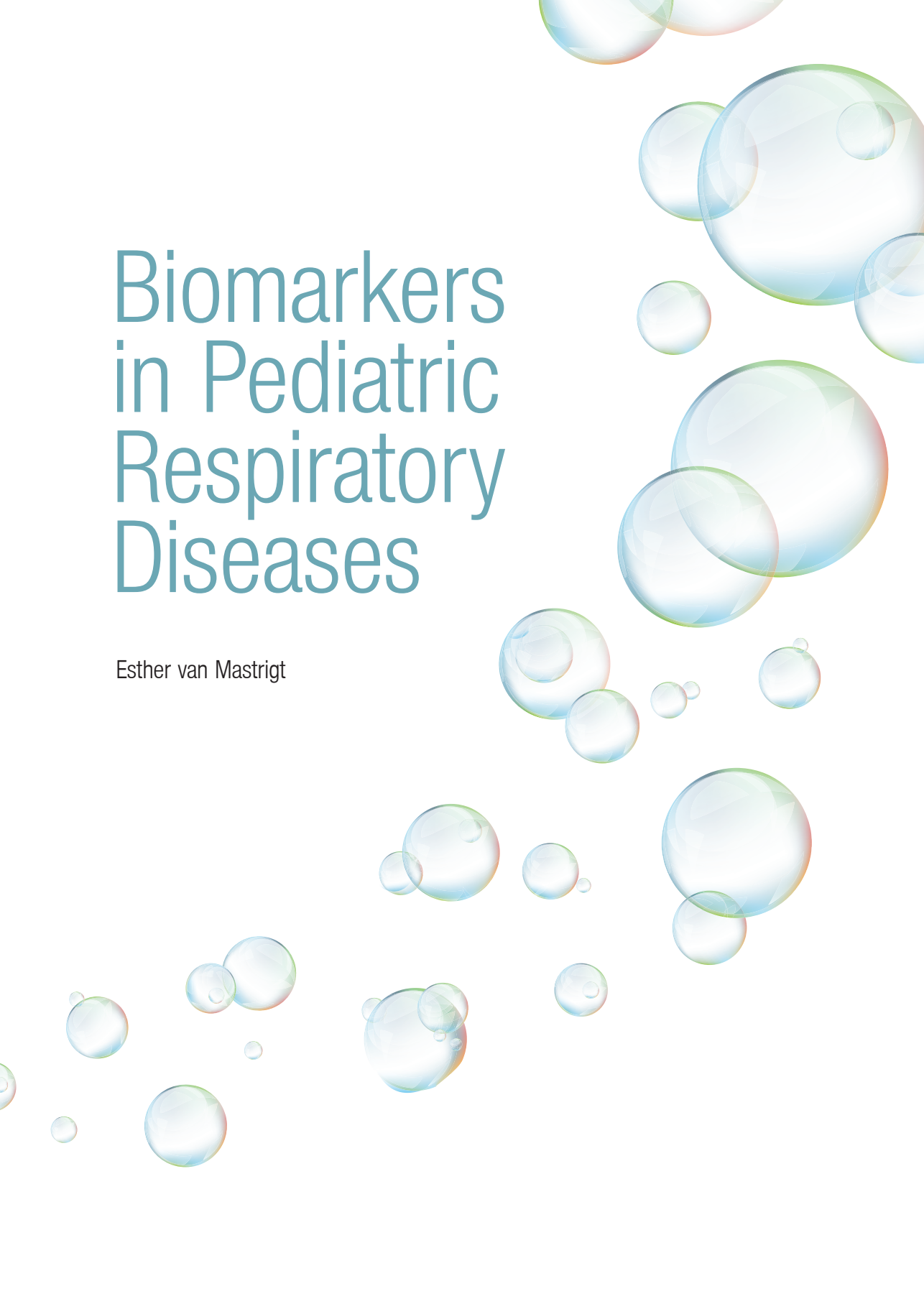
# Biomarkers in Pediatric Respiratory Diseases

Esther van Mastrigt



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# Biomarkers in Pediatric Respiratory Diseases

Het gebruik van biomarkers bij kinderen met longziekten

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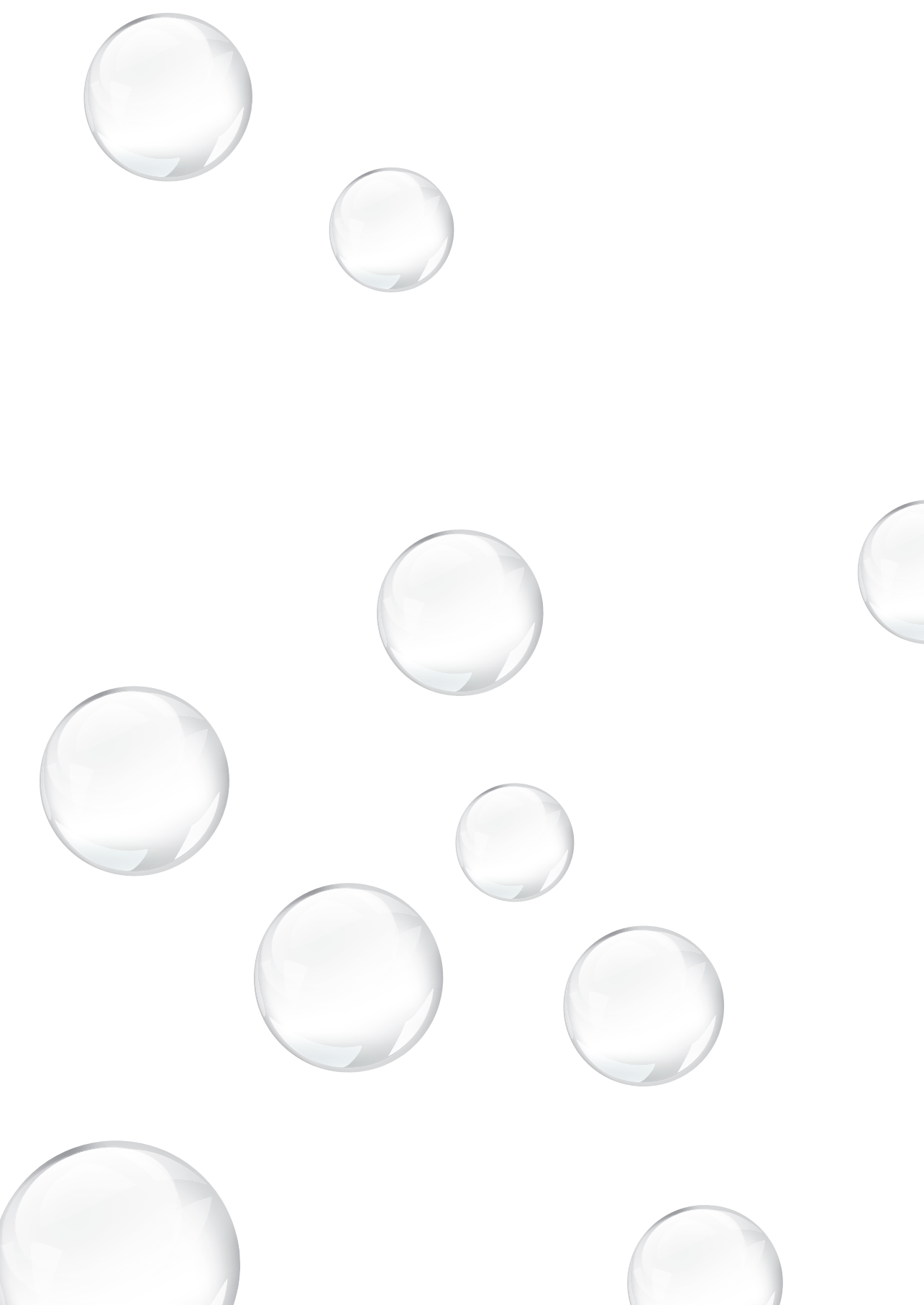
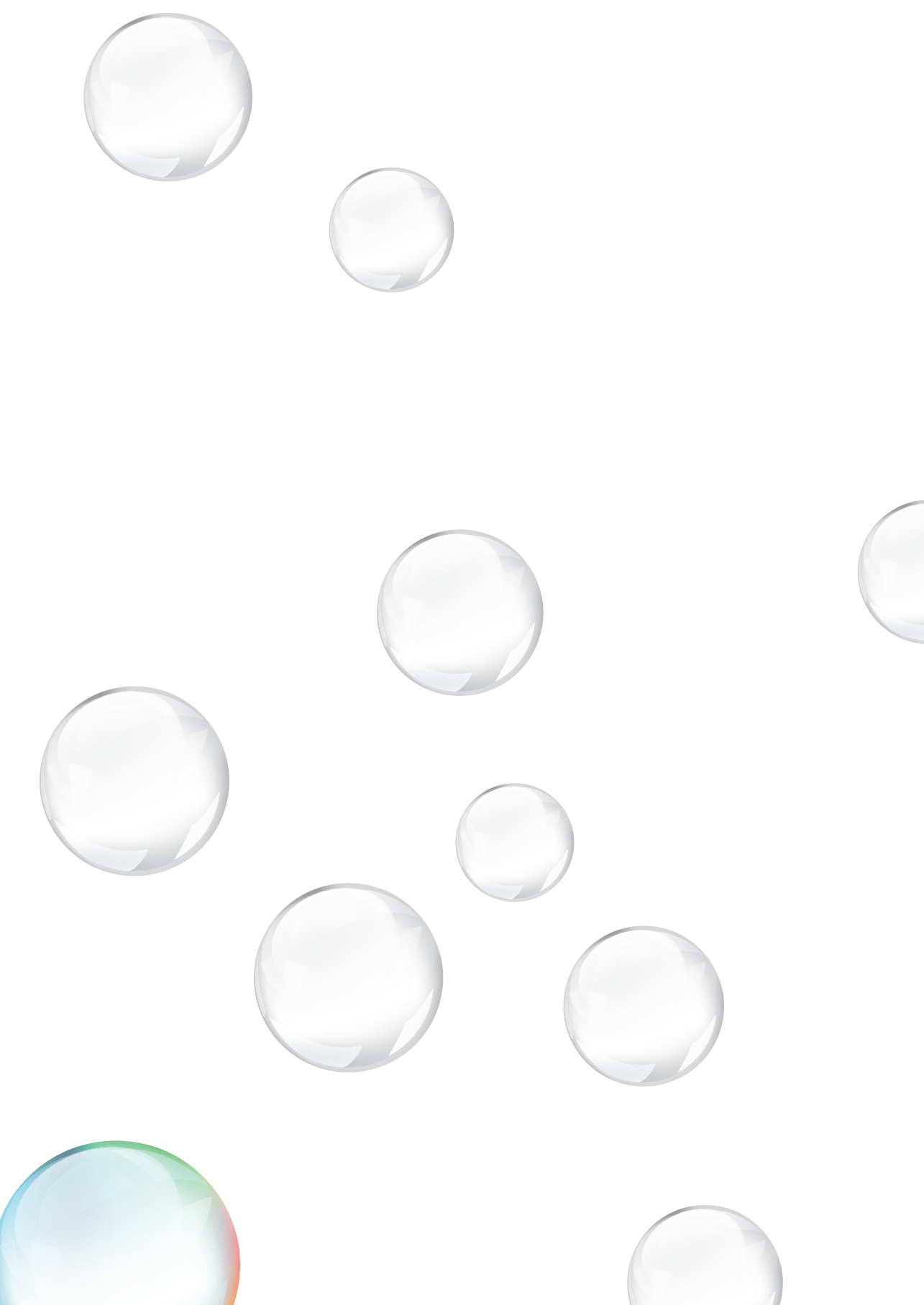


# Part I

## Introduction



# 1 General introduction

Worldwide hundreds of millions of people suffer from chronic respiratory diseases which have major personal, social and economic impact [1]. In the Netherlands about 1 million patients are diagnosed with a chronic respiratory disease. Most chronic respiratory diseases in adult life originate in early childhood [2]. Importantly, lung function is known to track over time: a low lung function during childhood will result in a lower lung function in later life [3]. Patients with a lower maximal lung function level, as a result of respiratory diseases during childhood, will reach lung function values associated with respiratory symptoms and disability much earlier than patients who achieved normal lung function at the start of adulthood (Figure 1) [4].

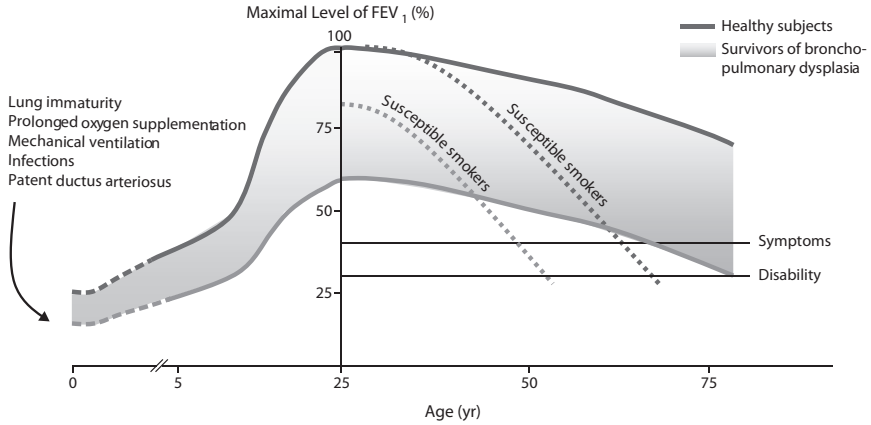



**Figure 1.** Theoretical model of changes in forced expiratory volume in 1 second in healthy subjects and in survivors of bronchopulmonary dysplasia according to age. Adapted from Baraldi *et al.* N Eng J Med 2007;357:1946-55.

Early intervention and improved management of pediatric respiratory diseases today will positively influence adult lung health tomorrow [5]. Therefore, early diagnosis and better monitoring of respiratory diseases in childhood may potentially alter lung growth and prevent lung function decline, thereby improving quality of life and reducing the risk of chronic lung disease in adulthood.

## ROLE OF BIOMARKERS IN THE DIAGNOSIS AND MONITORING OF PEDIATRIC RESPIRATORY DISEASES

The World Health organization defines a biomarker as "any substance, structure, or process that can be measured in the body or its products and influence or predict the incidence of outcome or disease" [6]. Biomarkers can be used as an early marker of disease and may play a role in the diagnostic process. Furthermore, they can give insight in the

pathogenesis of lung disease and may help in monitoring therapeutic effects with the ultimate goal to prevent or attenuate disease progression. Biomarkers in respiratory diseases can be measured systemically (e.g. in blood) or locally in the lung and airways (e.g. in endobronchial biopsies, bronchoalveolar lavage fluid (BALF), sputum, exhaled breath and exhaled breath condensate). Particularly in pediatrics, noninvasive ways to obtain biomarkers are preferred over invasive tests. Thousands of biomarkers have been identified in respiratory diseases, reflecting the complexity of processes in lung and airways. Obviously, single biomarkers are probably not able to reflect the biological complexity in health and disease. An 'omics approach' (e.g. proteomics, metabolomics) enables the identification and quantification of all biological compounds, without a priori hypothesis. Such a profile of biomarkers is the ultimate expression of (epi)genetic information and interaction with environmental agents, micro-organisms, nutritional factors, medication and toxic substances. Hence, this approach allows individual phenotyping and opens the door to personal individualized medicine.

