salam MT, Standl M, Curtin JA, et al. Fraction of exhaled nitric oxide values in childhood are associated with 17q11.2-q12 and 17q12-q21 variants. J Allergy Clin Immunol. 2014;134(1):46-55.

25. Fuchs O, Latzin P, Kuehni CE, Frey U. Cohort profile: the Bern Infant Lung Development Cohort. International journal of epidemiology. 2012;41(2):366-76.

26. Fuchs O, Latzin P, Thamrin C, Stern G, Frischknecht P, Singer F, et al. Normative data for lung function and exhaled nitric oxide in unsedated healthy infants. Eur Respir J. 2011;37(5):1208-16.

27. R Development Core Team. R Foundation for Statistical Computing. Vienna, Austria2006.

28. Aulchenko YS, Ripke S, Isaacs A, van Duijn CM. GenABEL: an R library for genome-wide association analysis. Bioinformatics. 2007;23(10):1294-6.

29. Purcell S, Neale B, Todd-Brown K, Thomas L, Ferreira MA, Bender D, et al. PLINK: a tool set for whole-genome association and population-based linkage analyses. American journal of human genetics. 2007;81(3):559-75.

30. Genomes Project C, Abecasis GR, Auton A, Brooks LD, DePristo MA, Durbin RM, et al. An integrated map of genetic variation from 1,092 human genomes. Nature. 2012;491(7422):56-65.

31. Purcell S, Cherny SS, Sham PC. Genetic Power Calculator: design of linkage and association genetic mapping studies of complex traits. Bioinformatics. 2003;19(1):149-50.

32. Ziebarth JD, Bhattacharya A, Cui Y. CTCFBSDB 2.0: a database for CTCF-binding sites and genome organization. Nucleic acids research. 2013;41(Database issue):D188-94.

33. Phillips JE, Corces VG. CTCF: master weaver of the genome. Cell. 2009;137(7):1194-211.

34. Ong CT, Corces VG. CTCF: an architectural protein bridging genome topology and function. Nature reviews Genetics. 2014;15(4):234-46.

35. Herold M, Bartkuhn M, Renkawitz R. CTCF: insights into insulator function during development. Development. 2012;139(6):1045-57.

36. Bodnar RJ. Epidermal Growth Factor and Epidermal Growth Factor Receptor: The Yin and Yang in the Treatment of Cutaneous Wounds and Cancer. Advances in wound care. 2013;2(1):24-9.

37. Roskoski R, Jr. The ErbB/HER family of protein-tyrosine kinases and cancer. Pharmacological research : the official journal of the Italian Pharmacological Society. 2014;79:34-74.

38. Dizier MH, Margaritte-Jeannin P, Madore AM, Esparza-Gordillo J, Moffatt M, Corda E, et al. The ANO3/MUC15 locus is associated with eczema in families ascertained through asthma. J Allergy Clin Immunol. 2012;129(6):1547-53 e3.

39. Pallesen LT, Pedersen LR, Petersen TE, Knudsen CR, Rasmussen JT. Characterization of human mucin (MUC15) and identification of ovine and caprine orthologs. Journal of dairy science. 2008;91(12):4477-83.

40. Valenzuela L, Kamakaka RT. Chromatin insulators. Annual review of genetics. 2006;40:107-38.

41. Holwerda S, de Laat W. Chromatin loops, gene positioning, and gene expression. Frontiers in genetics. 2012;3:217.

42. Li Y, Huang W, Niu L, Umbach DM, Covo S, Li L. Characterization of constitutive CTCF/cohesin loci: a possible role in establishing topological domains in mammalian genomes. BMC genomics. 2013;14:553.

43. Gosalia N, Neems D, Kerschner JL, Kosak ST, Harris A. Architectural proteins CTCF and cohesin have distinct roles in modulating the higher order structure and expression of the CFTR locus. Nucleic acids research. 2014;42(15):9612-22.

44. Muck JS, Kandasamy K, Englmann A, Gunther M, Zink D. Perinuclear positioning of the inactive human cystic fibrosis gene depends on CTCF, A-type lamins and an active histone deacetylase. Journal of cellular biochemistry. 2012;113(8):2607-21.

45. Blackledge NP, Carter EJ, Evans JR, Lawson V, Rowntree RK, Harris A. CTCF mediates insulator function at the CFTR locus. The Biochemical journal. 2007;408(2):267-75.

46. Franklin PJ, Turner SW, Mutch RC, Stick SM. Measuring exhaled nitric oxide in infants during tidal breathing: methodological issues. Pediatr Pulmonol. 2004;37(1):24-30.

47. Leipala JA, Williams O, Sreekumar S, Cheeseman P, Rafferty GF, Hannam S, et al. Exhaled nitric oxide levels in infants with chronic lung disease. European journal of pediatrics. 2004;163(9):555-8.

48. Williams O, Rafferty GF, Hannam S, Milner AD, Greenough A. Nasal and lower airway levels of nitric oxide in prematurely born infants. Early human development. 2003;72(1):67-73.

49. Bouzigon E, Nadif R, Thompson EE, Concas MP, Kuldanek S, Du G, et al. A Common variant in RAB27A gene is associated with fractional exhaled nitric oxide levels in adults. Clin Exp Allergy. 2014.

FIGURES

Figure 1. Flowchart of excluded study participants due to general exclusion criteria or quality control

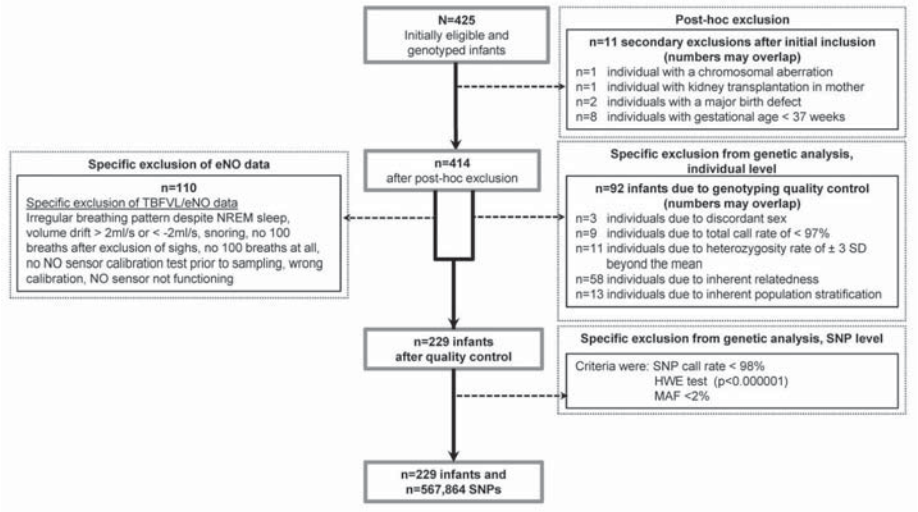
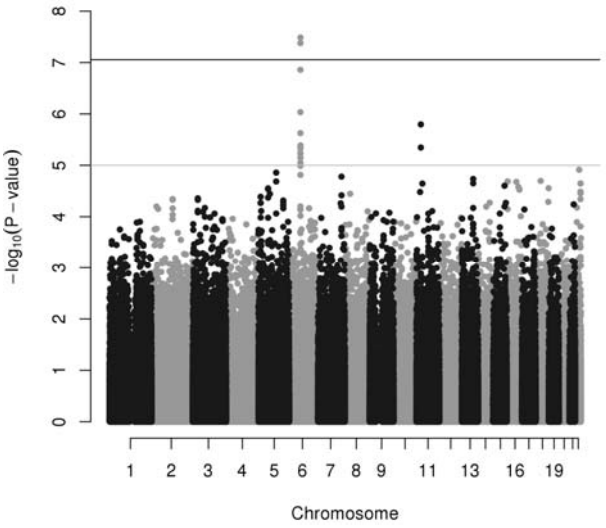


Figure 2, A and B. Manhattan plots displaying the associations of eNO (A) and V'NO values (B), final models

A.



B.

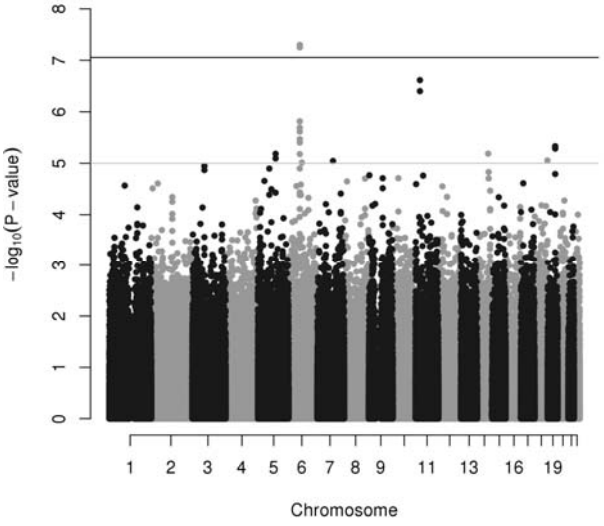


Figure 3: Association of the 6q12 locus with eNO and respiratory symptoms during the first year of life across genotypes

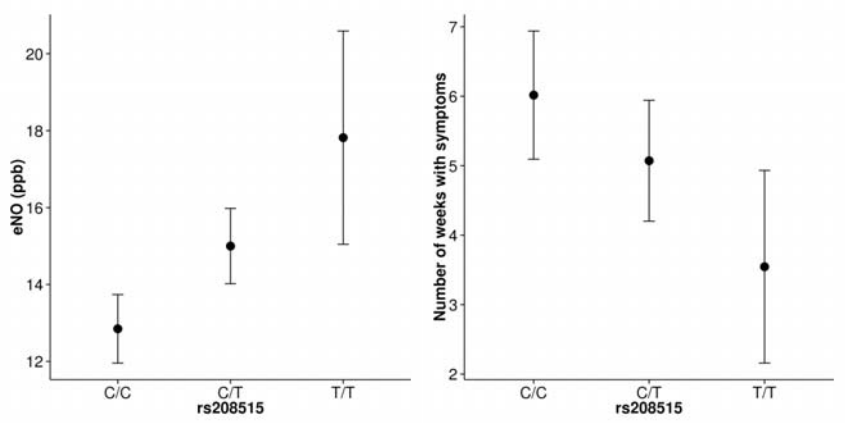


Figure 4: Association of the 11p14 locus with eNO and respiratory symptoms during the first year of life across genotypes