This thesis investigates the role of several biomarkers which may play a role in either inflammation, injury or growth and repair processes of the lungs and airways, which are the hallmarks of many respiratory diseases. The biomarkers studied in this thesis have been obtained from exhaled breath and tracheal aspirates of children and infants with respiratory diseases. The first part of this thesis focuses on diagnosing and monitoring of children with asthma and cystic fibrosis by a new laser-based spectroscopy technique for the detection of volatile organic compounds in exhaled breath. The second part focuses on the pathogenesis and prediction of bronchopulmonary dysplasia and investigates biomarkers involved in alveolar development and pulmonary angiogenesis in tracheal aspirates of preterm infants. In addition, infants who developed severe BPD are included in a clinical follow-up program at the Pediatric Chest Center of Sophia Children's Hospital. Data of this follow-up program are analyzed to investigate both lung structure and ventilator function in these children at 6 months corrected age.

## ASTHMA AND CYSTIC FIBROSIS: EXHALED BREATH ANALYSIS WITH LASER-BASED SPECTROSCOPY

### Asthma and cystic fibrosis

*Asthma*

Asthma is the most common chronic disease in children in the western world, affecting 5-10% of all school children worldwide and in most countries the prevalence is still increasing [7]. Asthma is defined by clinical symptoms like episodic wheezing, dyspnea and/or cough, and is characterized by variable airways obstruction, airways hyper-responsiveness and airways inflammation [8]. Today, treatment of childhood asthma mainly consists of anti-inflammatory drugs, with inhaled corticosteroids (ICS) as first choice, in combination with bronchodilators.

Asthma is a complex heterogeneous disease and despite effective medication many children with asthma suffer poor asthma control with frequent symptoms, nocturnal awakenings, exercise intolerance and impaired lung function. Future risks of asthma are impaired lung function development, exacerbations, emergency care visits, hospital admissions, and even fatal or near-fatal asthma attacks [9].

*Cystic fibrosis*

Cystic fibrosis (CF) is the most common life shortening genetic disease in the Caucasian population, with a prevalence of 70,000 patients worldwide. The disease is caused by impairment or absence of the CF transmembrane conductance regulator (CFTR) protein, which results in reduced or absent chloride secretion and increased sodium absorption, leading to an increase in mucus viscosity and impaired mucociliary clearance [10]. The main cause of both morbidity and mortality in CF is lung disease, characterized by chronic airway inflammation, recurrent airways infections, oxidative stress and fibrosis [11,12].

Like asthma, CF is a very heterogeneous disease. The most prevalent mutation in the CFTR gene in the Netherlands is the dF508 deletion, but nowadays over 2000 different mutations have been described [13]. However, there is poor correlation between genotype and phenotype [14]. The variability in clinical phenotypes and treatment response amongst children with chronic respiratory diseases like asthma and CF stresses the need for more individualized phenotyping and treatment.

## Asthma and CF: role of inflammation

In both asthma and CF chronic airways inflammation, infections and oxidative stress may result in structural changes of the large and small airways.

In asthma, chronic airway inflammation is characterized by the presence of various inflammatory cells (e.g. mast cells, macrophages, eosinophils, neutrophils and T cells) and the release of many inflammatory mediators in the airways [8,15]. Allergic asthma is characterized by infiltration of airway mucosal tissue with eosinophils [16]. The airway pathology in asthma may consist of accumulation of mucus, epithelial shedding, goblet cell hyperplasia, thickening of the epithelial reticular basement membrane and increase in airway smooth muscle mass [17]. Persistent airways inflammation in children with asthma may result in structural, irreversible changes in the airway wall, a complex process called remodeling which eventually may lead to irreversible airway obstruction in adulthood [17,18].

In CF airways inflammation is mainly characterized by persistent and excessive infiltration of airway mucosal tissue with neutrophils. Neutrophils release large quantities of reactive oxygen species (ROS), leading to increased oxidative stress. In addition, the production of proteases like neutrophil elastase causes direct damage to the airway wall by digesting elastin and other structural proteins [19]. Neutrophils and macrophages also generate and stimulate the production of pro-inflammatory cytokines and chemokines, while the airways of CF patients are relatively deficient in anti-inflammatory cytokines and anti-proteases [20,21]. The disruption of this balance causes structural damage, ultimately leading to structural changes of the bronchi, so called bronchiectasis. These structural changes in combination with the increased mucus viscosity creates an ideal environment for overgrowth of several pathogens. In summary, lungs of CF patients are subject to continuous inflammation and recurrent infections, creating a vicious cycle leading to damage of the airways and the lung parenchyma, which is the primary cause of morbidity and mortality.

Current monitoring of asthma is based on clinical history, physical examination, lung function tests, and in CF additionally microbiological studies and imaging of the lungs are used for monitoring. However, neither of these monitoring tools have shown a close association with airways inflammation, the hallmark of these diseases. Bronchoalveolar lavage, endobronchial biopsies and sputum induction are invasive techniques to obtain material from the airways and are not suitable for routine use. Hence, there is a need for noninvasive methods to assess airways inflammation in monitoring asthma and CF.

## Exhaled breath analysis

The sense of smell has been used as a medical diagnostic tool for thousands of years. Already in ancient times Hippocrates suggested that a specific breath odor could reflect a certain disease. Since the fraction of exhaled nitric oxide (FeNO) was recognized as a

noninvasive marker of eosinophilic airway inflammation [22], research into volatile organic compounds (VOCs) in exhaled breath has progressed rapidly. Exhaled breath is a mixture of water vapor, carbon dioxide, oxygen, nitrogen, and small amounts of inert gasses such as nitric oxide and carbon monoxide. In the 1970's Pauling *et al.* were the first to discover that exhaled breath also contains multiple VOCs in very low concentrations [23]. Currently more than 4500 different VOCs have been identified originating from the human body [24]. It is most likely that these VOCs result from metabolic fractioning of larger molecules, however the exact biological source of most VOCs is still unknown. Exhaled VOCs originate from both local (upper and lower airways) and systemic endogenous processes (diffusion from pulmonary capillary bed into the alveolar compartment). In addition, exhaled VOCs can also originate from exogenous sources (environmental contamination) that have been inhaled, which is one of the biggest disturbing factors in breath collection. VOCs in exhaled breath consist of different groups of molecules, e.g. acids, alcohols, aldehydes, esters, furans and ethers, hydrocarbons, halogen containing compounds, ketones, nitrogen, sulfer containing compounds and others [24]. An important group of VOCs in relation to airways inflammation are hydrocarbons. During inflammatory processes, ROS are produced, which react with lipid components in cell membranes resulting in lipid peroxidation and degradation of cell membranes. Hydrocarbons are the stable breakdown products of this lipid peroxidation. The detection of VOC profiles in exhaled breath has the potential to assess the degree of airways inflammation and therefore may have the potential for early detection of exacerbations, to detect the presence of specific micro-organisms and to evaluate treatment effects. Thus, exhaled breath profiling may provide a step towards personalized management of chronic inflammatory respiratory diseases like asthma and CF.

Several methods are available for exhaled breath analysis. Gas chromatography coupled to mass spectrometry (GC-MS) is most commonly used for the identification of VOCs in exhaled breath. This technique is applicable for broad-spectrum detection and quantification of specific VOCs at relatively low concentrations based on their mass-to-charge ratio. Disadvantages include the need for preconcentration of the samples, the fragmentation of molecules and the need for qualified technicians and expensive equipment. Another sensor-based technology is the 'electronic nose' (eNose) [25]. Combined sensor responses provide a real-time analysis of the complete spectrum of VOCs and can be analyzed by pattern recognition algorithms. This technique allows real-time measurement and is rapid, easy to use and relatively inexpensive, however it is not possible to identify separate molecules. We cooperated with the Technical University Delft (Delft, the Netherlands) to develop a broadband quantum cascade laser-based spectroscopy technique, with the aim to combine the advantages of both previously mentioned techniques, to identify and quantify VOCs with the future potential to be miniaturized.

## BRONCHOPULMONARY DYSPLASIA: EARLY MARKERS IN TRACHEAL ASPIRATES AND CLINICAL FOLLOW UP

### Bronchopulmonary dysplasia

Bronchopulmonary dysplasia (BPD) was first described in 1967 by Northway *et al*. The classic form of BPD, now referred to as 'old' BPD, was typically seen in preterm born infants exceeding 1200 gram and 30 weeks gestational age (GA) who received high concentration oxygen therapy and pressure regulated mechanical ventilation for the treatment of respiratory distress syndrome [26]. In these infants pathology was mostly characterized by emphysema, atelectasis, parenchymal fibrosis and airway inflammation [27]. Due to advances in perinatal and neonatal care including antenatal glucocorticoid therapy, surfactant replacement therapy and advanced ventilation strategies, this form of BPD is now rare in infants exceeding 1200 gram and 30 weeks GA [28]. However, the incidence of BPD did not decrease during the last decade due to increased survival of extremely preterm born infants.

*Bronchopulmonary dysplasia*

Bronchopulmonary dysplasia (BPD) is a chronic lung disease and is clinically defined in infants born before 32 weeks of gestational age (GA) as need for oxygen supplementation for ≥ 28 days at 36 weeks postmenstrual age [29]. In Europe the incidence of BPD ranges from 4% at a GA of 31 weeks to 56% at a GA of <26 weeks [30]. BPD is characterized by impaired lung development with fewer and larger alveoli as a result of interrupted alveolar septation and abnormal pulmonary vascular organization [31]. Treatment of infants with BPD is limited and symptomatic with diuretics, (inhaled) corticosteroids, vaccinations and supplemental oxygen as the most frequently prescribed treatments. Survivors of preterm birth and BPD experience long term cardio-respiratory complications, but also neurological and cognitive impairment are common [32].

The risk for BPD increases substantially with lower GA [33] and lower birth weight [34,35]. Extremely preterm infants (< 28 weeks GA) are born when lung development is still in the saccular stage, resulting in alveolar simplification. This 'new' form of BPD is characterized by an impaired lung development with fewer and larger alveoli as a result of interrupted alveolar septation and abnormal vascular organization, while inflammation and fibrosis are less prominent [31]. This suggests that poor lung development at the time of birth and during exposure to mechanical ventilation and supplemental oxygen is crucial for the development of 'new' BPD. The etiology is multifactorial and besides GA at birth, includes (epi)genetic predisposition and exposure to antenatal and postnatal environmental factors, such as chorio-amnionitis or preeclampsia, intrauterine growth

retardation, mechanical ventilation, oxygen exposure, postnatal infections, persistent ductus arteriosus and malnutrition. Even though progress has been made over the last decades in the management of extremely preterm infants with BPD, to date treatment modalities are still limited. Survivors of preterm birth and BPD have more respiratory symptoms, such as wheeze, cough and dyspnea than term and preterm controls without BPD [36-41]. Both preterm infants with and without BPD have obstructive lung function, although the level of obstruction is more pronounced in the children with BPD [42]. Lung CT imaging studies reveal structural abnormalities in almost all patients with BPD, both in the neonatal period [43-45] and in later life [46-51]. Severe BPD is also associated with pulmonary hypertension [52-54]. Besides cardio-respiratory sequelae also neurological and cognitive impairment are common in preterm born infants with BPD [32,39,55].

Many of the above mentioned follow-up studies describe populations born before 1990 and reflect the 'old' BPD population. The increasing rates of preterm birth and the improved survival of especially extremely preterm born infants (< 28 weeks GA) result in an increasing incidence of 'new' BPD. Therefore, it is important to gain more insight in the pathophysiology of this chronic lung disease as well as into the structural and functional respiratory changes across their life course.

**Impaired lung development in BPD**

Prenatal lung development consists of an embryonal and a fetal phase. The fetal phase is subdivided in 3 separate stadia: canalicular, saccular and alveolar phase (Figure 2). In preterm born infants, lung development is still in the saccular phase (24-36 weeks GA), during which lengthening and widening of the saccules distal to the terminal bronchioles take place. In the saccular phase the lungs are immature, which is characterized by insufficient gas exchange, insufficient surfactant production, poor airway supportive structures, reduced antioxidant mechanisms, reduced compliance and insufficient fluid clearance compared to lungs in the alveolar phase. The primary septa in this saccular phase form the basis for a process called secondary alveolar septation, which is crucial for the formation of alveoli. The alveolar phase follows the saccular phase and lasts until 2-3 years of age [56,57,58].

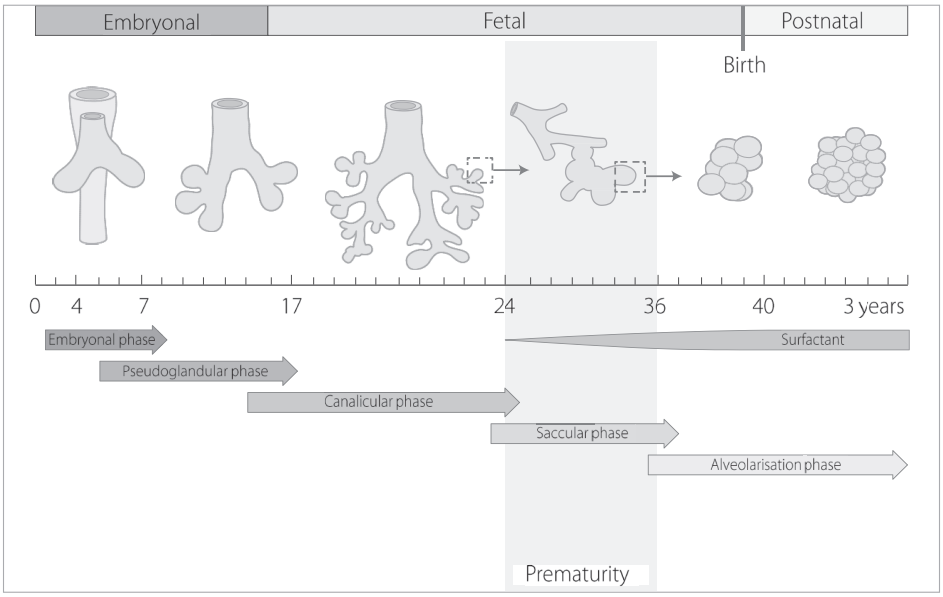



**Figure 2.** Different phases of lung development. Adapted from de Kleer *et al.* Kinderarts en Wetenschap 2014. Oct;11:13-19.

During alveolarisation secondary septa subdivide the immature saccules into smaller alveolar units to increase the surface area for gas exchange. Also, the capillary monolayer is formed reducing the septal thickness and thereby improving gas exchange properties [59]. The most prominent pathological finding in 'new' BPD is an impairment in this alveolar development, resulting in large and simplified airspaces with varying degrees of interstitial fibrosis. Since angiogenesis is a driving force of alveolarisation during normal lung development, disruption of microvascular development in premature lungs has recently been postulated as a critical factor in the alveolar development in BPD [60,61].

## Pathophysiological aspects of bronchopulmonary dysplasia: role of inflammation and angiogenesis

Inflammation is a hallmark of BPD development. Indeed, numerous reports have shown associations between increased cytokine concentrations in blood and BAL fluid and development of BPD, as reviewed elsewhere [62]. This inflammation is often already prenatally induced by maternal factors like chorio-amnionitis [63], and postnatally aggravated by exposure to mechanical ventilation and both hypoxemia and hyperoxia causing acute pulmonary injury followed by an inflammatory response. Both neutrophils and macrophages are immune cells involved in this inflammatory process [64-66]. Activated neutrophils and macrophages produce cytokines, proteases, elastase, collagenase, metallomatrix proteases (MMPs) and oxygen radicals (reactive oxygen species, ROS).

Proteases cause direct damage to the extracellular matrix, in which interstitial capillaries need to grow, hampering secondary alveolar septation. ROS cause direct damage inducing microvascular permeability and edema and inhibit protective anti-proteases. Additionally, the imbalance of oxidative and anti-oxidant properties increases the susceptibility of preterm infants for damage by ROS [67].

Besides the presence of inflammation, BPD is also characterized by abnormal pulmonary vascular development and interrupted septation [60,61,68]. Several pro-angiogenic and anti-angiogenic factors have shown to play a role in these processes: such as vascular endothelial growth factor (VEGF) [69,70], soluble fms-like tyrosine kinase-1 (sFlt-1) [63, 64], placenta growth factor (PlGF) [64, 65], insulin-like growth factor 1 (IGF-1) [71], angiopoietin 1 and 2 [72], platelet derived growth factor (PDGF)-AA [73,74] and transforming growth factor beta (TGF-β) [75,76]. Placental mediated pregnancy complications (e.g. gestational hypertension, preeclampsia and HELLP syndrome) with fetal consequences (e.g. fetal growth restriction) are associated with moderate to severe BPD development [77]. In these disorders increased blood levels of anti-angiogenic factors arising from the placenta have been found [78]. This suggests that an imbalance in circulating pro-angiogenic and anti-angiogenic factors can disrupt vasculogenesis in fetal lungs, which may lead to impaired lung development [79].

## Pathophysiological aspects of bronchopulmonary dysplasia: role of sphingolipids

Sphingolipids are important structure-bearing constituents of cell membranes and function as regulatory molecules in cell proliferation and cell death, endothelial barrier function, angiogenesis, and immune response (Figure 3) [80,81,82].

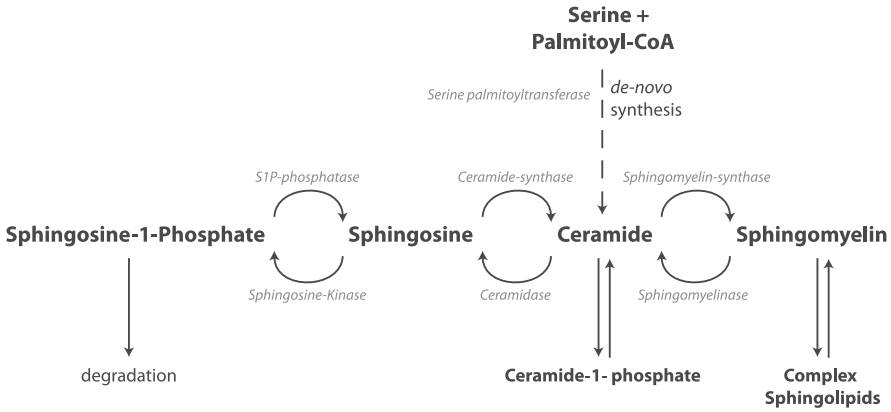



**Figure 3.** Pathway of sphingolipid metabolism. The regulation of sphingolipid metabolism is complicated and involves many enzymes, marked in italics. Adapted from Tibboel *et al.* Chest 2014 Jan;145(1):120-8.

Altered sphingolipid levels have been found in a variety of diseases such as atherosclerosis, chronic heart failure, diabetes mellitus, sepsis, but also in respiratory disorders like asthma, cystic fibrosis and chronic obstructive pulmonary diseases [82]. Sphingolipids are involved in lung development and damage and repair processes after lung injury [81]. Recently, we investigated the role of sphingolipids in a hyperoxia-induced mouse model of BPD [83]. In short, we observed a transient increase in long chain and very long chain ceramides in the first 2-4 weeks of hyperoxia [84]. Additionally, supplementation of D-sphingosine, a synthetic precursor of sphingosine-1-phosphate, during normoxia recovery of hyperoxia-induced lung damage accelerated normalization of ceramides and improved the hyperoxia-induced alveolar arrest in this neonatal mouse model of BPD [84]. We hypothesize that ceramides may be involved in the early stages of BPD development, can be used as an early biomarker for BPD, and might be a promising target for new therapies.

### Tracheal aspirate analysis

In the second part of this thesis we used tracheal aspirates to analyze inflammatory factors, angiogenic growth factors and ceramides in preterm infants who did or did not develop BPD. Tracheal aspirates are suitable substitutes for BALF samples in studies of newborn lung fluid [85,86]. Tracheal aspirates can be collected relatively noninvasively during routine suctioning procedures in children who are mechanically ventilated through an endotracheal tube. Numerous reports have shown the possibility to measure cytokines, chemokines and growth factors in the supernatant of lung-derived fluid including tracheal aspirates [62,87-89]. Over recent years, newer molecular techniques allow simultaneous detection of an increasing number of biomarkers in small samples of body fluid using multiplex assays. Both proteome analysis and multiplex immunoassays (Luminex) are examples of such techniques to identify and quantify multiple biomarkers simultaneously in a systematic matter.

## AIMS AND OUTLINE OF THIS THESIS

The aims of this thesis are:

- To review the use of biomarkers in exhaled breath and exhaled breath condensate in diagnosing and monitoring respiratory diseases in children.
- To study the potential of exhaled breath profiling using broadband quantum cascade laser-based spectroscopy in healthy children and children with asthma and cystic fibrosis.
- To identify early biomarkers for BPD development and possible new targets for therapeutic intervention in BPD.

- To review structural lung abnormalities that have been described on chest CT scans of children and adults with BPD and to propose a new quantitative CT scoring method.
- To investigate the structural and functional pulmonary outcomes of preterm born infants with severe BPD at 6 months corrected age.

**Part I** focuses on exhaled breath analysis in healthy children and children with asthma or CF. In **chapter 2** the current knowledge on the methodological issues and clinical applications of exhaled breath and exhaled breath condensate analysis in children with respiratory disorders is reviewed. In **chapter 3** we present our new broadband quantum cascade laser-based spectroscopy method to detect VOC profiles in exhaled breath. In **chapter 4** we describe the results of a pilot study investigating the clinical applicability of this new exhaled breath analyzing technique in healthy children and in children with asthma or CF.

**Part II** focuses on children with BPD. In **chapter 5** we study the role of inflammation and angiogenesis in BPD development in a prospective study performed in preterm born infants (≤ 32 weeks of GA). We assess whether profiles of monocytes/macrophages and their products in tracheal aspirates correlate with BPD development. In **chapter 6** we aim to evaluate our earlier findings in a mouse model of hyperoxia induced lung injury and investigate the presence of sphingolipids in tracheal aspirates of preterm born infants, and their role as possible early new biomarker for BPD development. At Sophia Children's Hospital Pediatric Chest Center, we follow preterm born children with severe BPD until adulthood in a multidisciplinary team of specialists. In **chapter 7** we review the current literature regarding chest CT scoring in BPD patients and propose a new quantitative scoring system, the 'Perth-Rotterdam Annotated Grid Morphometric Analysis', PRAGMA-BPD scoring system. In **chapter 8** we present follow-up data of severe BPD patients, combining information on lung structure, function and postnatal growth.

In **chapter 9** we discuss our main findings and conclusions and give recommendations for future research. In **chapter 10** our findings, as described in this thesis, are summarized in English and in Dutch.

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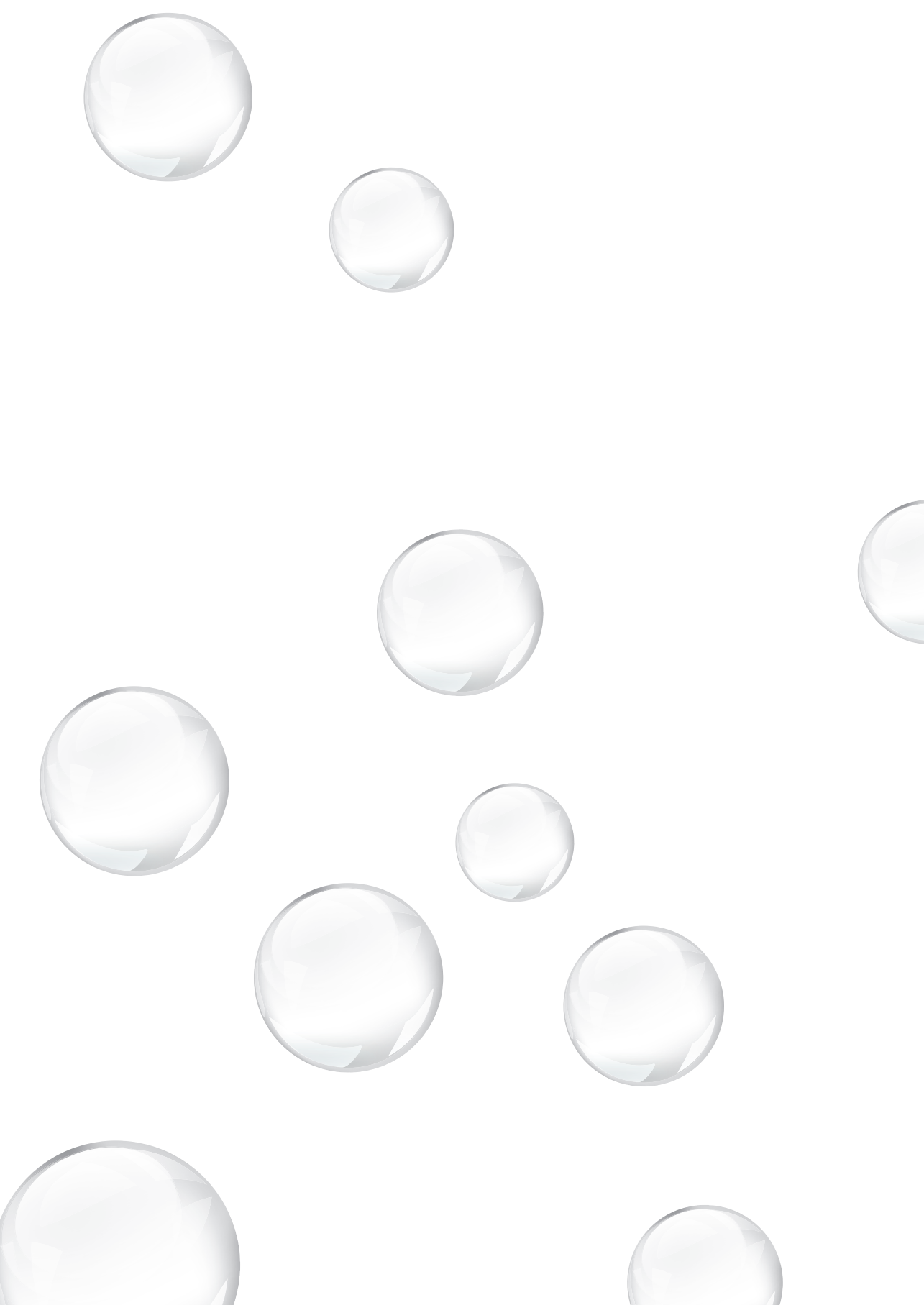
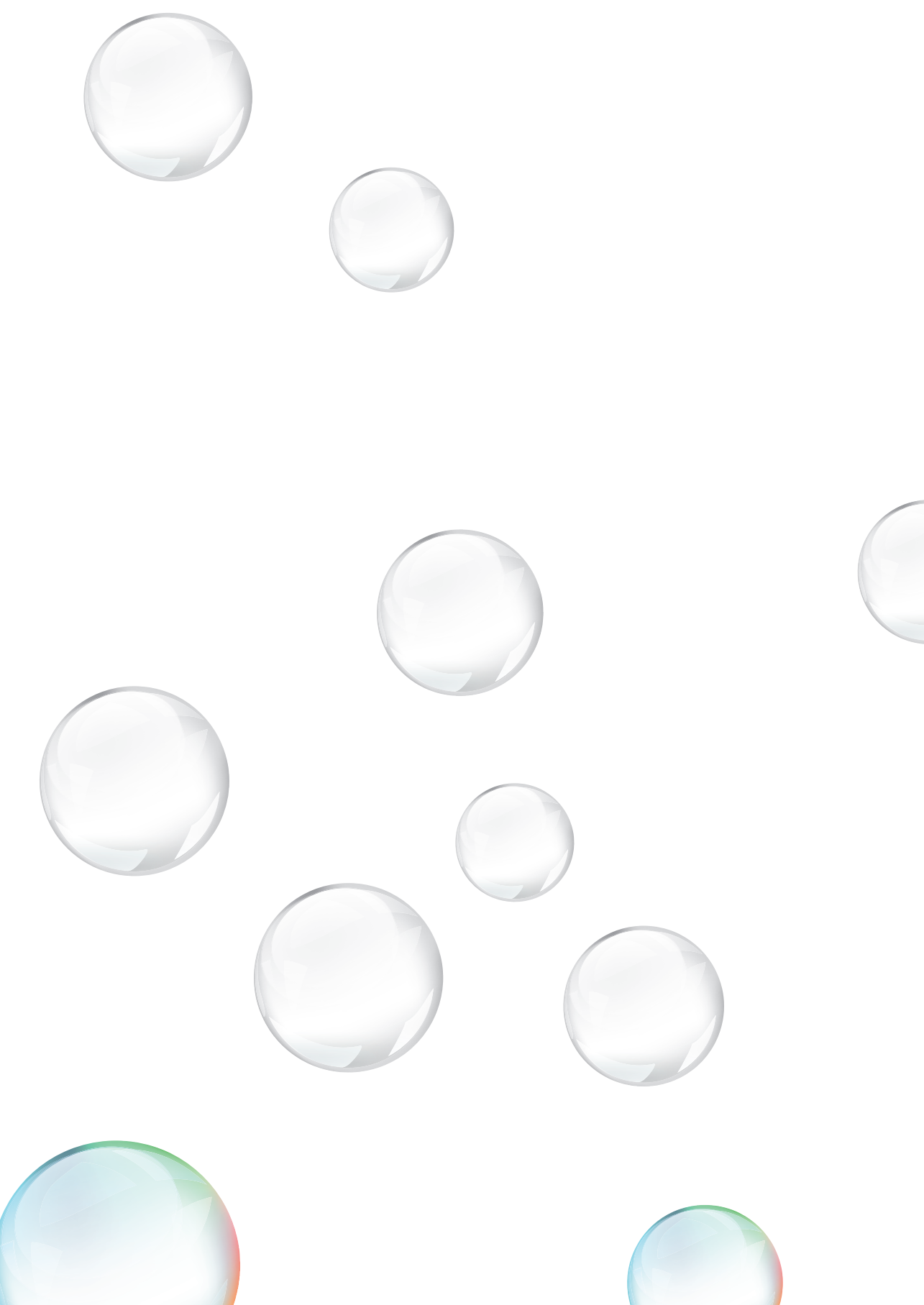


# Part II

## Asthma and cystic fibrosis: exhaled breath analysis with laser-based spectroscopy



# 2 The analysis of volatile organic compounds in exhaled breath and biomarkers in exhaled breath condensate in children

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## ABSTRACT

Current monitoring strategies for respiratory diseases are mainly based on clinical features, lung function and imaging. As airway inflammation is the hallmark of many respiratory diseases in childhood, noninvasive methods to assess the presence and severity of airway inflammation might be helpful in both diagnosing and monitoring pediatric respiratory diseases. At present, the measurement of fractional exhaled nitric oxide is the only noninvasive method available to assess eosinophilic airway inflammation in clinical practice.