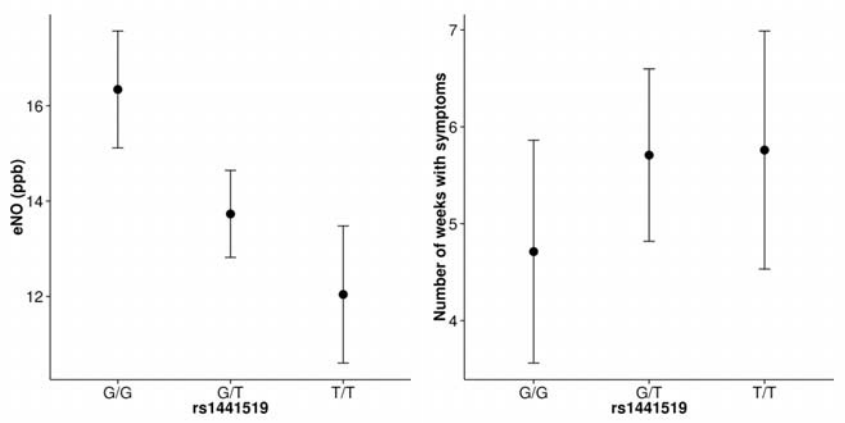


Figure 5, A and B: Fine mapping of eNO association signals on chromosomes 6 and 11

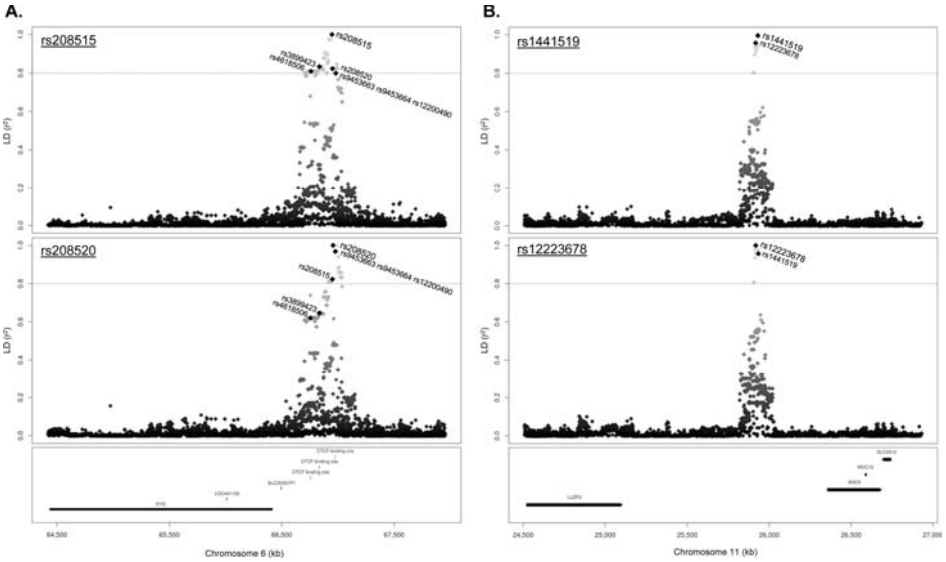


FIGURE LEGENDS

Figure 1. Flowchart of excluded study participants due to general exclusion criteria or quality control.

See text for details.

Abbreviations: (e)NO – (exhaled) nitric oxide, IBD – inheritance-by-descend, NREM – non rapid eye movement, SD – standard deviation, PCA – principal component analysis

Figure 2, A and B. Manhattan plots displaying the associations of eNO (A) and V'NO values (B), final models.

Each dot represents the p-value for the association of a SNP with the outcomes eNO and V'NO. For this, the $-\log_{10}$ of each SNP (y-axis) is plotted against the genomic position, i.e. chromosome (x-axis) adjusting for PC1 as well as known and possible confounders. These were study length, maternal smoking during pregnancy, maternal history of atopy, age at measurement, as well as V'E for eNO, and study weight, maternal smoking during pregnancy, maternal history of atopy, age at measurement, and V'E for V'NO in the final models for either outcome. The black lines display the genome-wide Bonferroni significance threshold $p<8.8\times10^{-8}$ (0.05/ 567,864). The light grey lines display the suggestive genome-wide significance level $p <1\times10^{-5}$.

Abbreviations: eNO – exhaled nitric oxide, PC1 – principal component 1, SNP – single nucleotide polymorphism, V'E – minute ventilation, V'NO – NO output

Figure 3: Association of the 6q12 locus with eNO and respiratory symptoms during the first year of life across genotypes

Figures display the effect of rs208515 on means of eNO on the left (y-axis, eNO in ppb) and weeks with respiratory symptoms during the first year of life on the right (y-axis in weeks

with symptoms) and their respective 95% confidence interval across genotypes (from left to right: homozygous for non-risk allele, heterozygous, homozygous for risk allele).
Abbreviation: (e)NO – (exhaled) nitric oxide, ppb – parts per billion.

Figure 4: Association of the 11p14 locus with eNO and respiratory symptoms during the first year of life across genotypes

Figures display the effect of rs1441519 on means of eNO on the left (y-axis, eNO in ppb) and weeks with respiratory symptoms during the first year of life on the right (y-axis in weeks with symptoms) and their respective 95% confidence interval across genotypes (from left to right: homozygous for non-risk allele, heterozygous, homozygous for risk allele).
Abbreviation: (e)NO – (exhaled) nitric oxide, ppb – parts per billion.

Figure 5, A and B: Fine mapping of eNO association signals on chromosomes 6 and 11.

LD analysis identified that no polymorphisms in linkage with the identified target SNPs at 6q12 (A: rs208515 and rs208520) and 11p14 (B: rs12223678 and rs1441519) are located in close vicinity to known genes (LD threshold $r2 \geq 0.8$). Database search identified that five tagged polymorphisms on chr.6 are located in CTCF binding sites (rs4618506, rs3899423, rs9453663, rs9453664, rs12200490; please note that only CTCF binding sites matching to analyzed SNPs are depicted). *In-silico* prediction proposed that the 11p14 locus contains cell type-specific chromatin structures called topologically associating domains, which encompass the *LUZP2* and *ANO3/MUC15* loci (domains are not specifically depicted due to their vast predicted extend of over 3,000kb in embryonic stem cells and human lung fetal myofibroblasts).The plots were prepared using R version 3.0.0.
Abbreviations: *ANO3* - Human anoctamin 3 gene locus, CTCF - 11-zinc finger protein or CCCTC- binding factor, *EYS* – Human eyes shut homolog gene locus, kb – kilo bases, LD –

linkage disequilibrium, *LUZP2* – Human leucine zipper protein 2 gene locus, MUC15 - Human mucin 15 gene locus, rs - reference SNP cluster code.

TABLES

Table 1: Main outcomes, as well as anthropometric and demographic characteristics for n=229 study participants with complete data on eNO measurements and GWAS

Variable	Mean (SD)	Median	IQR	Range
eNO, ppb	14.1 (5.2)	13.9	11.0-17.2	1.8-32.9
V'NO, nL/s	0.63 (0.23)	0.63	0.46-0.78	0.07-1.29
Minute ventilation, ml	1401.0 (251.1)	1401.0	1232-1565	786-2221
Anthropometric characteristics				
Gestational age, weeks	39.8 (1.14)	40.0	39.1-40.9	37.1-42.3
Postnatal age at measurement, days	35.2 (4.65)	34	32-38	25-53
Birth weight, kg	3.4 (0.42)	3.4	3.1-3.7	2.3-4.9
Birth length, cm	49.6 (2.03)	49.6	48.0-51.0	45.0-57.0
Study weight, kg	4.4 (0.52)	4.3	4.0-4.8	3.1-5.8
Study length, cm	54.9 (2.11)	54.8	53.2-56.6	50.0-59.6
	Number (%)			
Demographic and socioeconomic characteristics, distribution of known and possible confounders				
Male sex	115 (50.2%)			
Maternal asthma	27 (11.8%)			
Maternal atopy	79 (34.5%)			
Positive maternal skin prick test*	82 (37.8%)			
Maternal smoking during pregnancy	20 (8.7%)			
Paternal smoking during pregnancy	42 (18.4%)			
Parental smoking during pregnancy	46 (20.1%)			
Caesarean section	39 (17%)			

* data available for n=217 mothers only

Abbreviations: eNO – exhaled nitric oxide, GWAS – genome-wide association study, IQR – interquartile range, SD – standard deviation, V'NO – nitric oxide output.

9.2 Additional manuscript

False normal Lung Clearance Index in infants with cystic fibrosis due to software algorithms

Pinelopi Anagnostopoulou, Sophie Yammine, Anne Schmidt, Insa Korten, Elisabeth Kieninger, Ines Mack, Daniel Trachsel, Gaudenz Hafen, Alex Moeller, Carmen Casaulta, Philipp Latzin

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False Normal Lung Clearance Index in Infants With Cystic Fibrosis Due to Software Algorithms

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Summary. Background: Lung clearance index (LCI), a marker of ventilation inhomogeneity, is elevated early in children with cystic fibrosis (CF). However, in infants with CF, LCI values are found to be normal, although structural lung abnormalities are often detectable. We hypothesized that this discrepancy is due to inadequate algorithms of the available software package. Aim: Our aim was to challenge the validity of these software algorithms. Methods: We compared multiple breath washout (MBW) results of current software algorithms (automatic modus) to refined algorithms (manual modus) in 17 asymptomatic infants with CF, and 24 matched healthy term-born infants. The main difference between these two analysis methods lies in the calculation of the molar mass differences that the system uses to define the completion of the measurement. Results: In infants with CF the refined manual modus revealed clearly elevated LCI above 9 in 8 out of 35 measurements (23%), all showing LCI values below 8.3 using the automatic modus (paired *t*-test comparing the means, $P < 0.001$). Healthy infants showed normal LCI values using both analysis methods ($n = 47$, paired *t*-test, $P = 0.79$). The most relevant reason for false normal LCI values in infants with CF using the automatic modus was the incorrect recognition of the end-of-test too early during the washout. Conclusion: We recommend the use of the manual modus for the analysis of MBW outcomes in infants in order to obtain more accurate results. This will allow appropriate use of infant lung function results for clinical and scientific purposes. **Pediatr Pulmonol.**

Key words: cystic fibrosis; infant pulmonary function; multiple breath washout; ultrasonic flowmeter.