We aimed to evaluate whether the analysis of volatile organic compounds (VOCs) in exhaled breath (EB) and biomarkers in exhaled breath condensate (EBC) is helpful in diagnosing and monitoring respiratory diseases in children.

An extensive literature search was conducted in Medline, Embase and PubMed on the analysis and applications of VOCs in EB and EBC in children.

We retrieved 1165 papers, of which 9 contained original data on VOCs in EB and 84 on biomarkers in EBC. These were included in this review. We give an overview of the clinical applications in childhood and summarize the methodological issues.

Several VOCs in EB and biomarkers in EBC have the potential to distinguish patients from healthy controls and to monitor treatment responses. Lack of standardization of collection methods and analysis techniques hampers the introduction in clinical practise. The measurement of metabolomic profiles may have important advantages over detecting single markers. There is a lack of longitudinal studies and external validation in order to reveal whether EB and EBC analysis have added value in the diagnostic process and follow-up of children with respiratory diseases. In conclusion, the use of VOCs in EB and biomarkers in EBC as markers of inflammatory airway diseases in children is still a research tool and not validated for clinical use.

## INTRODUCTION

The analysis of volatile and non-volatile substances in exhaled breath (EB) and exhaled breath condensate (EBC) for diagnosis and management of respiratory and nonrespiratory diseases has raised great interest. Current monitoring strategies of chronic inflammatory lung diseases such as asthma and cystic fibrosis (CF) are mainly based on clinical features, lung function and imaging techniques. However, neither of these have shown a close association with airway inflammation, a hallmark of these diseases. Available techniques to quantify airway inflammation, including assessment of biomarkers in bronchoalveolar lavage fluid, induced sputum and endobronchial biopsies are invasive and not suitable for routine use. In pediatric pulmonary medicine, noninvasive methods to assess airway inflammation might be helpful in diagnosing and monitoring airway diseases. The analysis of EB and EBC has the potential to meet these expectations. Research into noninvasive assessment of airway inflammation has progressed rapidly since it was recognized that exhaled nitric oxide (eNO) is elevated in exhaled air of asthmatic patients as compared to healthy persons [1,2]. However, the use of only a single biomarker cannot be expected to reflect complex pathological processes, nor to monitor heterogeneous diseases such as asthma. A profile of several biomarkers has the potential to reflect disease phenotypes and may provide a key step towards personalized medicine. In this review we will give an overview of methodological issues and clinical applications of EB and EBC analysis in children with respiratory diseases.

## MATERIALS AND METHODS

We conducted a literature search on the clinical applications of volatile organic compounds (VOCs) in EB and EBC analysis in children, in Medline, Embase and PubMed. The last search was run on July 15, 2013. Key words/Mesh terms included: Breath Tests (Mesh), Volatile Organic Compounds (Mesh), Biological Markers (Mesh), breath analysis, Infant (Mesh), Pediatric (Mesh), Adolescent (Mesh). The search was limited to articles published in English. We retrieved 1165 articles. Reference lists were reviewed for additional references. To be included in this review, studies had to address original data, either VOC profiles in EB or at least one EBC marker, in children up to 18 years of age with a respiratory disease. Finally, we included 151 articles, of which 9 clinical studies regarding VOC profiles in exhaled breath and 84 clinical studies regarding biomarkers in EBC. This could be randomized controlled trials, case-control studies, observational studies and case reports. Fifty-eight additional papers, arguing methodological issues on EB and EBC analysis, were retrieved. The great majority of the reviewed articles did

not meet criteria for formal grading of quality, so we decided not to grade, but to focus on clinical applicability.

## EXHALED BREATH

Volatile organic compounds in exhaled breath most likely result from metabolic fractioning of larger molecules. However, the exact biological source of most VOCs is still unknown [3]. VOCs can originate not only from the lungs or upper airways but also from the circulation and diffuse from the lung capillary bed into the alveoli [4]. Therefore, the measurement of VOCs is a potential diagnostic tool not only for diseases of the respiratory tract, but also for nonrespiratory diseases. In this review we will focus on VOCs in respiratory disorders (Table 1).

### Methodology of exhaled breath collection and processing

The collection of VOCs is influenced by environmental, physiological and methodological factors. In general, these can be divided in precollection, collection and postcollection conditions.

*Precollection conditions*

Volatile organic compounds are abundant in ambient air, and significant linear relationships between ambient and exhaled concentrations of VOCs, such as ethane, propane, pentane and methanol, were demonstrated [5]. To minimize the influence of ambient VOCs, various techniques have been proposed. These include prior inhalation of purified air [6], tidal breathing through a charcoal filter [7,8] and correction for the calculated alveolar gradient [5,9-11]. None of these techniques has proven superior, but for every substance in EB contamination from ambient air should be considered. Also, intake of food and beverages, smoking habits and medication have been shown to influence exhaled VOC profiles. Not surprisingly, several studies have shown that both active and passive smoking influence components in EB [4,12]. Similarly, medication like inhaled corticosteroids (ICS) influences VOC patterns due to either exogenous contamination or indirectly by their effect on the disease process [6]. Patient characteristics, such as age and gender, also influence VOC patterns in EB as lung volume, pulmonary circulation and metabolism may contribute to variation in VOC patterns [6,13,14].

*Collection conditions*

Both breathing manoeuvres and sampling materials can potentially influence the composition of EB. EB is a mixture of alveolar air and environmental air from the dead space of the conducting airways. Alveolar air has undergone gaseous exchange with

**Table 1.** Summary of studies on VOC profiles in exhaled breath of children with respiratory diseases

| First author (year publication) | Groups (nr of children) | Age range (years) | Collection method | Storage | Analysis technique | No. of markers | Main results (sensitivity/specificity in %) |
|---|---|---|---|---|---|---|---|
| **Paff *et al.* (2013)** | CF (25), PCD (25) and healthy controls (23) | 5-18 | Tidal breathing: inspiration through VOC filter, expiration into spacer connected to eNose | Not applicable, online analysis | eNose (Cyranose 320) | N.S. | Breath prints significantly different between CF and healthy (84/65), PCD and healthy (88/52) and CF and PCD (84/60). In CF and PCD different breath prints in patients with and without exacerbation. |
| **Robroeks *et al.* (2013)** | Asthmatic children (40) prospective follow-up | 6-16 | 1 to 3 uncontrolled exhalations after deep inhalation | 5 L Tedlar bag, emptied over stainless steel sorption tubes containing active carbon | GC-ToFMS | 6 | Total 3434 different VOCs. Optimal prediction of exacerbations within patients based on 6 VOCs (100/93). 5 VOCs identified. |
| **van de Kant *et al.* (2013)** | Preschool with (202) and without (50) recurrent wheeze | 2-4.5 | Tidal breathing | 1 L Tedlar bag with non-rebreathing valve, emptied over stainless steel sorption tube | GC-ToFMS | 28 | Total 913 different VOCs with a prevalence > 7%. Discrimination between preschool children with and without wheeze based on 28 VOCs (84/80). 21 VOCs identified. |
| **Caldeira *et al.* (2012)** | Allergic asthma (32) and healthy controls (27) | 3-16 | One deep exhalation after 5 seconds breath holding | 1L Tedlar bag | HS-SPME/GC x GC-ToFMS | 9 | Distinguish patients with allergic asthma from healthy controls based on 9 VOCs (98/93). All 9 VOCs identified. |
| **Caldeira *et al.* (2011)** | Allergic asthma (35) and healthy controls (15) | 4-13 | One deep exhalation after 5 seconds breath holding | 1L Tedlar bag | HS-SPME/GC-qMS | 28 | Total 44 VOCs detected (31 identified). Based on 28 VOCs correct classification of allergic asthma in 88%. |
| **Dallinga *et al.* (2010)** | Asthma (63) and healthy controls (57) | 5-16 | 3 uncontrolled exhalations after deep inhalation | 5L Tedlar bag, within 1 hour emptied over stainless steel sorption tube | GC-ToF-MS | 6 | Able to discriminate between children with asthma and healthy based on 6 VOCs (89/95). All discriminative VOCs identified. |

**Table 1.** Summary of studies on VOC profiles in exhaled breath of children with respiratory diseases (continued)

| First author (year publication) | Groups (nr of children) | Age range (years) | Collection method | Storage | Analysis technique | No. of markers | Main results (sensitivity/specificity in %) |
|---|---|---|---|---|---|---|---|
| **Robroeks *et al.* (2010)** | CF (48) and healthy controls (57) | 5-25 | 3 uncontrolled exhalations | 5L Tedlar bag, within 1 hour emptied over stainless steel sorption tube | GC-MS | 1-26 | Total 6000 different VOCs: 1099 with a prevalence > 7%.<br>Correct discrimination with 1-26 VOCs (58-100/ 91-100).<br>10 most discriminative VOCs all identified.<br>100% correctly classification of CF patients with *Pseudomonas aeruginosa (Pa)* infection based on 14 VOCs vs CF without *Pa*. |
| **Barker *et al.* (2006)** | Cystic fibrosis (20) and healthy controls (20) | 8-29 | Deep inspiration, 5 second breath-hold followed by slow and complete exhalation over 10 seconds, the first 2 seconds are discarded | 6L canister of electro polished stainless steel with a fused silica inner lining | GC-FID-MS | 12 | Higher pentane and lower DMS in children with CF. Higher pentane in children with acute exacerbation or chronic *Pa* infection. Correlation with $FEV_1$. |
| **Delfino *et al.* (2003)** | Asthma (26) | 10-16 | 2 minute collection period, inhalation true activated-carbon-filter and exhalation into an evacuated stainless steel canister | Evacuated stainless steel canister | GC-MS | 8 | Possible association between benzene and symptom episodes. Multiple ambient VOCs (benzene, toluene, m,p-xylene and o-xylene) showed strong association with asthma symptoms. |

Definitions of abbreviations: CF: cystic fibrosis; PCD: primary ciliary dyskinesia; eNose: electronic nose; GC-ToFMS: gas chromatography-time of flight mass spectrometry; VOCs: volatile organic compounds; HS-SPME/GCxGC-ToFMS: head space solid phase micro extraction/two dimensional gas chromatography- time of flight mass spectrometry; qMS: fast quadruple mass spectrometry; GC-FID-MS: gas chromatography flame ionization detector; DMS: dimethyl sulphide. ; $FEV_1$: forced expiratory volume in 1 s.

the blood, and its composition may therefore specifically reflect systemic diseases. In contrast, components derived from the conducting airways provide information that is relevant to airway diseases. To determine the anatomical origin of exhaled breath, simultaneous measurement of $CO_2$ in exhaled air has been proposed [9,10,15]. In contrast to eNO which is highly flow dependent [16], only limited influence has been reported of both breathing pattern and expiratory flow on exhaled VOC profiles and current data do not suggest that breathing manoeuvres need to be standardized [6,17-19].

Several techniques to collect EB samples have been described. Breath samples can be collected for offline analysis via canisters, bags, syringes or sorbent tubes, or can be directly exhaled via inert tubing into an analyser for online measurement. Reservoirs carry the risk of being permeable for certain VOCs, absorb VOCs, or release VOCs themselves. A suitable material is Tedlar, which is chemically inert to most compounds, impermeable for gases and does not absorb molecules. However, Tedlar bags release (N,N-dimethyl) acetamide and phenol in relatively high concentrations [4]. Siliconized metal canisters and thermal desorption tubes have reliable storage properties for many VOCs, but are expensive [20]. Furthermore, one should be aware of contamination by disposable plastic components in mouth pieces, tubes and valves.

*Postcollection conditions*

Several techniques have been used for the detection and analysis of VOCs in exhaled breath samples. Gas chromatography coupled with mass spectrometry (GC-MS) or flame ionization detection (GC-FID) is most commonly used. These techniques can detect and quantify specific VOCs at relatively low concentrations. Disadvantages include the need for preconcentration of the samples, mainly by solid-phase micro-extraction, and the need for highly qualified technicians and expensive equipment [11]. Other techniques also make use of mass spectrometry as detection technique, such as selected ion flow tube (SIFT-MS) and proton transfer reaction (PTR-MS), and have the advantage to enable real time measurement. Ion mobility spectroscopy and laser spectroscopy also allow for real-time measurement, although detection is restricted to those substances which can be ionized or can absorb the laser within the specific wavelength spectrum. Breath samples can also be analysed using different sensor-based techniques, such as colorimetric sensor array, gold nanoparticle sensors and the electronic nose. These techniques make use of an array of specific sensors of which the optical, chemical or electrical properties change when they interact with the molecules present in EB samples [21]. Advantages of these techniques include that pretreatment of samples is not required, online real-time measurement is possible and that they are rapid, easy to use and relatively inexpensive. The combined sensor responses provide a spectrum of VOCs, but determination of individual molecular components is not possible. This is a major disadvantage, which limits the opportunity to understand the pathophysiology of diseases. Moreover, the results of

individual analysers are not comparable [22]. Presently, sensor-based techniques are being studied for their possible clinical applications.

### Clinical applications in pediatric respiratory diseases

*Asthma and allergy*

Overall, VOCs in EB samples can discriminate asthmatic from healthy children, and atopics from nonatopics (Table 1) with a relatively high sensitivity and specificity [23-25] and limited intra-individual variability [17,23,24]. A combination of six VOCs was able to predict exacerbations in asthmatic children [26]. Collection of VOCs is also feasible in preschool children, and it has been shown that VOC profiles differ between children with and without recurrent wheeze [27]. Overall, the best discriminating VOCs for asthma seem to be hydrocarbons [23-25,27]. However, each individual study demonstrates a different superior set of VOCs, and there clearly is a need for external validation. Also, further research is needed on the effects of disease severity and ICS on VOCs in children with asthma. Hence, VOCs in EB may be helpful for asthma diagnosis, but their role in asthma monitoring is still unclear.

*Cystic fibrosis*

Only one study investigated VOC profiles in 48 children with CF compared to 57 healthy controls. About 6000 different VOCs were identified and by using 22 VOCs, 100% correct identification of CF patients and controls was possible with good short-term reproducibility [17]. In addition, VOC profiles could discriminate CF patients with positive cultures for *Pseudomonas aeruginosa* (Pa) from patients with negative cultures and predict CF exacerbations [17]. As Pa colonization is associated with a less favourable prognosis in CF, early detection is important to facilitate early eradication; especially in young children who are not able to produce adequate sputum samples, the analysis of EB may prove useful.

The electronic nose was able to distinguish children with CF or primary ciliary dyskinesia (PCD) from healthy controls, and children with and without a pulmonary exacerbation; however data on longitudinal patterns are lacking [28].

## EXHALED BREATH CONDENSATE

Contrary to EB, EBC is thought to reflect the epithelial lining fluid and therefore, studies of EBC have been limited to respiratory disorders. EBC is collected by cooling the exhaled air of a tidally breathing subject [29]. Multiple biomarkers have been detected in EBC, such as acidity, nitrogen oxide-related compounds, oxidative stress markers, eicosanoids,

cytokines and others. Airway acidity is the simplest and best validated marker in EBC. EBC pH is reproducible [30,31] and normative data have been published [32].

With eNO as an established biomarker of eosinophilic airway inflammation, the measurement of metabolites of nitrogen oxides (NOx) in EBC, such as nitrate, nitrite, nitrosothiols, peroxynitrite and nitrotyrosine, may also be informative. In the presence of airway inflammation, increased amounts of reactive oxygen species (ROS), such as hydrogen peroxide ($H_2O_2$), are released and can be measured in EBC. ROS induce lipid peroxidation that results in the formation of isoprostanes. Other eicosanoids, such as prostaglandins, leukotrienes, hydroxyeicosatetraenoic acids (HETE), eoxins and lipoxins can also be measured in EBC [33]. Cytokine levels have been measured with varying success. In general, cytokine concentrations in EBC are at or below the detection limit in most samples.

Collection of EBC is simple, safe, noninvasive and highly repeatable. In an attempt to standardize EBC measurements and compare results between centres and between studies, the American Thoracic Society (ATS) and European Respiratory Society (ERS) published methodological recommendations for collection and analysis of EBC samples in 2005 and 2010, and the recommendations will be updated soon [34,35]. Today, several methodological issues, such as variability in collection devices, the issue of dilution and the sensitivity of available assay techniques, remain a challenge [36]. We will briefly discuss the methodology of EBC collection and processing, and focus on the clinical application of EBC markers in pediatric respiratory disorders (Table 2).

## Methodology of EBC collection and processing

Exhaled breath condensate can be easily collected during tidal breathing, even in very young children. Again several methodological factors are critical for the study of biomarkers in EBC, separated in precollection, collection and postcollection conditions.

### *Precollection conditions*

Most physiological and environmental factors, which can potentially influence the composition of EBC, have not been studied systematically. Overall, biomarkers in EBC show no relation with gender and age [32,37-40] except for cysteinyl leukotrienes and 8-isoprostane, which both increase with age, and pH, which decreases with age [41,42].

Active smoking influences biomarker composition of EBC [43]. The effect of passive smoking seems less clear. A study in healthy children showed no influence of passive smoking on $H_2O_2$ concentrations in EBC [44]. It was recommended to document smoking exposure and refrain from smoking at least 3 h before EBC sampling [34]. Also beverages significantly influence, for example, EBC acidity [45]. Similarly, the ATS/ERS taskforce advised to refrain from eating and drinking for a period of a few hours before collecting EBC [34].

**Table 2.** Exhaled breath condensate markers and potential clinical value in childhood respiratory disorders

| Marker | Pathophysiological background | Age (years) | Methodological issues | Potential clinical value in asthma | Potential clinical value in other respiratory diseases | References |
|---|---|---|---|---|---|---|
| ***Acidity*** | | | | | | |
| **pH** | Altered pH homeostasis in inflammatory diseases. Acidification of airway surface liquid leads to smooth muscle contraction, increased mucous viscosity, decreased ciliary beat frequency. | 0-18 | - Good repeatability<br>- Significantly higher pH values after deaeration<br>- Considerable overlap in pH values between patients and controls<br>- Reference values from healthy controls available [32] | - Lower EBC pH compared with healthy<br>- Steroid naïve patients lower values than steroid treated<br>- Atopy seems to have no influence on pH<br>- In some studies related to disease severity<br>- No correlation with lung function | - Lower pH in CF vs healthy<br>- During CF exacerbation lower pH compared with stable disease<br>- Conflicting results for changes in pH after treatment of CF exacerbation | Asthma: (30, 55, 62-71, 73-75, 88, 120, 139)<br>Other: (64, 121-123, 140) |
| **Ammonium** | Produced by glutaminase. Glutaminase expression and activity are increased by low pH and reduced by pro-inflammatory cytokines. Corticosteroids upregulate glutaminase. | 5-18 | - Correlated with pH | - Lower ammonium in asthma vs healthy<br>- Steroid naïve patients lower values than steroid treated<br>- No correlation with lung function | - Lower ammonium in CF vs healthy<br>- No influence of antibiotic treatment in CF | Asthma: (65, 69)<br>Other: (123) |
| ***NO metabolites*** | | | | | | |
| **Nitrite and nitrate** | In aqueous solutions NO reacts with ROS to form stable oxides of nitrogen, such as nitrite and nitrate. Acidity increases conversion of nitrite into nitrate. | 1.5-17 | - Correlation between nitrite and nitrate<br>- Considerable intra- individual variability<br>- Consider salivary contamination | - Elevated nitrite in asthma vs healthy<br>- Elevated nitrite in preschool wheezers, possible predictor of later asthma<br>- No difference between stable condition vs exacerbation<br>- Possible indicator for asthma severity<br>- Not predictive for exacerbations<br>- No correlation with lung function or FeNO | - Higher nitrite in CF vs healthy<br>- No difference in nitrate between CF and healthy | Asthma: (70, 73, 79, 80, 82)<br>Other: (39, 40, 83, 125, 126) |

**Table 2.** Exhaled breath condensate markers and potential clinical value in childhood respiratory disorders (continued)

| Marker | Pathophysiological background | Age (years) | Methodological issues | Potential clinical value in asthma | Potential clinical value in other respiratory diseases | References |
|---|---|---|---|---|---|---|
| **3-Nitrotyrosine** | Nitration of tyrosine leads to formation of 3-NT, a biomarker for the generation of reactive nitrogen metabolites. | 5-17 | | - Conflicting results regarding difference between asthma and healthy<br>- Not related to asthma severity and no effect of steroids<br>- No correlation with FeNO | - Single study showed no difference between CF, asthma or healthy | Asthma: (77, 78, 81) Other: (81) |
| ***Oxidative stress*** | | | | | | |
| **$H_2O_2$** | Superoxide is rapidly metabolized to $H_2O_2$. Multiple sources, not limited to inflammatory cells. Associated with different lung diseases. | 5-18 | - Different $H_2O_2$ concentration in different lung compartments<br>- Reference values in healthy children available [37] | - Elevated in patients with asthma<br>- Not related to asthma severity<br>- Not useful to predict exacerbations<br>- No effect of ICS<br>- No correlation with lung function | - Conflicting results in CF<br>- Single study found decrease after antibiotic treatment in CF | Asthma: (37, 73, 84-88) Other: (125, 128) |
| **Gluthatione** | Anti-oxidant | 6-18 | - Corticosteroids increase gluthatione synthase | - Lower values in asthma<br>- Not related to asthma severity<br>- Increased during asthma exacerbation<br>- Decreased after treatment with oral corticosteroids | - Reduced in allergic rhinitis, similar values as in patients with asthma | Asthma: (91-93) Other: (93) |
| **Malon-dialdehyde** | End product of lipid peroxidation | 6-18 | | - Increased in asthma<br>- Not related to asthma severity | - Increased in allergic rhinitis, similar values as in patients with asthma | Asthma: (91-93) Other: (93) |
| **ADMA** | L-arginine analogue:<br>- inhibits NOS resulting in reduction of NO synthesis<br>- promotion superoxide formation<br>- higher levels peroxynitrite | 5-16 | - Single study | - Elevated in asthma | Not described. | Asthma: (90) |

**Table 2.** Exhaled breath condensate markers and potential clinical value in childhood respiratory disorders (continued)

| Marker | Pathophysiological background | Age (years) | Methodological issues | Potential clinical value in asthma | Potential clinical value in other respiratory diseases | References |
|---|---|---|---|---|---|---|
| **Free radicals** | Consist of ROS and RNS, both oxidants. | 6-17 | - Single study | Not described. | - Elevated in CF, but not different from healthy | Other: (129) |
| **Urate** | Anti-oxidant, inhibits peroxidation by binding ROS. | 5-15 | - Single study | - Increased urates in controlled asthma compared to exacerbation and healthy controls | Not described | Asthma: (141) |
| ***Eicosanoids (prostaglandins)*** | | | | | | |
| **8-Isoprostane** | Produced due to lipid peroxidation of arachidonic acid, catalyzed by ROS. Specific marker for oxidative stress. Possible role in inducing bronchial smooth muscle contraction. | 1.5-8 | - Chemically stable marker<br>- 8-Isoprostane levels increase with age<br>- Variation in detection limits | - Increased in asthma vs healthy<br>- Levels related to asthma severity<br>- Not increased in preschool wheezers<br>- Conflicting data regarding influence corticosteroids<br>- No correlation with lung function | - Increased in CF and PCD<br>- Might be indicator for CF exacerbation<br>- No correlation with lung function | Asthma: (30, 46, 64, 73, 82, 94-100, 102, 107, 108, 142) Other: (64, 125, 133) |
| **PGE2** | Derivate of arachidonic acid. PGE2 has a broncho protective effect and an inhibitory effect on inflammatory cells. | 5-16 | | - No difference in asthma vs healthy<br>- Not related to severity | - Found to be increased in allergic rhinitis vs healthy | Asthma: (33, 96, 102, 107, 108) Other: (143) |
| **PGD2, PGEM PGF1α, 2α, 9α, and 11β 5-HETE** | All derive from arachidonic acid. | 12.4 (3.1)* | - Limited publications | - Single study reported increased levels of PGD2, PGEM, PGF1α, 5HETE in asthma | Not described | Asthma: (33, 107) |

**Table 2.** Exhaled breath condensate markers and potential clinical value in childhood respiratory disorders (continued)

| Marker | Pathophysiological background | Age (years) | Methodological issues | Potential clinical value in asthma | Potential clinical value in other respiratory diseases | References |
|---|---|---|---|---|---|---|
| ***Eicosanoids (leukotrienes, eoxins, lipoxins)*** | | | | | | |
| **cysLTs (LTC4, LTD4, LTE4)** | Derived from arachidonic acid trough the 5-lipoxygenase pathway. Potent bronchoconstrictors and pro-inflammatory mediators. Generated mainly by mast cells and eosinophils. | 1.5-18 | - LTC4 frequently below detection limit | - Increased in asthma<br>- Mainly LTE4 seems to decrease on treatment with ICS, oral corticosteroids and/or LTRA<br>- Correlated with LTB4 | Not described | Asthma:<br>(30, 41, 79, 82, 95, 97, 102-105, 107-112, 142) |
| **LTB4** | Chemotactic action on airway neutrophils. | 5-14 | - Large variation in analysis techniques between studies | - Increased in preschool wheezers (single study)<br>- Increased in asthma, mainly in steroid naïve patients<br>- Conflicting results regarding relation with asthma severity<br>- No correlation with lung function | - Increased in CF and in community acquired pneumonia<br>- Possible predictor for CF exacerbation<br>- In pneumonia patients decline after antibiotics<br>- No correlation with lung function | Asthma:<br>(30, 41, 62, 64, 82, 98, 102, 104, 105, 110, 113, 142-144)<br>Other:<br>(64, 121, 133, 145) |
| **Lipoxins** | Lipoxins are derived from 15-lipoxygenase pathway. Potential counter regulatory mediator of inflammation. | 7-17 | - LX A4 only lipoxin measured in EBC, single study | - Lower levels in status asthmaticus vs healthy | Not described | Asthma:<br>(100) |
| **Eoxins** | End products from 15-lipoxygenase pathway. Formed in eosinophils. Pro-inflammatory properties. | 6-18 | - Eoxins C4, D4 and E4 measured in a single study | - Increased in asthma<br>- Highest levels in problematic severe asthma, but not significantly related to severity | Not described | Asthma:<br>(110) |

**Table 2.** Exhaled breath condensate markers and potential clinical value in childhood respiratory disorders (continued)

| Marker | Pathophysiological background | Age (years) | Methodological issues | Potential clinical value in asthma | Potential clinical value in other respiratory diseases | References |
|---|---|---|---|---|---|---|
| ***Cytokines and chemokines*** | | | | | | |
| **IL-4** | Th2 cytokine. Induces B-cell class switching to IgE producing B cells. | 2-18 | - Concentrations close to detection limits of assays | - Increased in asthma<br>- Related with asthma severity<br>- Increased in preschool wheezers, not predictive of asthma | - Detectable in CF<br>- IL-4/IFN-γ ratio higher in atopic dermatitis vs healthy | Asthma: (67, 73, 75, 116, 119, 120, 146-149)<br>Other: (148) |
| **IL-5** | Th2 cytokine. Key regulator in local infiltration and activation of eosinophils. | 2-18 | - Concentrations close to detection limits of assays | - Conflicting results, might be elevated in asthma<br>- Increased levels in preschool wheezers, not predictive for asthma<br>- Possible predictor asthma exacerbation and indicator asthma control | - Increased in atopic dermatitis vs healthy | Asthma: (63, 73, 75, 88, 117, 120, 148)<br>Other: (63) |
| **IL-8** | Produced by several cell types, mainly macrophages. Induces neutrophilic chemotaxis and phagocytosis. | 2-4.5 | - Concentrations close to detection limits of assays | - Increased in preschool wheezers, not predictive for asthma | - Not increased in PCD<br>- Increased in CF with Pseudomonas aeruginosa<br>- In CF significant decrease after antibiotics | Asthma: (68, 117, 118)<br>Other: (64, 121, 122, 133) |
| **IL-10** | Anti-inflammatory cytokine | 2-18 | - Concentrations close to detection limits of assays | - Increased in asthma<br>- Increased in preschool wheezers, not predictive for asthma | - Detectable in CF | Asthma: (67, 73, 75, 88, 118, 120, 147, 148)<br>Other: (148) |