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INTRODUCTION

Multiple breath washout (MBW) is an established lung function test that measures ventilation inhomogeneity, a marker for small airway function.[1] The lung clearance index (LCI), its main outcome parameter, seems to be a sensitive marker of mild lung disease in children with cystic fibrosis (CF).[2–4] It has been shown to be elevated earlier than conventional spirometry (FEV1) in children with CF,[3–5] and to be a useful tool for monitoring and prediction of disease course.[6,7] The technique requires only passive cooperation and minimal coordination, therefore the measurements are feasible from infancy on.[8,9]

Recent studies using CT scans revealed structural abnormalities and air trapping in the lung in a significant proportion of infants with CF.[10,11] Thus, one would expect longer washout times and elevated LCI values, similar to older patients with CF.[2] However, several working groups that performed MBW measurements in infants with CF have reported LCI values within the normal range.[12–14]

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We hypothesized that this discrepancy is due to the current software algorithms, as they are inadequate to obtain accurate LCI values in infants. To test this hypothesis, we assessed the validity of results obtained from the automatic analysis in the available software. We compared results from multiple breath washout (MBW) measurements analyzed using the current software algorithms ("automatic modus") with those analyzed using refined software algorithms ("manual modus"). We further examined underlying reasons for differences between algorithms.

METHODS

Study Subjects

For our methodological study we included MBW measurements of 17 infants with CF diagnosed by newborn screening[15] and 24 healthy term-born infants recruited from the Bern Infant Lung Development (BILD) cohort.[16] The study protocol was approved by local ethics committees, and parents gave written consent.

MBW Measurements

MBW measurements were performed in infants during natural sleep, in accordance with current ERS/ATS standards.[1] Flow, volume, and molar mass (MM) were measured by an ultrasonic flowmeter (Exhalyzer D, Eco Medics AG, Duernten, Switzerland). Main stream MM was used for indirect measurement of tracer gas concentration. We used 4% sulfur hexafluoride (SF_6) as the tracer gas, as previously described.[17] A detailed description of the ultrasonic flowmeter and the MM measurements is available in the on-line supplement (OLS).

Measurements were analyzed using software provided by the manufacturer (WBreath Version 3.28.0.0, ndd Medizintechnik AG, Zuerich, Switzerland). 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.[17]

We selected one to three high-quality measurements per subject based on visual quality control and the following criteria: no leaks, no sighs, no irregularities in breathing pattern and complete washin and washout as identifiable by visual control of original traces.

Calculation of MM Step

The MM signal acquired through the ultrasonic flowhead is influenced by differences in temperature, airway pressure, and gas composition.[18] The calibration of the device and the temperature model simulation correction[17] are intended to minimize the effect of the two first parameters. Hence, changes in MM signal should reflect changes in gas composition. Indeed, the MM signal is increased during expiration, due to the presence of carbon dioxide (CO_2). The MM signal at the end of expiration [end-expiratory molar mass (EEMM)] is a sum signal of the actual concentration of the tracer gas and CO_2 at the end of expiration (see Fig. 1). At the end of a complete washin or washout phase, SF_6 is equally distributed within the lungs or completely washed out of the lungs, respectively, and the difference in MM signal between inspiration and expiration reflects again the CO_2 exchange. Therefore, in an ideal measurement where washin and washout are complete, the change in EEMM (EEMM-step) between end of washin and end of washout is expected to be equal with the change in end-inspiratory MM (EIMM-step) between end of washin and end of washout. Inequality between these two values reflects an insufficient SF_6 washin or washout, given that breathing pattern and CO_2 expirogram remain stable[18,19] (see further details in the OLS).

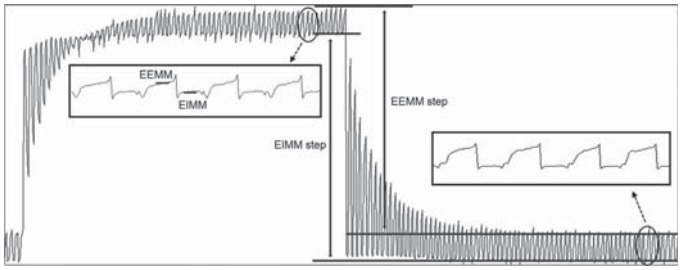


Fig. 1. Original molar mass tracing curve of a SF6-MBW from an infant with CF. The enlarged parts at the end of washin and the end of the washout show the end-expiratory molar mass (EEMM) and end-inspiratory molar mass (EIMM). The difference of molar mass (MM) between the end of washin and the end of washout is called MM-step; EEMM-step and EIMM-step, respectively.

Calculation of FRC and LCI

The software calculates functional residual capacity (FRC) and LCI at 2.5% of the initially defined 100% step in gas concentration. The current automatic modus of analysis uses the EEMM-step to define this initial 100% step. In the manual modus, we used the EIMM-step instead to define this step (Fig. 1). In contrast to the EEMM, the EIMM signal is determined during inspiration, and reflects changes of the tracer gas only. Applying the EIMM-step thus enables a more thorough evaluation of whether the tracer gas was completely washed in and out during the test without further influence by other gases (see further details in the OLS).

Procedure of Using the EIMM-Step

We determined the EIMM-step of each measurement by using the automatic modus of analysis. The value of the EIMM-step is shown in the results-box, under the name "deltaEIMM". To perform the refined manual modus of analysis, we selected "FRC analysis" under the "Analysis" option. We then unselected the automatic MM modus and included the EIMM value manually in the box "delta MM [g/mol]". We then repeated the analysis using this manual modus. To assess differences, we first compared the EEMM- and EIMM-steps from measurements of healthy infants and infants with CF. Second, we compared the primary MBW outcomes (FRC and LCI) derived from the automatic modus with the ones obtained by the manual modus, that is, when the EEMM-step was replaced by the EIMM-step for each measurement.

Statistical Analysis

Data were analysed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA). We performed statistical analyses using Student's paired and unpaired *t*-test, and $P < 0.05$ was accepted to indicate statistical significance. The upper limit of normal was defined as 7.9 for LCI.[20]

RESULTS

We analyzed 35 measurements of 17 asymptomatic infants with CF, at median (IQR) age of 7.0 (2.7) weeks, and 47 measurements of 24 healthy term born infants at 5.1 (0.8) weeks. The clinical characteristics of the subjects included in the study are reported in Table 1.

Using the manual modus, we found elevated LCI in 8 out of 35 measurements (23%) in infants with CF, whereas all but one measurement were within the normal range using the automatic modus (Fig. 2A, Table 2, and E-Fig. 3A, paired *t*-test comparing the means, $P < 0.001$). In contrast, both modes of analysis resulted in similar LCI values in healthy infants ($n = 47$, Fig. 2A, Table 2, and E-Fig. 3A, paired *t*-test, $P = 0.79$).

If the washout phase is complete, both EIMM- and EEMM-steps are similar, as mentioned above. This was the case in all measurements of healthy infants (Table 2, paired *t*-test, $P = 0.54$). In infants with CF, however, the EIMM- and EEMM-steps were significantly different (Table 2, paired *t*-test, $P < 0.001$). The EIMM-step was similar in healthy infants and infants with CF (Table 2, unpaired *t*-test, $P = 0.24$), as this is based on the concentration of the tracer gas only. The difference between EIMM- and EEMM-step was positively correlated to the tracer gas concentration at the end of washout in the manual modus, confirming that similar EIMM- and EEMM-steps indicate completion of the washout (Fig. 3A). It was also associated with the differences in LCI values (Fig. 3B and OLS E-Fig. 2).

Using the manual modus, we found higher FRC values in infants with CF compared to the automatic modus (Fig. 2B, Table 2, and E-Fig. 3B, paired *t*-test, $P < 0.001$). In healthy infants FRC remained unchanged (Fig. 2B, Table 2, and E-Fig. 3B, paired *t*-test, $P = 0.78$). The LCI derives from the cumulative expiratory volume (CEV) divided by the FRC.[1] The comparison between automatic and manual modus reveals a LCI increase that is proportionally higher than the FRC increase. According to the equation, this is thus attributed to the higher CEV.

TABLE 1— Demographic Characteristics of Study Subjects, Data Are Given in Median (IQR)

	Healthy infants	Infants with cystic fibrosis
Number, male/female	24 (10/14)	17 (8/9)
Age (wks)	5.1 (0.8)	7.0 (2.7)
Weight (kg)	4.6 (0.6)	4.4 (0.6)
Height (cm)	55.0 (2.6)	55.0 (4.1)
BMI	14.7 (1.9)	14.7 (2.6)
BMI, z-score	−0.2 (1.0)	−0.7 (2.1)
Respiratory rate (min^{-1})	39.4 (8.8)	42.6 (7.2)
Tidal volume (ml)	33.3 (3.8)	33.5 (6.1)

Age in weeks (wks), weight in kg, height in cm, body mass index (BMI), BMI z-score, respiratory rate in min^{-1} and tidal volume in ml are reported for healthy infants ($N = 24$) and infants with CF ($N = 17$). The BMI z-scores are calculated according to the WHO child growth standards [Group WMGRS. WHO Child Growth Standards based on length/height, weight and age. In: de Onis M, Garza, C., Onyango, A.W., Martorell, R., editor. WHO Child Growth Standards. Acta Paediatrica, 2006;95:76-85 (Suppl 450)].

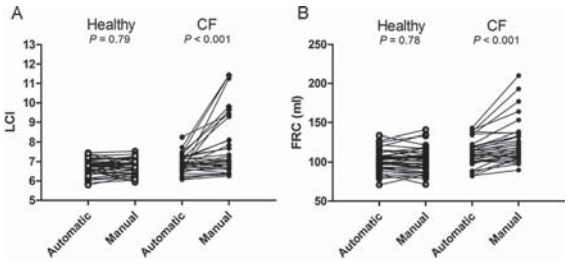


Fig. 2. Impact of the automatic and manual analysis modus on MBW outcomes. (A) LCI values and (B) FRC values (in ml) in 47 measurements from 24 healthy infants, and 35 measurements from 17 infants with CF calculated using the automatic (based on the EEMM-step) and manual modus (based on the EIMM-step). *P* values obtained from paired *t*-test. The manual modus revealed 3 incomplete washouts, and for these measurements the LCI (◆) was calculated at an earlier time-point (see OLS).

Indeed, as shown in the LCI breath number (LCIBrNr), the end of the test is determined much later in CF infants using the manual modus (Table 2, paired *t*-test, $P = 0.0003$, compared with the automatic modus). In healthy infants, however, it remains unchanged (Table 2, paired *t*-test, $P = 0.95$).