**Table 2.** Exhaled breath condensate markers and potential clinical value in childhood respiratory disorders (continued)

| Marker | Pathophysiological background | Age (years) | Methodological issues | Potential clinical value in asthma | Potential clinical value in other respiratory diseases | References |
|---|---|---|---|---|---|---|
| **IFN-y** | Pro-inflammatory effect and stimulates epithelial cells in releasing inflammatory cytokines. | 2-18 | - Concentrations close to detection limits of assays | - Conflicting results, seems increased in asthma<br>- Possible indicator asthma control<br>- Increased in preschool wheezers, not predictive for asthma | - Detectable in CF<br>- IL-4/IFN-γ ratio higher in atopic dermatitis vs healthy | Asthma: (67, 73, 75, 88, 116, 120, 148)<br>Other: (67, 148) |
| **Others** | IL-1α, IL-2, IL-13, TNF-α, RANTES, eotaxin, chemokines, soluble adhesion markers, sICAM1 | 2-18 | - Concentrations close to detection limits of assays | - IL-2 increased in preschool wheezers, not predictive for asthma | - IL-2 detectable in CF | Asthma: (68, 73, 75, 88, 115, 118-120, 147, 148)<br>Other: (148) |
| ***Other markers*** | | | | | | |
| **MMP-9 and TIMP-1** | Type IV collagenase. Key mediator in metabolism extracellular matrix and airway remodeling. Ability to cleave structural proteins (elastin, collagens). | 8-15 | - Correlated to IL-4 and IL-10 | - MMP-9 higher in asthma<br>- Related to severity<br>- Inversely correlated to lung function (FEV1 and PEF)<br>- No differences in TIMP-1 between asthma and healthy | - Higher MMP-9 in bronchiectasis vs healthy<br>- Inverse correlation with lung function (FVC, $FEV_1$)<br>- No difference in TIMP-1 | Asthma: (114)<br>Other: (132) |
| **Purines (adenosine, AMP, ATP)** | Released by resident airway cells. Act as signaling molecules to regulate host defense. | 4-19 | - Described as ratio with urea (dilution marker) | - Higher adenosine in asthma vs healthy | - Higher adenosine in CF vs healthy<br>- Higher ATP levels in CF<br>- Decrease in AMP and ATP after antibiotics for exacerbation | Asthma: (59)<br>Other: (59, 150) |
| **Magnesium and calcium** | Broncho constrictor and -dilatator | 7-14 | - No difference in absolute values between groups | - Decreased magnesium/calcium ratio in asthmatics vs healthy | Not described | Asthma: (141) |

**Table 2.** Exhaled breath condensate markers and potential clinical value in childhood respiratory disorders (continued)

| Marker | Pathophysiological background | Age (years) | Methodological issues | Potential clinical value in asthma | Potential clinical value in other respiratory diseases | References |
|---|---|---|---|---|---|---|
| **Iron and ferritin** | Ferritin protects to iron-induced oxidative stress. | 5-16 | - Single study | - Lower iron in controlled asthma vs healthy | Not described | Asthma: (151) |
| **Growth factors** | VEGF, PDGF-AA, EGF | 7-18 | - Single study | - Increased PDGF-AA in asthma with low $FEV_1$ | Not described | Asthma: (149) |
| **Albumin** | Reaches the epithelial lining fluid by microvascular leakage in the airway epithelium. | 6-16 | - Single study | - Rarely detectable in asthma<br>- Not detectable in healthy controls | Not described | Asthma: (68) |

Definition of abbreviations: CF: cystic fibrosis; NO: nitric oxide; ROS: reactive oxygen species; 3-NT: 3-nitrotyrosine; $H_2O_2$: hydrogen peroxide; ICS: inhaled corticosteroids; ADMA: asymmetric dimethylarginine; NOS: nitric oxide synthase; RNS: reactive nitrogen species; PGE2: prostaglandin E2; PGD2 prostaglandin D2; PGF2α, 9α, and 11β: prostaglandin F2α, 9α, and 11β; 5-HETE: 5-hydroxyeicosatetraenoic acids; cysLTs: cysteinyl leukotrienes; LTC4: leukotriene C4; LTD4: leukotriene D4; LTE4: leukotriene E4; LTB4: leukotriene B4; LTRA: leukotriene receptor antagonist; LX A4: lipoxin A4; Th2: T helper 2 cells; IL: interleukine; IFN-γ: interferon-γ; TNF-α; tumor necrosisfactor-α; RANTESor CCL-5: chemokine C-C motif ligand 5; sICAM1: soluble intracellular adhesion molecule-1; MMPs: matrix metalloproteases; TIMP-1: tissue inhibitors metalloproteinases; $FEV_1$: forced expiratory volume in 1 s; PEF: peak expiratory flow; FVC: forced vital capacity; AMP: adenosine monophosphate; ATP: adenosine triphosphate; VEGF: vascular endothelial growth factor; PDGF-AA: platelet derived growth factor-AA; EGF: epidermal growth factor.

The effect of exercise on EBC has been considered in a number of studies, as exercise may increase oxidative stress. No differences have been found in 8-isoprostane, pH and cysLT before and after exercise, however these studies all lacked power [46,47]. While better studies are awaited, it seems prudent to avoid exercise immediately before EBC collection [48].

*Collection conditions*

Exhaled breath condensate collecting devices have in common that subjects breathe tidally via a mouthpiece through a two-way nonrebreathing valve. The exhaled air is cooled into a condenser and EBC is collected in a container attached to the free end of the tube. Commercially available devices include the Ecoscreen® (Jaeger, Hoechberg/Wurzburg, Germany), the RTube® (Respiratory Research Inc., Charlottesville, VA, USA) and the 'Transportable Unit for Research on Biomarkers Obtained from Disposable Exhaled Condensate Collection Systems' (TURBO DECCS®). An important difference between these three collection devices is the cooling temperature. The Ecoscreen and TURBO DECCS both collect EBC at a stable cooling temperature of respectively -10 to -15$^0$C, and -5$^0$C. In contrast, the R-tube uses a precooled -20$^0$C aluminium sleeve, which is placed over the collection tube; therefore, the collection temperature gradually increases during the procedure, which affects the amount of EBC volume collected [49].

Other collection conditions can influence EBC volume and biomarkers, such as sampling material, expiratory flow and contamination by saliva or ambient air. Current condenser chambers are made of glass, silicone, Teflon, aluminium, polystyrene or polypropylene. Loss of EBC biomarkers due to adhesive properties of these materials can influence results [50]. Coating of all surfaces with bovine albumin to avoid adherence of proteins (such as cytokines) and with Tween 20 (polysorbate 20) to prevent adherence of fatty acid derivatives (such as leukotrienes and prostaglandins) has been suggested to overcome this problem [51].

Expiratory flow may be an important factor influencing the size and number of respiratory droplets formed, which might increase variability of biomarker concentrations [52]. Indeed, both minute ventilation and tidal volumes significantly affect EBC volume [53,54]. However, to date, no studies have shown that minute ventilation or tidal volumes influence specific EBC biomarkers [53,55].

Most biomarkers in EBC are also present in much higher concentrations in saliva. Therefore, it is important to avoid contamination with saliva, which can be achieved with either a saliva trap, rinsing of the mouth prior to collection, or regular swallowing during the collection procedure. In addition, samples can be tested for the presence of salivary amylase. Exposition of EBC samples to room air should be avoided, as ambient air may influence the composition of EBC through direct contribution or indirect via chemical reaction with molecules in EBC.

*Postcollection conditions*

The dilution of respiratory droplets by water affects biomarker concentrations in EBC, and several ways to correct for dilution have been suggested, including conductivity, total cations or urea concentration [56-59]. All of these have disadvantages and may introduce more variability. Presently, there is no generally accepted method to correct for dilution, and it is not recommended to apply any correction. Furthermore, storage time and conditions may influence biomarker concentrations in EBC. The ATS/ERS task force recommended to freeze EBC samples immediately after collection and to store samples at -70°C until analysis [34]. Data are available regarding the stability of EBC pH [60], $H_2O_2$ [37,44] and nitrite [61], but there is a lack of data on the long-term stability of other biomarkers.

## CLINICAL APPLICATIONS OF EBC ANALYSIS IN PEDIATRIC RESPIRATORY DISEASES

### Asthma and allergy

*Acidity*

Airway acidity is one of the best validated markers in EBC, at least in adults, and normative data for healthy children aged 0-20 years have been published, with a median (IQR) pH of 8.0 (7.8-8.1) [32].

In children with stable asthma, lower pH in EBC was demonstrated compared to healthy controls, and children with severe asthma had lower pH compared to mild asthmatics [30,55,62-67]. In addition, asthmatic children treated with ICS had higher pH values than steroid naïve patients [65,67], and patients with an acute exacerbation had significantly higher pH values after 1 week of treatment with inhaled budesonide [66]. No association between EBC pH and other measures of asthma control such as symptoms, lung function, airway hyperresponsiveness or FeNO has been shown [30,68-71]. However, when interpreting results, one should take into account that gas standardization by means of removing $CO_2$ does have a significant effect on EBC pH [72]. Indeed, studies measuring pH directly after collection without de-aeration found no difference in pH between asthmatic children and healthy controls [68-70,73,74], and pH did not reflect asthma severity nor changed after treatment [68,69,75].

Ammonium, thought to be a buffer of airway epithelial lining fluid, was found to be significantly lower in stable asthmatic children compared to healthy controls. However, there was a considerable overlap, and ammonium levels did not decrease further during an acute exacerbation or show any change during treatment [69].

*Nitrogen oxide related-compounds*

Conflicting results on EBC NOx have been found in asthmatic children [40,61,70,73,76-82]. Overall, EBC NOx were not related to asthma severity [40,61,77,81], did not differentiate between stable disease and exacerbations [79] and were not influenced by ICS [77,81]. In addition, most studies did not show a correlation between EBC NOx and either symptoms [70,83], lung function [39,70,76,78,83] or FeNO [61,76-78]. These conflicting results may be due to the high inter- and intra-individual variability, and perhaps to contamination [52,61].

*Oxidative stress*

Exhaled breath condensate $H_2O_2$ is increased in asthmatic children, especially during exacerbations [84-86] and decreases after treatment with ICS, supporting the hypothesis that $H_2O_2$ reflects airway inflammation [84,86]. On the contrary, others found no differences in $H_2O_2$ between stable asthmatic children and controls, and $H_2O_2$ did not predict exacerbations in childhood asthma [73,87,88]. Normal values differ between studies, most likely due to different methodologies [89].

Another potential biomarker of oxidative stress in asthma is asymmetric dimethylarginine (ADMA). ADMA is a L-arginine analogue, which inhibits NOS, resulting in a decreased synthesis of NO and increased synthesis of superoxide. Higher levels of ADMA have been found in asthmatic children compared to healthy controls, with no difference between asthmatic children with or without ICS treatment [90]. In addition to increased oxidative stress, several authors also found a reduction of the antioxidant defence system, as reflected by lower glutathione in EBC of children with asthma [91-93].

*Eicosanoids*

Children with asthma have increased EBC 8-isoprostane compared with healthy controls [64,94-97]. Particularly high levels have been found in patients with severe asthma or with an asthma exacerbation [98-100]. Moreover, 8-isoprostane predicted asthma control and severity [73]. Contrastingly, no difference could be found between preschool children with and without recurrent wheeze symptoms [82]. Overall, there was no correlation between 8-isoprostane and lung function or eNO [46,94,95]. Interestingly, 8-isoprostane was hardly affected by ICS [94,96,97,101,102], but did decrease after oral corticosteroids in children with asthma exacerbations [95].

In children with asthma also, increased cysteinyl leukotrienes (CysLT) have been found compared with healthy controls with higher levels during exacerbations [41,97,102-111]. Also, in wheezing preschool children, increased levels of LTB4 and LTE4 were found [82]. Although some authors reported a significant reduction in cysLTs following 5 days of oral corticosteroids or 6 months of ICS [95,109] others reported no response of CysLT levels following ICS treatment [102,106,107,111]. A significant reduction in cysLTs after treatment with the cys-LT1 receptor inhibitor montelukast was reported [104,108,112]. In general, only weak

correlations have been found between lung function, FeNO and cysLTs and LTB4 in EBC [41,95,98,102,104,105,107,113].

*Cytokines*

Overall, children with asthma have increased levels of T helper 2 (Th2) cytokines and decreased T helper 1 (Th1) cytokines compared with healthy controls [67,73,114-116]. Also in preschool wheezing children, several Th2-derived cytokines were increased in EBC (IL-1α, IL-2, IL-4, IL-5, IL-8, IL-10 and IL-13) [117,118]. IL-4 was a significant predictor for an asthma diagnosis and IL-5 predicted asthma exacerbations [88]. Also increased levels of Th1 (IL-2 and IFN-γ) and proinflammatory cytokines (IL-6) have been reported in children with asthma [119]. In multivariate models, both IL-4 and IFN-γ were able to assess asthma control. Contradictory results have been reported on the effect of ICS treatment on EBC cytokine levels [67,75,120]. Higher levels of several chemokines and soluble adhesion molecules have been reported in asthmatic children [115,119].

*Other markers*

Extracellular adenyl purines, such as adenosine triphosphate (ATP) and metabolites, are important signalling molecules on airway surfaces and are thought to be released in reponse to inflammation. One study reported elevated levels of adenosine-urea ratio in children with asthma [59].

During chronic airway inflammation, activated neutrophils release proteolytic enzymes, such as matric metalloproteases (MMPs), which probably contribute to airway damage. Increased MMP-9 levels in EBC have been found in children with persistent asthma compared to children with intermittent asthma and healthy controls [114].

## Cystic fibrosis

*Acidity*

In children with CF, lower pH compared to healthy subjects was found, although the lowest pH values were observed in CF children without bacterial infection [64,121]. This is in contrast with what one would expect and it has been suggested that certain bacterial products may buffer airway pH. However, others found an increase in pH after 2 weeks of antibiotic treatment in children with a CF exacerbation [122], or observed lower pH during CF exacerbations, while pH did not change after antibiotic treatment [123]. Hence, the interpretation of EBC acidity in children with CF is not straightforward, and based on the available data, EBC pH cannot be used as a marker of airway inflammation in CF.

*Nitrogen oxide-related compounds*

In children with CF, a discrepancy exists between low eNO in EB and high NOx in EBC [39,40,76,124,125]. One possible explanation could be an increased metabolism of NO to

reactive nitrogen metabolites or interaction with ROS resulting in an increased formation of nitrotyrosine. The presence of abundant airway secretions in CF may impair diffusion of NO to the airway lumen and stimulate chemical conversion to nonvolatile NO metabolites. Another explanation could be that high EBC nitrite levels in patients with CF originate from the oropharynx as antibacterial mouthwash reduced the difference between patients with CF and controls substantially [126], and nitrite was almost exclusively found in orally collected EBC compared with EBC obtained via a tracheostomy [127]. Contradictory results have been found regarding the predictive value of nitrite levels for CF exacerbations [83,125].

*Oxidative stress*

Children with CF have increased EBC $H_2O_2$, especially during exacerbations, which decrease after antibiotic treatment [128]. Rosias *et al.* detected free radicals in EBC as another potential biomarker for oxidative stress and reported a trend to more free radicals in children with CF compared to healthy controls [129].

*Eicosanoids*

Children with CF have high EBC 8-isoprostane compared with healthy controls [125,130]. These levels further increase in unstable patients with CF and seem to be predictive for CF exacerbations [130].

*Cytokines*

In patients with CF high levels of IL-8 are reported compared with healthy controls and IL-8 decreased significantly after antibiotic treatment [119]. IL-8 is known to play a role in both chemotaxis and activation of neutrophils [64,121, 122].

*Other markers*

In children with CF, elevated ATP levels were found during exacerbations, with a threefold decrease after treatment [131]. In addition, the AMP-to-urea ratio was elevated in patients with CF, with a negative relationship between changes in lung function and AMP-to-urea ratio after treatment for an exacerbation [59]. Also, in a longitudinal study by the same authors, adenosine-to-urea ratio correlated negatively with lung function in patients with CF [131].

MMP-9 levels are higher in children with CF- and non-CF-bronchiectasis and are related to pulmonary infections, lung function and abnormalities on computed tomography [132].

**Other respiratory diseases**

In pediatrics, the search for biomarkers in EBC has mainly focused on asthma and CF. Some individual biomarkers have been studied in relation to other respiratory disorders

(Table 2). In children with PCD and in prematurely born adolescents 8-isoprostane was found to be increased compared with controls [133,134]. However, no difference was found between prematurely born adolescents with and without bronchopulmonary dysplasia (BPD) [134]. LTB4, associated with neutrophilic inflammation, was increased in children with a community acquired pneumonia and levels returned to levels of healthy controls after antibiotic treatment [135]. On the contrary, in children with PCD, LTB4 concentrations were not different compared with healthy controls, despite the presence of sputum neutrophilia [133].

## METABOLOMIC PROFILING

Single biomarkers may not be able to reflect complex pathological processes, nor monitor complex and heterogeneous diseases such as asthma. Metabolomic profiling is a nonselective approach, without *a priori* hypothesis, enabling the identification and quantification of all metabolites in a biological system. The metabolomic profile is the ultimate expression of genetic information, and interaction with environmental agents, micro-organisms, nutritional factors, medication and toxic substances. Hence, with metabolomic profiles, disease phenotypes might be discerned and this might open the door to personalized medicine. Although identification of all molecules in metabolomic profiles may be essential to unravel disease pathways, this may be difficult, time-consuming and costly. Expert statistical analysis is needed to account for multiple observations, for example by applying discriminant analysis with cross-validation. Also, data from single studies need external validation in independent datasets.

Currently, metabolomic profiling is mainly used for the detection of VOC profiles. In chapter 1, the results of metabolomics in pediatric respiratory medicine have been summarized. Only in the last years, metabolomic profiling instead of measuring individual biomarkers has been applied to EBC in childhood respiratory disorders. Several research groups were able to distinguish asthmatic children from healthy controls [33,74,136,137], differentiate between asthma severities [136,137] and between patients with unstable or stable CF and healthy controls based on a metabolomic approach [138].

## CONCLUSION

Exhaled breath and EBC analysis are noninvasive methods to assess potential biomarkers of airway inflammation that may be useful in the assessment of respiratory diseases in childhood. Standardization of biomarker analysis in EB and EBC remains problematic. High variability and low reproducibility in exhaled biomarkers may be explained by dif-

ferences in a large number of precollection, collection and postcollection conditions. Hence, the interpretation of results is seriously hampered by the lack of standardization. Furthermore, biomarker detection in EB and EBC remains a challenge as concentrations are close to the detection limits of currently available analysis techniques. New techniques, such as the multiplex immunoassay in which multiple inflammatory markers can be measured simultaneously in low volume samples with increased sensitivity and reliability, might improve analysis of, for example, cytokines in EBC.

Despite these limitations, several biomarkers have shown the potential to distinguish patients with various diseases from healthy controls. In EBC, especially the measurement of acidity, $H_2O_2$ and 8-isoprostane seems promising. However, in the cross-sectional studies that have appeared until now, patient groups and controls have been clearly defined, which will give a flattered impression of their diagnostic performance. In real life, populations will be more heterogeneous, which will induce variability in exhaled biomarker profiles and concentrations. Recently, metabolomic approaches were applied to both EB and EBC, with the potential to restrain this problem by detecting complete profiles instead of single markers. These studies are still in an early phase. Besides rigorous standardization of procedures and more longitudinal studies, particularly external validation of specific breath metabolomic profiles for certain diseases or disease phenotypes is needed. In the future, the analysis of EB and EBC might prove of additional value to clinical features, lung function and imaging techniques. Whether or not these technically demanding methods pay off in terms of feasibility, discriminatory power and reproducibility, and in future will be used in clinical practice remains to be shown.

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# 3 Multicomponent gas analysis using broadband quantum cascade laser spectroscopy

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## ABSTRACT

We present a broadband quantum cascade laser-based spectroscopic system covering the region between 850 and 1250 $cm^{-1}$. Its robust multipass cavity ensures a constant interaction length over the entire spectral region. The device enables the detection and identification of numerous molecules present in a complex gas mixture without any pre-treatment in two minutes. We demonstrate that we can detect sub-ppmv concentration of acetone in presence of 2% of water at the same wavenumber region.

## INTRODUCTION

Detection of trace levels of gases is an integral part of life in modern societies. The ability to detect trace gases in real time or offline plays a major role in industrial processes, environmental monitoring, medical diagnostics [1], security, air quality monitoring besides other applications. For this reason and to be able to identify specific molecules, many optical techniques have been developed based on spectroscopy like long path absorption spectroscopy, cavity enhanced spectroscopy, cavity ring down spectroscopy, photoacoustic spectroscopy to name a few. The main advantage offered by spectroscopy is that specific molecules can be identified using their distinctive fingerprints. This can be beneficial when several molecular species are present simultaneously in the gas being analyzed. The devices which have been developed for this purpose can be based on high sensitivity, looking for parts per million by volume (ppmv), parts per billion by volume (ppbv) and even parts per trillion by volume (pptv) concentrations of a specific gas, or on a broad band technique which looks for many species simultaneously. These optical techniques are noninvasive and very little preprocessing is needed in most cases. Most devices for spectroscopic gas detection are based on absorption by the molecular species as described by the Beer-Lambert law. Therefore to optimize the sensitivity of the device, the illumination source wavelength and the interaction length have to be carefully chosen. Many systems are based in the near to mid-infrared region of the electromagnetic spectrum. This is mainly because the fundamental rovibrational absorption modes of the molecules produce strong spectroscopic fingerprints in this region [2]. In the near infrared region of the spectrum a host of sources of varying complexity, size and price exist because of the needs of the telecommunication industry for which this is the favored wavelength. However the spectroscopic fingerprints of molecules found in this region are 1000 times weaker than the fundamental absorption modes located in the mid-infrared. Sources in the mid-infrared are scattered over the entire wavelength range (3-24 μm) and are of varying types such as gas lasers, color center lasers, difference frequency generation, optical parametric oscillators, lead salt diodes and the recently developed quantum cascade lasers (QCLs), among others. The quantum cascade lasers which have been developing very fast in recent years are rapidly filling the holes in the wavelength axis, making them an attractive light source for gas analysis [3, 4]. Most QCLs have a well-defined central wavenumber and a narrow linewidth that allow an accurate molecular identification.

The next important element is the optimization of the interaction length between the light and the gas volume. In this case considering that sometimes the gas volume is limited, the choice is generally made to concentrate the light in the volume confining the gas using specifically designed optical cavities. The cavities are designed with two different methodologies namely, they can be resonant cavities or multipass cavities.

The resonant cavities offer the possibility to obtain interaction distances in the order of kilometers inside volumes lower than a liter. However the resonators have strong constraints that make their implementation difficult. They require mirrors with reflectivity's higher than 99.9% to achieve the needed finesse [5, 6]. Although such mirrors are available with the right reflectivity, their bandwidth is still limited to a few nanometers in the mid-infrared. Another restriction resonators have is the need of a feedback system to correct the position of the mirrors because they are susceptible to small mechanical and thermal changes. Furthermore it is necessary to couple the transversal mode of the laser to the cavity mode. Multipass cavities, on the other hand, only allow interaction distances of tens of meters but their requirements are less demanding. The mirrors' reflectivity is lower, but their working bandwidth is much broader. Multipass cavities are more robust to mechanical and thermal changes eliminating the need of a feedback system.

The combination of QCLs' relatively high power and adequate optical cavities has been successfully used to implement highly sensitive spectroscopic techniques such as cavity ring down, photoacoustic spectroscopy, wavelength modulation spectroscopy and integrated cavity output spectroscopy [6, 7]. Some of these techniques have demonstrated sensitivities in the orders of ppmv, ppbv and pptv levels. However, these techniques only focus on the detection of one or two selected molecules mainly because they only use a small range of the QCLs' tunability bandwidth. By using the full QCLs' tunability it is possible to detect and differentiate the components present in a more complex molecular mixture [8].

In this work we report a broad band spectroscopic trace gas analysis device that covers the atmospheric window between 850 and 1250 $cm^{-1}$ (8-11.76 μm) [9]. Our approach targets the simultaneous detection and identification of several molecules present in the gas volume, exploiting the fact that the molecular spectra in this region have a broad profile. We compare the detected spectral profile of the gas mixture with well-established molecular databases, like HITRAN [10] and the Pacific Northwest National Laboratory (PNNL) database [11] and identify the specific molecules. Furthermore, the multipass cavity employed in our system allows us to detect concentrations in the ppmv and sub-ppmv levels.

## EXPERIMENTAL SETUP

A schematic overview of the experimental setup is shown in Figure 1. Its main components are: a laser source, a multipass cavity to enhanced the absorption and two detectors to monitor the laser intensity and to measure the absorption signal after the multipass cavity. A visible laser distance-meter (Leica DISTO D2) co-aligned with the la-

ser is used as guiding beam and to measure the interaction distance inside the multipass cavity.

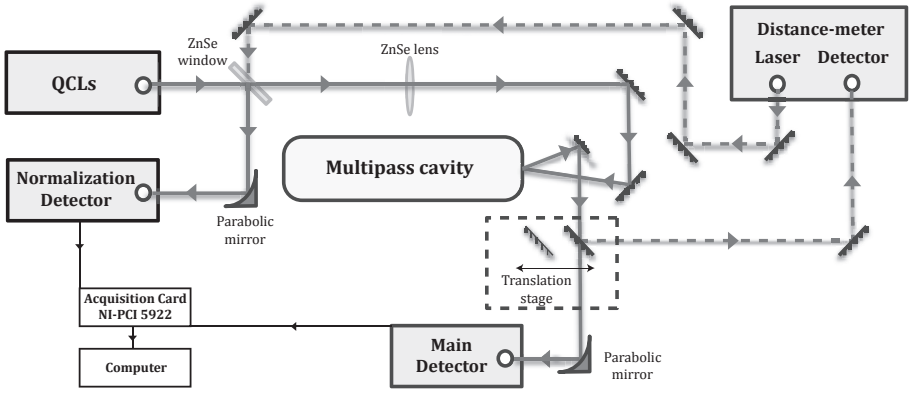


**Figure 1.** Schematic overview of the experimental setup. The translation stage allows to switch between the cavity length determination and the measurement configurations.

The laser source is part of a Laser Scope unit from Block Engineering. It consists of two co-aligned tunable QCLs. One QCL covers the region between 850 $cm^{-1}$ and 1010 $cm^{-1}$ and the second QCL emits in the region between 1010 $cm^{-1}$ and 1250 $cm^{-1}$. The QCLs use diffraction gratings in a Littrow configuration with back extraction to tune the wavenumber [12]. The angular position of the grating is controlled using a piezoelectric component. Therefore, the emitted wavenumber is calibrated with the voltage applied to the piezo. The QCLs wavenumber accuracy is 0.1 $cm^{-1}$. The QCLs' average power varies between 0.5 and 12 mW depending on the emitted wavenumber. Both QCLs are pulsed, with a pulse repetition rate of 200 kHz and the pulses have a temporal width of 208 ns. These parameters are optimum for the emission of the QCLs in terms of intensity and stability. We use pulsed QCLs because they have stable operation at room temperature. The multipass cavity is based on the modified Herriot configuration described by McManus *et al.*[13]. It consists of two 2.5 in. (1 in. = 2.54 cm) astigmatic mirrors (Aerodyne Research, Inc.) with a reflectivity $r > 0.983$ over the full spectral range of the QCLs. The distance between the mirrors measured along the optical axis is 32.4 cm. The mirrors are placed on custom made mounts inside a stainless steel vacuum cell. The cell has an input ZnSe window (Thorlabs WG71050-F) with a transmission $t > 0.95$ in the working spectral range. The number of reflections $n$ inside the cavity determines the interaction path-length achievable but it also defines the amount of light coming out of the cavity through the intensity reduction coefficient $k$,

$$k = t^2 r^n \tag{1}$$

Even though this type of cavities allow interaction lengths of up to 100 meters [13] we prefer a configuration with an interaction length of 54.36 meters, which is long enough to detect molecules in concentrations levels of few ppmv and at the same time provides a strong output signal. The power coming out of the multipass cell is 5.5% of the laser power sent into the cell. The total volume of the cell is 0.6 liters. The multiple reflections and the compact configuration allows the laser to travel through most of the volume of the cell, which allows a homogeneous interaction with the entire gas sample. We use a pressure controller (Bronkhorst, P-702CV-1K1A-RAD-22-V) and a diaphragm vacuum pump (Vacuubrand MD 1) to control the pressure inside the cavity. The pressure measurements are particularly important for molecules whose spectra contain sharp absorption lines. Such lines can suffer broadening because of different effects, for example collisional or Doppler broadening. To control the injection of samples inside the cell we use a set of mass flow controllers (Bronkhorst, F-201CV-1K0-RAD-22-V), which allows us to prepare samples with the desired concentrations using single and double dilution techniques [14]. For the detection we use two thermo-electric cooled (TEC) Mercury Cadmium Telluride (MCT) detectors and a fast acquisition card (NI-PCI 5922). As depicted in Figure 1 the main detector collects the signal emerging from the multipass cavity. The 100 MHz cut-off frequency of this detector (VIGO System, PVI-4TE-10.6-0.3x0.3-TO8-BaF2) and the 10 mega samples per second (MS/s) sampling rate of the acquisition card allows to detect the peak power of each pulse, increasing the signal to noise ratio (SNR) of the measurement [9]. A second detector (Laser Scope unit, Block Engineering) is necessary to monitor the laser intensity directly outside the QCLs and normalize the intensity of each pulse. This normalization minimizes the pulse to pulse intensity variation of the QCLs and increases the sensitivity of the system [15, 16].