In order to examine the ability of the automatic modus to detect an incomplete measurement in detail, we further challenged the software by performing FRC and LCI calculations at different cut-off points within the same measurement (Fig. 4). Using the EEMM-step (i.e., the automatic modus of the software), an incomplete washout was not detected, even when an artificial end was set very early during the washout. The software provided false low LCI values for all earlier ends. The manual modus overcomes this weakness, as the EIMM-step enables reliable detection of insufficient washout traces (Fig. 4). Being uninfluenced by the breathing pattern, an additional advantage of the EIMM-tracing is that it remains more stable throughout the measurement compared with the EEMM-tracing (Fig. 4 and OLS).

DISCUSSION

Summary

In this study we show that the current automatic modus used for the analysis of MBW measurements in infants can result in false normal LCI values. This happens unnoticed to the user and thus makes this way of analysis useless for clinical or research applications. We present

TABLE 2— Mean (SD) Values of MM-Step (g/mol), FRC (ml), FRC Difference (Calculated as FRC_{EIMM}-FRC_{EEMM} in ml), LCI, LCI Difference (Calculated as LCI_{EIMM}-LCI_{EEMM}), LCI Intrasubject Variability (IV, for Infants With Two Measurements) and Coefficient of Variation (CV, for Infants With 3 Measurements) in %, LCI Breath Number (LCI BrNr), and Test Duration Between the Beginning of the Washin and the Time-Point Where the Washout Reaches the 2.5% of the Initial SF6 Concentration in Sec in 24 Healthy Infants (n = 47 Measurements) and 17 Infants With CF (n = 35 measurements) Using the Automatic (EEMM) Modus and the Manual (EIMM) Modus

	Healthy infants		Infants with cystic fibrosis	
	Automatic EEMM	Manual EIMM	Automatic EEMM	Manual EIMM
MM-step (g/mol)	−5.52 (0.04)	−5.52 (0.04)	−5.48 (0.08)	−5.54 (0.07)
FRC (ml)	103 (15)	103 (17)	111 (16)	125 (27)
FRC difference (ml)	0.3 (7.0)		13.3 (15.8)	
LCI	6.7 (0.4)	6.7 (0.4)	6.9 (0.5)	7.7 (1.5)
LCI difference	0.01 (0.3)		0.8 (1.3)	
LCI IV/CV (%)	6.1 (4.1)/2.9[a]	5.1 (2.8)/0.8[a]	5.5 (3.2)/3.1 (1.0)	5.3 (10.0)/7.8 (2.8)[b]
LCI BrNr	86 (11)	86 (11)	93 (19)	100 (24)
Test duration (sec)	121 (15)		134 (22)	

[a]Based on one subject.
[b]Based on four subjects, including one outlier.

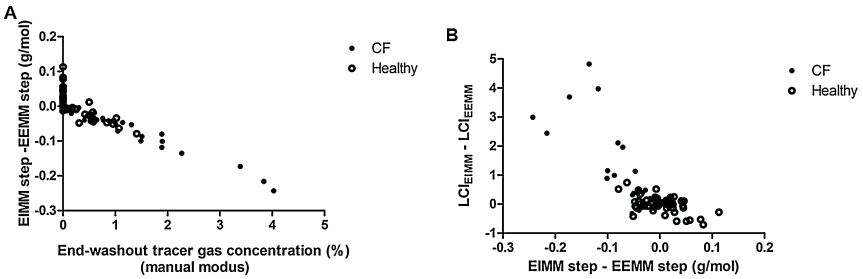
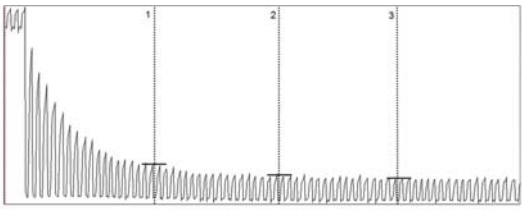


Fig. 3. (A) End-washout tracer gas concentration and differences between EIMM- and EEMM-step. An incomplete washout phase, detected by elevated end-washout tracer gar concentrations using the manual modus, is associated with large differences between EIMM-step and EEMM-step. Plot of the differences between the EIMM-step and the EEMM-step against the end washout tracer gas concentration (%) determined using the manual modus in measurements from healthy infants (n = 47) and infants with CF (n = 35). (B) Differences between EIMM- and EEMM-step and differences in LCI values. Differences between EIMM- and EEMM-step are associated with differences in LCI between the manual and the automatic modus. Plot of the differences between the manual LCI (LCI_{EIMM}) and the automatic LCI (LCI_{EEMM}) against the differences between EIMM-step and EEMM-step in 47 measurements from 24 healthy infants and 35 measurements from 17 infants with CF.



		DeltaMM (ΔMM in g/mol)	Difference EEMM-EIMM (in g/mol)	FRC (ml)	LCI	EndConc (in %)
	EEMM	-4.904		94	5.0	0
1	EIMM	-5.496	0.592	149	0	8.9
	EEMM	-5.372		131	6.5	0
2	EIMM	-5.537	0.165	159	0	2.7
	EEMM	-5.413		137	6.7	0
3	EIMM	-5.557	0.144	170	11.0	2.4

Fig. 4. Earlier stops of washout within an original molar mass tracing curve of a SF6-MBW from an infant with CF. In order to challenge the current software, we artificially defined the end of washout at different points within the same measurement. These points are displayed using lines 1, 2 and 3. For each of these points, molar mass difference [ΔMM (g/mol)], FRC (ml), LCI and End Concentration [EndCon(%)] values are shown, using the automatic (EEMM) and the manual (EIMM) modus. When EndCon is higher than 2.5%, LCI is not reached and thus reported as "0" by the software.

explanations for this fault and we further provide an alternative method to analyse the MBW measurements that leads to more precise results. In some infants with CF, the new manual modus reveals pathological LCI values found to be normal with the automatic modus.

A more detailed analysis revealed that the automatic modus recognizes the end-of-test criteria too early, thus calculating MBW outcomes in incomplete washouts. This was the main cause for false normal LCI values in CF lungs with ventilation inhomogeneity. This was proven by modelling artificially earlier ends during the washout (Fig. 4). The manual modus, however, depends only on the concentration of the inert gas and, thus, provides more accurate information about the completion of the measurement.

Interpretation

Our findings indicate that the higher the difference between EEMM- and EIMM-step, the more incomplete is the measurement (see OLS). As mentioned above, the measurements were carefully selected using quality criteria. The greatest difference between the EEMM- and EIMM-step within our study corresponds to a SF_6 concentration of 0.17%, which is not possible to see visually, neither during the performance of the measurement, nor during the analysis. The fact that this small SF_6 concentration cannot be detected by the user online is a clear disadvantage of this software package (see also OLS). In infants with CF, where the potential underlying pathology of the small airways will prolong washin and washout times, these small differences in gas concentration can have a high impact on final LCI results.

Interestingly, the analysis using the manual modus did not change MBW outcomes of the healthy infants. This supports our hypothesis that the non-detection of an incomplete washout was the most relevant reason for artificially normal LCI values in infants with CF, rather than a systematic mistake in the algorithm of the software.

The manual modus also changed the FRC values in infants with CF. This is explained by the fact that the MM-step affects the initial relative SF_6 fraction on which the calculation for gas volumes is based.[18] Interestingly, the effect on CEV was even larger than on FRC, otherwise LCI values would have been stable. The finding of higher FRC values in infants with CF in comparison to the healthy infants is, however, not surprising. The ventilation inhomogeneity found in CF lung disease is partially provoked by air trapping and hyperinflation.[10,13] Thus, LCI but also FRC values are expected to be increased.

The MM-step divided by the MM of SF_6 provides the SF_6 fraction.[18] Based on this step, the software algorithms will then calculate the FRC and the LCI. The accuracy of MM-step is, thus, of great importance for the MBW outcomes. Of note, the software algorithm used in the manual modus uses the EIMM-step to define the 100% concentration of SF_6 and from this uses the EEMM-step to calculate the FRC and determine when 2.5% of the initial 100% concentration is reached. The automatic software algorithm, instead, uses the EEMM-step for both. This leads to false calculations of values at earlier stops (Fig. 4).

It is important to mention that the optimised algorithms used by the software to correct for environmental parameters (increased temperature and humidity) change the MM signal only during expiration, thus affecting the EEMM value.[17] During inspiration, the air passes through the flowmeter under constant temperature and humidity conditions, so no changes have to be applied to the MM signal. Thus, the optimised algorithms do not affect the EIMM value.

Our study raises specific questions regarding the completion of MBW measurements. For example, the identification of a complete washin phase is a crucial issue, as this determines the validity of the washout outcomes. However, this issue is not examined so far, also with regards to other SF_6 systems.[4,21] Additionally, there is no specific recommendation on the analysis approach of incomplete washin measurements. Especially for infants with ventilation inhomogeneity, where the washin time is expected to be prolonged, a short washin will miss the poorly ventilated airway zones, leading to false MBW outcomes. We show that the manual modus can identify incomplete washin periods but we cannot make any recommendation upon the analysis of those. Further examination towards this direction is needed, in order to clarify how to deal with measurements with incomplete washin.

In theory a steeper phase III slope, as shown in older patients with CF,[22] could also contribute to differences between EEMM- and EIMM-steps in infants with CF. However, we did not observe steep slope III phase in any measurements of infants with CF, even in those with elevated LCI. This might be partly due to the fact that the side chamber of the mainstream ultrasonic flowmeter is washed in and washed out during the measurement and thus a formal SnIII analysis cannot be performed, or simply reflect less steep phase III slopes in infants with less pronounced lung disease compared to older patients with CF. In any case, our findings cannot be attributed to steeper phase III slopes in infants with CF.

Relevance

The device and the software package we used are currently the only commercially available equipment for MBW measurements in infants. Several working groups have reported FRC and LCI outcomes in healthy infants and infants with CF obtained with this method.[9,12,13,20,23,24] Moreover, this equipment together with

this software package have been used for the evaluation of lung function in preterm infants,[25–28] infants with congenital diaphragmatic hernia[29,30] and ventilated infants and children.[30–32] Therefore, our findings have broad implications not only for ongoing and future studies, but also for the interpretation of existing data. Hall et al., e.g., report normal LCI values in infants with CF despite air-trapping in chest CT.[13] It is tempting to speculate that this lack of association is due to the inability of the automatic modus to detect pathological LCI values.

Because of its high sensitivity, there is an increasing interest of the research community on using LCI as a primary outcome in clinical studies.[6] The incorporation of infant groups in those studies is of high priority, therefore our analysis method will serve as a useful tool for ongoing and future studies in infants with respiratory diseases, using MBW measurements as outcome.[33]

Of note, these findings account only for the present device and the ultrasonic flowmeter. It is beyond the scope of this study to determine how sensitive other measurement devices and software packages for SF_6-MBW, such as the mass spectrometer[4,34,35] and the Innocor photoacoustic gas analyser,[21,36] are to detect differences of such small concentrations. In fact, these two devices measure directly changes in tracer gas concentration[4,21] while the ultrasonic flowmeter measures them indirectly, by measuring changes in MM.[18] However, the detection of incomplete washout traces is an issue that accounts for all MBW techniques, independent of the methodological approach. Especially for infants, where the fast respiratory rate, the low tidal volume as well as the proportionally increased dead-space create a priori different conditions on performing MBW measurements,[17,37] we recommend a closer look at the end-of-test criteria and, if necessary, a critical reevaluation of the cut-off that the software uses to define differences in tracer gas concentrations.

CONCLUSION

Assessing ventilation distribution using the MBW technique is an important tool for monitoring early lung disease. SF_6-MBW measurements in infants are widely used in therapeutic trials[34] and epidemiological surveillance studies.[11] We show that the method used to determine the initial MM-step has a significant impact on both FRC and LCI results. Therefore we strongly recommend the refined manual modus using the EIMM-step to overcome the potential underestimation of LCI. Only the EIMM modus will allow an accurate determination of MBW outcomes in infancy. In infants with CF, this represents an important window of opportunity for interventions.[33]

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REFERENCES

1. Robinson PD, Latzin P, Verbanck S, Hall GL, Horsley A, Gappa M, Thamrin C, Arets HG, Aurora P, Fuchs SI, et al. Consensus statement for inert gas washout measurement using multiple- and single- breath tests. Eur Respir J 2013;41:507–522.
2. Owens CM, Aurora P, Stanojevic S, Bush A, Wade A, Oliver C, Calder A, Price J, Carr SB, Shankar A, et al. Lung Clearance Index and HRCT are complementary markers of lung abnormalities in young children with CF. Thorax 2011;66:481–488.
3. Aurora P, Bush A, Gustafsson P, Oliver C, Wallis C, Price J, Stroobant J, Carr S, Stocks J. Multiple-breath washout as a marker of lung disease in preschool children with cystic fibrosis. Am J Respir Crit Care Med 2005;171:249–256.
4. Gustafsson PM, Aurora P, Lindblad A. Evaluation of ventilation maldistribution as an early indicator of lung disease in children with cystic fibrosis. Eur Respir J 2003;22:972–979.
5. Singer F, Kieninger E, Abbas C, Yammine S, Fuchs O, Proietti E, Regamey N, Casaulta C, Frey U, Latzin P. Practicability of nitrogen multiple-breath washout measurements in a pediatric cystic fibrosis outpatient setting. Pediatr Pulmonol 2013;48: 739–746.
6. Kent L, Reix P, Innes JA, Zielen S, Le Bourgeois M, Braggion C, Lever S, Arets HG, Brownlee K, Bradley JM, et al. Lung clearance index: evidence for use in clinical trials in cystic fibrosis. J Cyst Fibros 2014;13:123–138.
7. Aurora P, Stanojevic S, Wade A, Oliver C, Kozlowska W, Lum S, Bush A, Price J, Carr SB, Shankar A, et al. Lung clearance index at 4 years predicts subsequent lung function in children with cystic fibrosis. Am J Respir Crit Care Med 2011;183:752–758.
8. Ramsey KA, Ranganathan S. Interpretation of lung function in infants and young children with cystic fibrosis. Respirology 2014;19:792–799.
9. Kieninger E, Singer F, Fuchs O, Abbas C, Frey U, Regamey N, Casaulta C, Latzin P. Long-term course of lung clearance index between infancy and school-age in cystic fibrosis subjects. J Cyst Fibros 2011;10:487–490.
10. Sly PD, Brennan S, Gangell C, de Klerk N, Murray C, Mott L, Stick SM, Robinson PJ, Robertson CF, Ranganathan SC. Lung disease at diagnosis in infants with cystic fibrosis detected by newborn screening. Am J Respir Crit Care Med 2009;180: 146–152.

11. Mott LS, Park J, Murray CP, Gangell CL, de Klerk NH, Robinson PJ, Robertson CF, Ranganathan SC, Sly PD, Stick SM. Progression of early structural lung disease in young children with cystic fibrosis assessed using CT. Thorax 2012;67:509–516.
12. Belessis Y, Dixon B, Hawkins G, Pereira J, Peat J, MacDonald R, Field P, Numa A, Morton J, Lui K, et al. Early cystic fibrosis lung disease detected by bronchoalveolar lavage and lung clearance index. Am J Respir Crit Care Med 2012;185:862–873.
13. Hall GL, Logie KM, Parsons F, Schulzke SM, Nolan G, Murray C, Ranganathan S, Robinson P, Sly PD, Stick SM, et al. Air trapping on chest CT is associated with worse ventilation distribution in infants with cystic fibrosis diagnosed following newborn screening. PLoS ONE 2011;6:e23932.
14. Kieninger E, Yammine S, Proietti E, Casaulta C, Regamey N, Frey U, Latzin P. Group TSCFNS. Normal lung function in infants with cystic fibrosis shortly after birth. Eur Respir J 2013;42 (Suppl 57):5052.
15. Barben J, Gallati S, Fingerhut R, Schoeni MH, Baumgartner MR, Torresani T. Retrospective analysis of stored dried blood spots from children with cystic fibrosis and matched controls to assess the performance of a proposed newborn screening protocol in Switzerland. J Cyst Fibros 2012;11:332–336.
16. Fuchs O, Latzin P, Kuehni CE, Frey U. Cohort profile: the Bern infant lung development cohort. Int J Epidemiol 2012;41:366–376.
17. Latzin P, Sauteur L, Thamrin C, Schibler A, Baldwin D, Hutten GJ, Kyburz M, Kraemer R, Riedel T, Frey U. Optimized temperature and deadspace correction improve analysis of multiple breath washout measurements by ultrasonic flowmeter in infants. Pediatr Pulmonol 2007;42:888–897.
18. Schibler A, Henning R. Measurement of functional residual capacity in rabbits and children using an ultrasonic flow meter. Pediatr Res 2001;49:581–588.
19. Schibler A, Hall GL, Businger F, Reinmann B, Wildhaber JH, Cernelc M, Frey U. Measurement of lung volume and ventilation distribution with an ultrasonic flow meter in healthy infants. Eur Respir J 2002;20:912–918.
20. Fuchs O, Latzin P, Thamrin C, Stern G, Frischknecht P, Singer F, Kieninger E, Proietti E, Riedel T, Frey U. Normative data for lung function and exhaled nitric oxide in unsedated healthy infants. Eur Respir J 2011;37:1208–1216.
21. Gonem S, Singer F, Corkill S, Singapuri A, Siddiqui S, Gustafsson P. Validation of a photoacoustic gas analyser for the measurement of functional residual capacity using multiple-breath inert gas washout. Respiration 2014;87:462–468.
22. Bigler A, Yammine S, Singer F, Riedel T, Latzin P. Feasibility of automated slope III and Scond analysis in children. Pediatr Pulmonol 2014; Published online first 2014 Jul 23. DOI: 10.1002/ppul.23080. [Epub ahead of print].
23. Gray DM, Willemse L, Alberts A, Simpson S, Sly PD, Hall GL, Zar HJ. Lung function in African infants: A pilot study. Pediatr Pulmonol 2015;50:49–54.
24. Stahl M, Joachim C, Blessing K, Hammerling S, Sommerburg O, Latzin P, Mall MA. Multiple breath washout is feasible in the clinical setting and detects abnormal lung function in infants and young children with cystic fibrosis. Respiration 2014;87:357–363.
25. Latzin P, Roth S, Thamrin C, Hutten GJ, Pramana I, Kuehni CE, Casaulta C, Nelle M, Riedel T, Frey U. Lung volume, breathing pattern and ventilation inhomogeneity in preterm and term infants. PLoS ONE 2009;4:e4635.
26. Hutten GJ, van Eykern LA, Latzin P, Thamrin C, van Aalderen WM, Frey U. Respiratory muscle activity related to flow and lung volume in preterm infants compared with term infants. Pediatr Res 2010;68:339–343.
27. Sinhal S, Galati J, Baldwin DN, Stocks J, Pillow JJ. Reproducibility of multiple breath washout indices in the unsedated preterm neonate. Pediatr Pulmonol 2010;45:62–70.
28. Hulskamp G, Lum S, Stocks J, Wade A, Hoo AF, Costeloe K, Hawdon J, Deeptha K, Pillow JJ. Association of prematurity, lung disease and body size with lung volume and ventilation inhomogeneity in unsedated neonates: a multicentre study. Thorax 2009;64:240–245.
29. Dotta A, Palamides S, Braguglia A, Crescenzi F, Ronchetti MP, Calzolari F, Iacobelli BD, Bagolan P, Corchia C, Orzalesi M. Lung volumes and distribution of ventilation in survivors to congenital diaphragmatic hernia (CDH) during infancy. Pediatr Pulmonol 2007;42:600–604.
30. Hofmann SR, Stadler K, Heilmann A, Hausler HJ, Fitze G, Kamin G, Nitzsche KI, Hahn G, Dinger J. Stabilisation of cardiopulmonary function in newborns with congenital diaphragmatic hernia using lung function parameters and hemodynamic management. Klin Padiatr 2012;224:e1–e10.
31. von Ungern-Sternberg BS, Hammer J, Schibler A, Frei FJ, Erb TO. Decrease of functional residual capacity and ventilation homogeneity after neuromuscular blockade in anesthetized young infants and preschool children. Anesthesiology 2006;105:670–675.
32. von Ungern-Sternberg BS, Petak F, Hantos Z, Habre W. Changes in functional residual capacity and lung mechanics during surgical repair of congenital heart diseases: effects of preoperative pulmonary hemodynamics. Anesthesiology 2009;110:1348–1355.
33. Stick SM, Sly PD. Exciting new clinical trials in cystic fibrosis: infants need not apply. Am J Respir Crit Care Med 2011; 183:1577–1578.
34. Subbarao P, Stanojevic S, Brown M, Jensen R, Rosenfeld M, Davis S, Brumback L, Gustafsson P, Ratjen F. Lung clearance index as an outcome measure for clinical trials in young children with cystic fibrosis. A pilot study using inhaled hypertonic saline. Am J Respir Crit Care Med 2013;188:456–460.
35. Hoo AF, Thia LP, Nguyen TT, Bush A, Chudleigh J, Lum S, Ahmed D, Balfour Lynn, I, Carr SB, Chavasse RJ, et al. Lung function is abnormal in 3-month-old infants with cystic fibrosis diagnosed by newborn screening. Thorax 2012;67:874–881.
36. Horsley AR, Gustafsson PM, Macleod KA, Saunders C, Greening AP, Porteous DJ, Davies JC, Cunningham S, Alton EW, Innes JA. Lung clearance index is a sensitive, repeatable and practical measure of airways disease in adults with cystic fibrosis. Thorax 2008;63:135–140.
37. Robinson PD, Latzin P, Gustafsson P. Multiple-breath washout. In: Frey U, Merkus PJFM, editors. Eur Respir Mon. UK: European Respiratory Society; 2010. p 87–104.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher's web-site.

9.3 Additional manuscript
Ventilatory response to nitrogen multiple-breath washout in infants

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Ventilatory Response to Nitrogen Multiple-Breath Washout in Infants

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Summary. Background: Nitrogen multiple-breath washout (N_2MBW) using 100% oxygen (O_2) has regained interest to assess efficiency of tracer gas clearance in, for example, children with Cystic Fibrosis (CF). However, the influence of hyperoxia on the infants' respiratory control is unclear. We assessed safety and impact on breathing pattern from hyperoxia, and if exposure to 40% O_2 first induces tolerance to subsequent 100% O_2 for N_2MBW. Methods: We prospectively enrolled 39 infants aged 3–57 weeks: 15 infants with CF (8 sedated for testing) and 24 healthy controls. Infants were consecutively allocated to the protocols comprising of 100% O_2 or 40/100% O_2 administered for 30 breaths. Lung function was measured using an ultrasonic flowmeter setup. Primary outcome was tidal volume (V_T). Results: None of the infants experienced apnea, desaturation, or bradycardia. Both protocols initially induced hypoventilation. V_T temporarily declined in 33/39 infants across 10–25 breaths. Hypoventilation occurred independent of age, disease, and sedation. In the new 40/100% O_2 protocol, V_T returned to baseline during 40% O_2 and remained stable during 100% O_2 exposure. End-tidal carbon dioxide monitored online did not change. Conclusion: The classical N_2MBW protocol with 100% O_2 may change breathing patterns of the infants. The new protocol with 40% O_2 induces hyperoxia-tolerance and does not lead to changes in breathing patterns during later N_2 washout using 100% O_2. Both protocols are safe, the new protocol seems an attractive option for N_2MBW in infants. **Pediatr Pulmonol 2014; 49:342–347.** © 2013 Wiley Periodicals, Inc.

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BACKGROUND

Multiple-breath inert tracer gas washout (MBW) is an easy and sensitive lung function test assessing efficiency of inert tracer gas clearance across multiple tidal breaths.[1–4] In children with cystic fibrosis (CF), impaired tracer gas clearance quantified by the lung clearance index (LCI) correlates to other biomarkers of small airways disease,[5–8] and detects pulmonary function response upon short-term interventions.[9–11]

To overcome the need for expensive inert tracer gases such as sulfur hexafluoride, nitrogen (N_2) MBW using pure oxygen (O_2) has regained interest and seems attractive for MBW in infants. However, there is scanty evidence as to whether 100% O_2 for the purpose of MBW is applicable in infants.[12–14] A regular breathing pattern is a prerequisite for reliable MBW testing. By unloading peripheral chemoreceptors, hyperoxia may change tidal volume (V_T) if 100% O_2 is used.[15] We hypothesized that 40% O_2 may induce "tolerance" of the infants' respiratory control to subsequent 100% O_2 exposure N_2MBW. Breathing 40% O_2 would allow later washout of the lungs' remaining N_2 fraction using pure O_2. In the current study, we measured tidal breathing indices when infants

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were exposed to hyperoxia during the classical 1-step N_2MBW protocol with a switch from ambient to 100% O_2 gas, and the new 2-steps protocol introducing an intermediate 40% O_2 step, that is, 40/100% O_2. Our aim was to assess safety and impact of hyperoxia (40% or 100% O_2) on breathing patterns in infants with CF and healthy controls, and to compare tidal breathing parameters from both N_2MBW protocols. Primary outcome was change in V_T upon 40% or 100% O_2 exposure.

METHODS

We prospectively enrolled 40 infants (16 with CF) from two ongoing studies, the *Bern Infant Lung Development* (BILD) cohort and the *Swiss CF Newborn Screening Study*.[16–18] The following inclusion criteria applied: white ethnicity, term delivery (37 weeks), and no known other diseases likely to affect lung function. General exclusion criteria were clinical signs of lower respiratory tract infection. The population is described in Table 1. The study was approved by the Ethics Committee of the Canton of Bern, Bern, Switzerland. Parents provided written informed consent for this study. Parents from one child withdrew consent.

We consecutively allocated 23 infants (7 with CF of which 4 were sedated) to protocol I and 16 (8 with CF of which 4 were sedated) to protocol II. The sequence of tidal breathing measurements in protocol I was (i) 1 baseline test during ambient air exposure (21% O_2) and (ii) 1 test during breathing 100% O_2. Protocol II implied (i) 1 baseline test during ambient air exposure, (ii) 1 test during breathing 40% O_2, and (iii) 1 test during breathing 100% O_2. Hyperoxia was administered for 30 breaths which reflect approximately twice the cumulative expired volume (CEV) of average N_2MBW in infants.[12] Oxygen saturation, heart rate, and the exhaled carbon dioxide (CO_2) fraction were monitored online.

TABLE 1— Anthropometric Characteristics of the Study Population (n = 39)

	Younger infants	Older infants
N	31 (7 with CF)	8 (8 with CF)
Protocol I/II	19/12	4/4
Males/females	16/15	3/5
Age	5 (5–6) weeks	13 (13–14) months
Weight	4,150 (3,600–4,960) g	9.3 (8.7–9.5) kg
Height	55 (52–58) cm	77 (73–78) cm

Anthropometric data from the 24 healthy infants and 15 infants with CF. Numeric data are described as median and interquartile range. Protocol I = classical 1-step nitrogen multiple-breath washout protocol using 100% oxygen; Protocol II = new 2-steps nitrogen multiple-breath washout protocol using 40–100% oxygen. Lung function was measured during natural quiet sleep in younger infants and during sedation in older infants.

Lung function was measured in infants in supine position with the head midline via an infant mask (size 1; Homedica AG, Huenenberg, Switzerland), according to the ERS/ATS standards of infant lung function testing.[19–21] Thirty-one young infants, age range 3–13 weeks, 7 with CF, were studied during behaviorally defined natural sleep. Measurements followed regular feeding of the infants, usually resulting in quiet (non-rapid eye movement) sleep in this age group. Eight older infants with CF aged 13–14 months were studied during chloral–hydrate sedation. Flow was measured using a mainstream ultrasonic flow meter (Exhalyzer D; Eco Medics AG, Duernten, Switzerland).[17] Data were used if no apparent volume drift was present, defined as a change exceeding 2 ml s^{-1}. Tidal breathing indices derived from tidal flow were measured from all breaths. Main outcome was V_T which directly affects end-expiratory lung volume, the functional residual capacity (FRC). Secondary outcomes were respiratory rate (RR), minute ventilation (V_E), mean tidal expiratory flow (MTEF), and end-tidal CO_2 monitored online. Tidal breathing indices within children were compared by paired *t*-test. *P*-values < 0.05 were considered significant (STATA 11; College Station). Variability of V_T was estimated by the coefficient of variation (CV = SD/mean %) within each test and per child.

RESULTS

Thirty-eight infants completed the study according to protocol (Table 1). None of the infants experienced apnea, desaturation ($\leq$92% O_2), bradycardia ($<$60 min^{-1}) or change in end-tidal CO_2 levels. One non-sedated infant woke up during 100% O_2 exposure (protocol II). In total, V_T, RR, V_E, and MTEF were measured in 4,063 breaths from 132 tests.

In both protocols, initial hyperoxia (40% or 100% O_2) induced hypoventilation. In 33/39 infants, V_T declined after 2–4 breaths, remained low during 10–25 breaths, and then returned to baseline (Fig. 1). Similarly V_E and MTEF but not RR decreased and increased again (Table 2). V_T decreased irrespective of disease, age, or sedation. We stratified children according to disease, age, and sedation: Mean (95% CI) V_T decrease was 2.9 (1.4; 4.4) ml in the 24 controls, and 6.7 (2.9; 10.4) ml in the 15 infants with CF; 3.0 (1.7; 4.2) ml in the 31 non-sedated (younger) infants, and 9.7 (3.3; 16.2) ml in the eight sedated infants (older, all with CF). Intra-individual variability of V_T increased during initial hyperoxia (Table 2). In both protocols, mean (SD) CV rose from 8.8 (3.6)% at baseline to 15.1 (7.3)%, $P < 0.001$, during initial hyperoxia. Variability of V_T increased irrespective of age, sedation, or disease ($P \leq 0.001$ for all).

The infants' respiratory control apparently adapted to hyperoxia (Figs. 1 and 2). Here, we report ventilatory

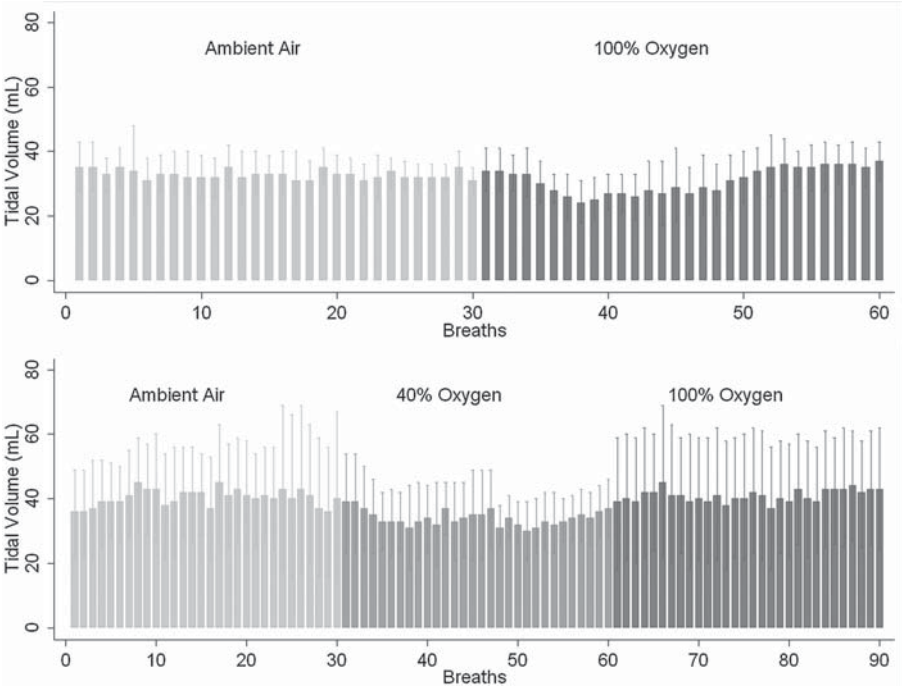

Fig. 1. Tidal breathing pattern during the classical and new nitrogen MBW protocol. The course of tidal volumes (V_T) from 39 infants (16 with CF of which 8 were sedated) across both protocols illustrates transient hypoventilation induced by hyperoxia (40% or 100% O_2). The bars give median and inter-quartile ranges of V_T. V_T slowly returns to baseline during both 100% O_2 (protocol I: 23 infants, 4 sedated; upper panel) and 40% O_2 exposure (protocol II: 16 infants, 4 sedated; lower panel) and remains stable during subsequent 100% O_2 exposure in protocol II.

dynamics from infants in protocol (II). After initial V_T decrease during 40% O_2, V_T returned to baseline during 100% O_2 exposure. Mean (SD) baseline V_T was 51.4 (29.2) ml, V_T during 40% O_2 exposure was 46.4 (26.8) ml, $P = 0.007$ and 51.5 (29.2) ml, $P > 0.9$ during 100% O_2 exposure. Similarly V_E and MTEF returned to baseline while RR remained unchanged. Similarly end-tidal CO_2 levels monitored online during hyperoxia remained stable. V_T variability remained slightly elevated (Table 2).

DISCUSSION

Summary

This is the first study to report on a new infant N_2MBW protocol inducing tolerance to hyperoxia. Infant N_2MBW protocols using 100% O_2 or both 40% and 100% O_2 applied sequentially are safe. Both protocols initially lead to hypoventilation and increased variability of V_T. These V_T dynamics were apparently independent of age, disease, and natural or induced sleep in infants younger than 14 months. Of importance, the infants' hypoventilation is temporary. The new protocol comprising 40% O_2 exposure prior to N_2MBW using 100% O_2 induces tolerance to hyperoxia. Breathing 40% O_2 allows later washout of the lungs' remaining N_2 fraction using pure O_2. The new protocol seems applicable for future N_2MBW studies in healthy infants and infants with CF.

Physiological Aspects of Nitrogen Multiple-Breath Washout in Infants

The principle of N_2MBW is based on lung inherent N_2 washout via breathing pure O_2. It appears that temporary

TABLE 2— Impact of the Classical and New Nitrogen MBW Protocol on Tidal Breathing

	Baseline	Protocol I	Protocol II	
	Ambient air	100% O_2	40% O_2	100% O_2
N	39	23	16	15
	Mean [range]	Mean diff. (95% CI)	Mean diff. (95% CI)	Mean diff. (95% CI)
V_T (ml)	44.7 [23.4–121.0]	−3.9 (−6.1; −1.7)#	−4.9 (−8.0; −1.9)#	0.1 (−2.2; 2.4)*
CV_{VT} (%)	8.8 [4.3–23.1]	8.7 (5.9; 11.5)#	2.2 (−0.8; 5.1)*	2.1 (−0.8; 5.1)*
RR (min^{-1})	39 [24–59]	−1 (−3; 1)*	1 (−2; 4)*	0 (−3; 3)*
V_E (ml min^{-1})	1,575 [980–2,993]	−177 (−246; −108)#	−141 (−251; −31)##	−9 (−102; 119)*
MTEF (ml s^{-1})	46.1 [27.1–80.5]	−6.5 (−8.9; −4.0)#	−3.4 (−7.1; 0.3)*	0.9 (−2.7; 4.5)*

Tidal breathing indices from baseline are given as mean [range], and as mean difference (95% confidence intervals) between (…) baseline and later tests. One infant woke up during breathing 100% O_2. V_T, tidal volume, CV_{VT}, intra-subject coefficient of variation from tidal volume; RR, respiratory rate; V_E, minute ventilation; MTEF, mean tidal expiratory flow. Paired *t*-tests:
#$P \leq 0.003$;
##$P = 0.015$;
*$P \geq 0.100$.

hyperoxia does not influence respiratory control in children older than 4 years,[22] but may trigger the infants' peripheral chemoreceptors which then activate the brainstem respiratory pattern generator network.[15] Hyperoxia may also induce temporary vasoconstriction of cerebral circulation, an effect that theoretically could have contributed to the observed change in ventilation by the induced local increase in tissue CO_2. Alternative pathways mediated by systemic hypercapnia seem less likely as end-tidal CO_2 levels monitored throughout the studies remained stable. During hyperoxia, the respiratory control adapted. Administration of 40% O_2 induced tolerance to hyperoxia leading to normal but slightly more variable V_T during 100% O_2 exposure. Of note, breathing pattern changed in some but not all infants (Fig. 2). Respiratory responses were heterogeneous and apparently independent of age, disease, and sedation.

Methodological Aspects of Nitrogen Multiple-Breath Washout in Infants

Previous MBW techniques based on sulfur hexafluoride or helium are safe and have been frequently used in children. However, sulfur hexafluoride is a potent greenhouse gas and not available in many countries.[1] Helium is highly volatile which increases the risk of gas

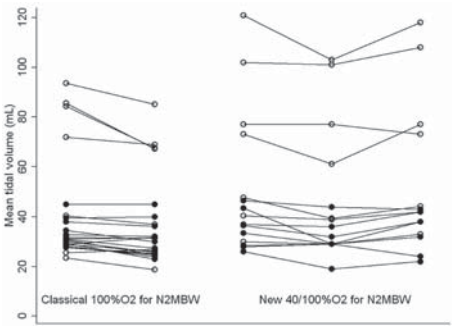

Fig. 2. Impact of the classical and new nitrogen MBW protocol on tidal volumes. Mean tidal volumes during nitrogen multiple-breath washout (N_2MBW) in 23 healthy infants (closed circles) and 16 infants with CF (hollow circles) of which 8 were sedated. In the classical 1-step protocol, infants were exposed to ambient air and 100% oxygen (O_2). In the new 2-steps protocol, infants were exposed to ambient air, 40% O_2, and 100% O_2. Classical protocol on the left panel: 23 infants, 4 sedated; new protocol on the right panel: 16 infants, 4 sedated.

leakage during infant MBW. N_2MBW has regained interest for lung function testing in infants and young children as N_2MBW seems appropriate for widespread use. N_2MBW setups are available and O_2 supply is easily accessible. Modern N_2MBW setups provide online tracing of expired N_2 and flow-volume loops.[22,23] These technical features are also recommended by the new ERS/ATS consensus statement on inert gas washout testing[1] and could make the new N_2MBW protocol attractive for early CF lung disease assessment.[12,22,24,25] Previous studies[12,13] reported initial hypoventilation during classical N_2MBW (100% O_2) in healthy infants and infants with CF, bronchopulmonary dysplasia, or wheezy bronchitis. However analysis of the breathing pattern was presumably done post hoc as online V_T control in previous N_2MBW setups was not available.

Strengths and Limitations of the Study

The ventilatory response to hyperoxia was similar in older sedated sleeping CF infants and younger non-sedated sleeping CF infants. However, carotid chemoreceptor and ventilatory response to hypoxia are known to undergo significant changes early in life. Due to ethical considerations, we did not sedate younger infants as MBW success rate is excellent without sedation in this age group.[26] We acknowledge that consecutive allocation hampered the assessment of the magnitude of the ventilatory chemoreflex and possible interactions between ventilatory response and age and sedation. Allocation ensured equal distribution of healthy and diseased infants and older infants requiring sedation in both protocols.

The current N_2MBW setup is accurate for measuring infant lung volumes as recently shown in a plastic lung model.[23] Due to expected software adaptations for 40% O_2, we could not measure classical in vivo N_2MBW outcomes such as the LCI which may limit the interpretation of the current data. However, because of the complex dynamic respiratory control and the likely change in FRC, changes in, for example, LCI seem also likely (LCI = CEV/FRC).[13] Furthermore, the observed initial depression of the first few breaths when hyperoxia is administered is of importance. The depression in ventilation affects our potential to derive alternative indices of gas mixing efficiency based on phase III slopes (Sacin and Scond). The latter assumptions are supported by recent preliminary data from school-aged children suggesting a significant decrease in gas mixing efficiency upon change in breathing pattern.[27] The impact from both N_2MBW protocols on FRC and LCI in infants nevertheless remains to be determined. Before this is done, MBW tests using foreign inert tracer gases seem preferable in infants.

CONCLUSION

Taken together, we provide new perspectives for future N_2MBW infant studies. Hyperoxia for the purpose of pulmonary function testing is safe but influences ventilatory control in infants. The new 40/100% O_2 protocol comprising 40% O_2 exposure prior to N_2MBW using 100% O_2 may induce tolerance to hyperoxia. The current technique requires adaption for gradual O_2 administration in infants. We assume that continued 100% O_2 exposure, for example for 60 breaths, would similarly induce tolerance to hyperoxia but would not allow subsequent N_2MBW. The new 40/100% O_2 protocol seems an attractive option for N_2MBW in infants.

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REFERENCES

1. Robinson PD, Latzin P, Verbanck S, Hall GL, Horsley A, Gappa M, Thamrin C, Arets HG, Aurora P, Fuchs SI, King GG, Lum S, Macleod K, Paiva M, Pillow JJ, Ranganathan S, Ratjen F, Singer F, Sonnappa S, Stocks J, Subbarao P, Thompson BR, Gustafsson PM. Consensus statement for inert gas washout measurement using multiple- and single-breath tests. Eur Respir J 2013;41:507–522.
2. Kieninger E, Singer F, Fuchs O, Abbas C, Frey U, Regamey N, Casaulta C, Latzin P. Long-term course of lung clearance index between infancy and school-age in cystic fibrosis subjects. J Cyst Fibros 2011;10:487–490.
3. Gustafsson PM, Aurora P, Lindblad A. Evaluation of ventilation maldistribution as an early indicator of lung disease in children with cystic fibrosis. Eur Respir J 2003;22:972–979.
4. Fuchs SI, Toussaint S, Edlhaimb B, Ballmann M, Gappa M. Short-term effect of physiotherapy on variability of the lung clearance index in children with cystic fibrosis. Pediatr Pulmonol 2010;45: 301–306.

5. Gustafsson PM, De Jong PA, Tiddens HA, Lindblad A. Multiple-breath inert gas washout and spirometry versus structural lung disease in cystic fibrosis. Thorax 2008;63:129–134.
6. Owens CM, Aurora P, Stanojevic S, Bush A, Wade A, Oliver C, Calder A, Price J, Carr SB, Shankar A, Stocks J. Lung Clearance Index and HRCT are complementary markers of lung abnormalities in young children with CF. Thorax 2011;66:481–488.
7. Hall GL, Logie KM, Parsons F, Schulzke SM, Nolan G, Murray C, Ranganathan S, Robinson P, Sly PD, Stick SM. Air trapping on chest CT is associated with worse ventilation distribution in infants with cystic fibrosis diagnosed following newborn screening. PLoS ONE 2011;6:e23932.
8. Aurora P, Stanojevic S, Wade A, Oliver C, Kozlowska W, Lum S, Bush A, Price J, Carr SB, Shankar A, Stocks J. Lung clearance index at 4 years predicts subsequent lung function in children with cystic fibrosis. Am J Respir Crit Care Med 2011;183:752–758.
9. Amin R, Subbarao P, Lou W, Jabar A, Balkovec S, Jensen R, Kerrigan S, Gustafsson P, Ratjen F. The effect of dornase alfa on ventilation Inhomogeneity in patients with Cystic Fibrosis. Eur Respir J 2011;37:806–812.
10. Amin R, Subbarao P, Jabar A, Balkovec S, Jensen R, Kerrigan S, Gustafsson P, Ratjen F. Hypertonic saline improves the LCI in paediatric patients with CF with normal lung function. Thorax 2010;65:379–383.
11. Horsley AR, Davies JC, Gray RD, Macleod KA, Donovan J, Aziz ZA, Bell NJ, Rainer M, Mt-Isa S, Voase N, Dewar MH, Saunders C, Gibson JS, Parra-Leiton J, Larsen MD, Jeswiet S, Soussi S, Bakar Y, Meister MG, Tyler P, Doherty A, Hansell DM, Ashby D, Hyde SC, Gill DR, Greening AP, Porteous DJ, Innes JA, Boyd AC, Griesenbach U, Cunningham S, Alton EW. Changes in physiological, functional and structural markers of cystic fibrosis lung disease with treatment of a pulmonary exacerbation. Thorax 2013;68:532–539.
12. Schibler A, Schneider M, Frey U, Kraemer R. Moment ratio analysis of multiple breath nitrogen washout in infants with lung disease. Eur Respir J 2000;15:1094–1101.
13. Gustafsson PM, Källman S, Bhiladvala M, Fletcher ME. N2-washout with pure oxygen reduces tidal volumes markedly in sedated infants. Eur Respir J 1997;10(Suppl. 25):165s.
14. Hertzberg T, Lagercrantz H. Postnatal sensitivity of the peripheral chemoreceptors in newborn infants. Arch Dis Child 1987;62: 1238–1241.
15. Chardon K, Telliez F, Bach V, Leke A, Delanaud S, Bouferrache B, Libert JP, Gaultier C. Effects of warm and cool thermal conditions on ventilatory responses to hyperoxic test in neonates. Respir Physiol Neurobiol 2004;140:145–153.
16. Fuchs O, Latzin P, Kuehni CE, Frey U. Cohort profile: the Bern infant lung development cohort. Int J Epidemiol 2012;41:366–376.
17. Fuchs O, Latzin P, Thamrin C, Stern G, Frischknecht P, Singer F, Kieninger E, Proietti E, Riedel T, Frey U. Normative data for lung function and exhaled nitric oxide in unsedated healthy infants. Eur Respir J 2011;37:1208–1216.
18. Barben J, Gallati S, Fingerhut R, Schoeni MH, Baumgartner MR, Torresani T. Retrospective analysis of stored dried blood spots from children with cystic fibrosis and matched controls to assess the performance of a proposed newborn screening protocol in Switzerland. J Cyst Fibros 2012;11:332–336.
19. Frey U, Stocks J, Coates A, Sly P, Bates J. Specifications for equipment used for infant pulmonary function testing. ERS/ATS Task Force on Standards for Infant Respiratory Function Testing. European Respiratory Society/American Thoracic Society. Eur Respir J 2000;16:731–740.
20. Frey U, Stocks J, Sly P, Bates J. Specification for signal processing and data handling used for infant pulmonary function testing. ERS/ATS Task Force on Standards for Infant Respiratory Function Testing. European Respiratory Society/American Thoracic Society. Eur Respir J 2000;16:1016–1022.
21. Bates JH, Schmalisch G, Filbrun D, Stocks J. Tidal breath analysis for infant pulmonary function testing. ERS/ATS Task Force on Standards for Infant Respiratory Function Testing. European Respiratory Society/American Thoracic Society. Eur Respir J 2000;16:1180–1192.
22. Singer F, Kieninger E, Abbas C, Yammine S, Fuchs O, Proietti E, Regamey N, Casaulta C, Frey U, Latzin P. Practicability of nitrogen multiple-breath washout measurements in a pediatric cystic fibrosis outpatient setting. Pediatr Pulmonol 2012; doi: 10.1002/ppul.22651
23. Singer F, Houltz B, Latzin P, Robinson P, Gustafsson P. A realistic validation study of a new nitrogen multiple-breath washout system. PLoS ONE 2012;7:e36083.
24. Verbanck S, Paiva M, Paeps E, Schuermans D, Malfroot A, Vincken W, Vanderhelst E. Lung clearance index in adult CF patients: the role of convection-dependent lung units. Eur Respir J 2012; doi: 10.1183/09031936.00125312
25. Gappa M, Fletcher ME, Dezateux CA, Stocks J. Comparison of nitrogen washout and plethysmographic measurements of lung volume in healthy infants. Am Rev Respir Dis 1993;148:1496–1501.
26. Latzin P, Roth S, Thamrin C, Hutten GJ, Pramana I, Kuehni CE, Casaulta C, Nelle M, Riedel T, Frey U. Lung volume, breathing pattern and ventilation inhomogeneity in preterm and term infants. PLoS ONE 2009;4:e4635.
27. Yammine S, Singer F, Latzin P. One size does not fit all—impact of the one liter tidal volume breathing protocol on indices from nitrogen multiple-breath washout in children. Eur Respir J 2013; 40:259s.

9.4 Abstract of additional manuscript

Preterm birth, infant weight gain, and childhood asthma risk: a meta-analysis of 147,000 European children

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Background: Preterm birth, low birth weight, and infant catch-up growth seem associated with an increased risk of respiratory diseases in later life, but individual studies showed conflicting results. Objectives: We performed an individual participant data meta-analysis for 147,252 children of 31 birth cohort studies to determine the associations of birth and infant growth characteristics with the risks of preschool wheezing (1-4 years) and school-age asthma (5-10 years).

Methods: First, we performed an adjusted 1-stage random effect meta-analysis to assess the combined associations of gestational age, birth weight, and infant weight gain with childhood asthma. Second, we performed an adjusted 2-stage random-effect meta-analysis to assess the associations of preterm birth (gestational age <37 weeks) and low birth weight (<2500 g) with childhood asthma outcomes.

Results: Younger gestational age at birth and higher infant weight gain were independently associated with higher risks of preschool wheezing and school-age asthma (P <.05). The inverse associations of birth weight with childhood asthma were explained by gestational age at birth. Compared with term-born children with normal infant weight gain, we observed the highest risks of school-age asthma in children born preterm with high infant weight gain (odds ratio [OR], 4.47; 95%CI, 2.58-7.76). Preterm birth was positively associated with an increased risk of preschool wheezing (pooled odds ratio [pOR], 1.34; 95%CI, 1.25-1.43) and school-age asthma (pOR, 1.40; 95%CI, 1.18-1.67) independent of birth weight.Weaker effect estimates were observed for the associations of low birth weight adjusted for gestational age at birth with preschool wheezing (pOR, 1.10; 95%CI, 1.00-1.21) and school-age asthma (pOR, 1.13; 95%CI, 1.01-1.27).

Conclusion: Younger gestational age at birth and higher infant weight gain were associated with childhood asthma outcomes. The associations of lower birth weight with childhood asthma were largely explained by gestational age at birth.

9.5 Abstract of additional manuscript

Early growth characteristics and the risk of reduced lung function and asthma: a meta-analysis of 25,000 children

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Background Children born preterm or with a small-size-for-gestational-age are at increased risk for childhood asthma.

Objective To assess the hypothesis that these associations are explained by reduced airway patency.

Methods We used individual participant data of 24,938 children from 24 birth cohorts to examine and meta-analyze the associations of gestational age, size-for-gestational-age, and infant weight gain with childhood lung function and asthma (age range 3.9 19.1 years). Second, we explored whether these lung function outcomes mediated the associations of early growth characteristics with childhood asthma.

Results Children born with a younger gestational age had a lower FEV_1 (forced expiratory volume in 1 second), FEV_1/FVC (FEV_1/forced vital capacity), and FEF_{75} (forced expiratory volume after exhaling 75% of vital capacity), whereas those born with a smaller size-for-gestational-age at birth had lower FEV_1 but higher FEV_1/FVC (p-values$<$0.05). Greater infant weight gain was associated with higher FEV_1, but lower FEV_1/FVC and FEF_{75} in childhood (p-values$<$0.05). All associations were present across the full range and independent of other early life growth characteristics. Preterm birth, low birth weight and greater infant weight gain were associated with an increased risk of childhood asthma (pooled odds ratio (95%CI): 1.34 (1.15, 1.57), 1.32 (1.07, 1.62) and 1.27 (1.21, 1.34), respectively). Mediation analyses suggested that FEV_1, FEV_1/FVC and FEF_{75} may explain 7 (2, 10) % to 45 (15, 81) % of the associations between early growth characteristics and lung function.

Conclusions Younger gestational age, smaller size-for-gestational-age, and greater infant weight gain were across the full ranges associated with childhood lung function. These associations explain to a substantial extent the risk of childhood asthma.

10 Curriculum vitae

Curriculum vitae available in print version