## MULTIPASS CHARACTERIZATION

### Robustness

In most multipass cavities the allowed input angle of the incoming laser beam has a small tolerance. Our configuration increases the tolerance using a 500 mm ZnSe lens in front of the multipass cavity. As explained before, the broadband tunability of our QCLs is obtained using a Littrow configuration with back extraction, which reduces the wavenumber-dependent angular displacement of the laser beam to less than 3 mrad. To demonstrate the robustness of our device we produced pulses with a temporal width of 112 ns and measured the normalization signal and the signal from the cavity using one detector. For this experiment the configuration of Figure 1 was modified to place both signals on the main detector. We only used one detector to have the same detection response and avoid any electronic delay. The signal was analyzed using a RIGOL DS1102E

oscilloscope. We repeated this measurement for three different central wavenumbers as shown in Figure 2. In all cases the delay between signals is 185 ns when the cavity is empty, confirming that all pulses travel the same distance inside the cavity. In particular the angular displacement between the pulses of Figure 2(b) and (c) is maximum, which confirms the tolerance of the multipass cell.



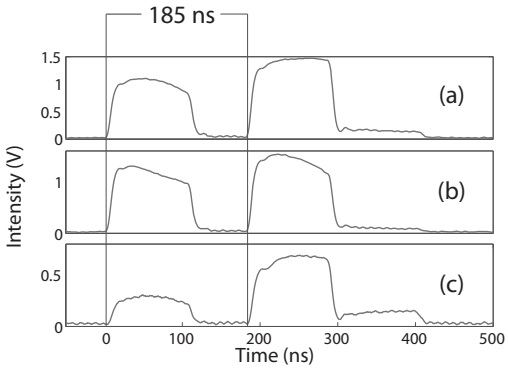


**Figure 2.** Delay time between the normalization and the main signals for three different wavenumbers. **(a)** 950 $cm^{-1}$, **(b)** 1000 $cm^{-1}$ and **(c)** 1150 $cm^{-1}$.

## Internal path-length determination

To measure the path-length inside the multipass cavity we used two methods. First we used the laser distance meter, averaged 50 measurements and subtracted the path the laser travels outside the cavity, getting a path-length of 54.36 meters. Then we used the 185 ns pulse delay between the normalization and the main signal shown in Figure 2. Subtracting the distance traveled by the laser outside the cavity we got a measured distance of 54.33 meters. These two independent measurements are consistent. The 3 cm difference between them represents a delay of 100 ps which we cannot resolve using our oscilloscope.

## Measurements

For the absorption measurements we first build a reference spectra $I_0(\nu)$, where $\nu$ is the wavenumber. For that we record the transmission of a laser scan through the multipass cavity with vacuum or filled with a neutral gas which has no absorption lines in the spectral region of our device. Then we average the result of ten laser scans. Immediately after, we introduce the gas sample inside the cavity to minimize the effects on our measurements induced by atmospheric variations outside the cavity. Then we scan the laser ten times and average the signal to construct a measurement spectrum $I_m(\nu)$. Using the Beer-Lambert law [17] we obtain the absorbance $A(\nu)$ of the sample,

$$A(\nu) = - \log_{10} \left( \frac{I_m(\nu)}{I_0(\nu)} \right) \qquad (2)$$

The absorbance can also be written in terms of the molecular concentration C in ppmv, the interaction path-length between the light and the molecules (l = 54.36 meters in the case of the present device) and the molecular absorption coefficient ε(ν) in $ppmv^{-1}m^{-1}$, which is an intrinsic property of the material,

$$A(\nu) = \varepsilon(\nu)Cl \tag{3}$$

Using this last relation we can determine the molecular concentration from the strength of the absorbance.

**Noise determination and sensitivity analysis**

The noise level of our system is determined comparing two reference spectra. As they are taken with an empty cavity the variations are only induced by the laser and detector fluctuations. The ratio between two reference spectra is a good comparison and as we are interested in the absorbance we can use directly Equation (2). From this result the noise level is established by taking the absorbance root mean square (rms) value. For our system it has a value of 0.02.

To determine the performance of our system we used the formalism developed by Moyer *et al.*[18]. The minimum detectable absorption per scan (MDAps) is given by

$$MDA_{ps} = \left(\frac{\Delta P}{P}\right)_n \sqrt{n}\ \sqrt{T_{scan}} \tag{4}$$

Where $\Delta P/P$ correspond to the rms level calculated above, the number of scans integrated, $n$, is equal to 10 and the time a single scan takes, $T_{scan}$, is equal to 5.3 s. The $MDA_{ps}$ for our system has a value of 0.14 $Hz^{-1/2}$. To compare with systems that scan over a narrower or broader spectral region is important to define the minimum detectable absorption per point ($MDA_{pp}$). As we have a well-defined number of data points, $\#pts = 8000$, we use the data acquisition rate, $r_{data} = \#pts/Tscan$ as our relevant bandwidth. In this case the $MDA_{pp} = MDA_{ps}/(\#pts)^{-1/2}$ and has a value of $1.6 \times 10^{-3} Hz^{-1/2}$. The comparison with systems with different interaction path lengths is better done using the noise equivalent absorption sensitivity (NEAS) [18,19]. Since the interaction distance inside the cavity, $l$, was determined to be 54.36 meters the $NEAS = MDA_{pp}/l$ and gives a value of $2.99 \times 10^{-7} cm^{-1} Hz^{-1/2}$.

**Specific gas measurements**

In this section we present a series of measurements to show the performance of our setup. In these measurements the reference spectrum was taken with vacuum of 0.10 mbar inside the cavity. In all cases we use an atmosphere of $N_2$ as buffer for the sample gas mixture because $N_2$ does not have absorption lines in the spectral range of the present

device. The first two measurements correspond to controlled mixtures of acetone and ethanol. The mixtures were obtained using a double dilution process [14]. This way we could cross-check the concentration of the samples. We choose these molecules because they have strong and smooth absorption profiles in our spectral region. Then we prepared a single dilution mixture of $CO_2$ to study the wavenumber resolution and the sensitivity of our system, because $CO_2$ has a lower absorption in our region. Finally we present the measurement of two complex mixtures to show the applications of our method.



### Acetone, ethanol and $CO_2$ spectra

In Figure 3(a) the measured spectrum of acetone with a concentration of 22 ppmv at 900 mbar in an atmosphere of $N_2$ is shown in red. The black line corresponds to the acetone spectrum in the same wavelength region from the PNNL database. In this case there are broad features that can be easily identified, they are centered at 900 $cm^{-1}$ and 1218 $cm^{-1}$. In Figure 3(b) we present the measured spectrum of ethanol with a concentration of 60 ppmv at 900 mbar in an atmosphere of $N_2$, in red. The black line corresponds to the ethanol spectrum in the same wavelength region from the PNNL database. In this spectrum the experimental setup captures the finer spectral details, such as the peaks at 1028, 1038, 1058 and 1066 $cm^{-1}$. In this case there is a mismatch in the region between 980 $cm^{-1}$ and 1020 $cm^{-1}$ corresponding to the transition between the two QCLs. But this

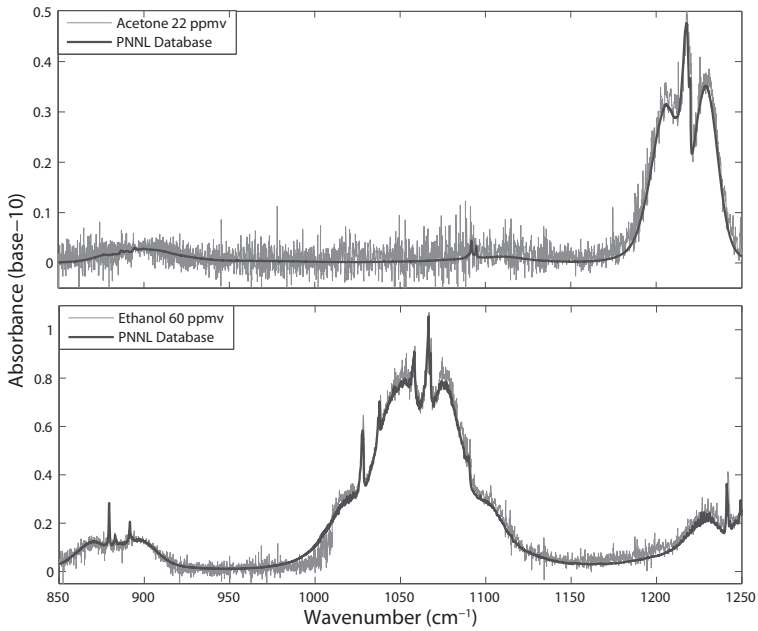


**Figure 3. (a)** Absorption spectrum of 22 ppmv of acetone and **(b)** 60 ppmv of ethanol. Both at 900 mbar in an atmosphere of $N_2$.

is not a limitation to detect the desired molecules since the main spectroscopic features are clearly identifiable.

Furthermore, from a measurement of a 15% concentration of $CO_2$ at 500 mbar in an atmosphere of $N_2$ we could resolve the rovibrational spectrum as shown in Figure 4(a). The observed $CO_2$ absorption lines are four orders of magnitude weaker than the strongest absorption lines of $CO_2$. However, when we compare the spectrum with the theoretical information contained in the HITRAN database we notice that the strength of the absorbance does not match with the information contained in the database, Figure 4(b). This effect is due to the wavenumber accuracy of our device, which is too large to correctly retrieve the profile of each absorption line. Therefore, it is not possible to directly measure the strength of each absorption line and the sample concentration. To determine the sample concentration we first fit a Voigt profile to each individual absorption line, then we compare the maxima of the peaks with the HITRAN database using Equation (3) to obtain a concentration value for each peak. We thus have several concentration values calculated for different lines of the same molecule. The mean value of these concentrations give us the sample concentration. From this analysis we obtained a 15% concentration of $CO_2$ with a standard deviation of 5%.

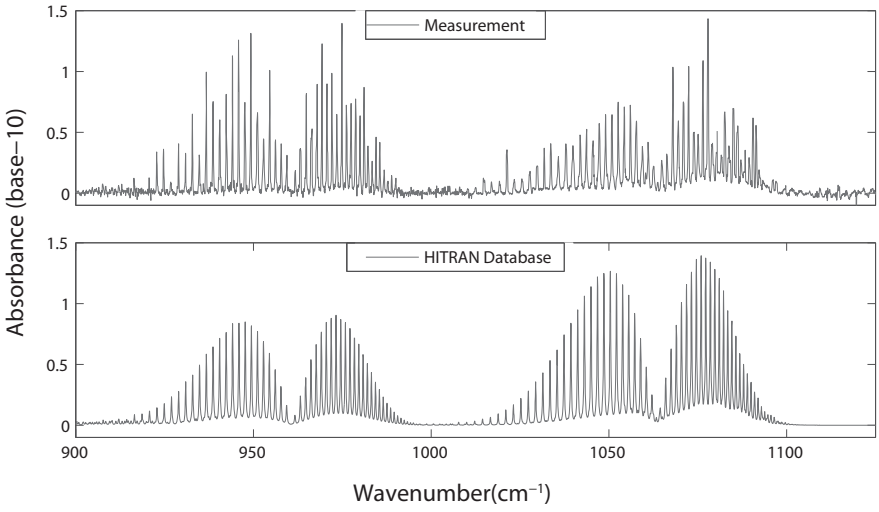


**Figure 4.** Spectrum of 15% of $CO_2$ in an atmosphere of $N_2$ at 500 mbar. **(a)** Experimental measurement. **(b)** HITRAN database.

These measurements show some of the system's advantages and limitations. The broadband coverage of the system allows the molecular identification of molecules with a smooth spectral profile because all the available absorption features are used, this permits to determine the sample concentration in a couple of minutes. For molecules

with sharp spectral features the identification is possible but the concentration determination strongly depends on the absorption strength.

*Lab air spectrum*

An easy test for our setup is the measurement of the environment from our lab. For this purpose we filled the multipass cavity with lab air and obtained the absorbance depicted in Figure 5. In this spectrum we can clearly observe five absorption lines of water at 1136 $cm^{-1}$, 1175 $cm^{-1}$, 1187 $cm^{-1}$, 1212 $cm^{-1}$ and 1225 $cm^{-1}$. As in the case of CO2 there is a mismatch between the strength from some measured spectral lines and the HITRAN database. Again the main reasons for this mismatch is the fact that we are observing absorption lines three orders of magnitude weaker than the strongest water absorption lines and the wavenumber accuracy mentioned previously. However, using the method described above we determined a concentration of 1% with a standard deviation of 0.7%, which reflects how the magnitude of the absorption strength of the molecule improves the concentration determination.

To obtain an independent measurement of the water concentration in the lab air we used a Vaisala transmitter (PTU300). It allowed us to measure the atmospheric pressure P = 1001.0 mbar, the relative humidity RH = 31.0% and the temperature T = 23.0°C in the lab. With this information we calculated the saturated pressure of water vapor p*w using the relation [20],

$$P^*w = \left[1.0007 + (3.46 \times 10^{-6}P)\right] \times (6.1121) \exp\left[\frac{17.502T}{240.97 + T}\right] \quad (5)$$

and then we calculated the concentration in percentage,

$$C_w = \frac{P^*_w RH}{P} \quad (6)$$

From this independent measurement we obtained a concentration of 0.9%. This value is consistent with the spectroscopic measurement obtained using our device. The main source of error is the uncertainty in the strength of the absorption signal due to the wavenumber spacing.

*Breath sample*

Finally we measured a breath sample from a healthy volunteer from our research group. The pressure inside the multipass cavity was 900 mbar and there was a continuous flow of the sample at 500 milliliter normal per minute ($ml_n$/min) [22]. As seen in Figure 6, we could resolve the absorption lines of $CO_2$ with a concentration of 3% and water at 2%. An interesting feature is the shift upwards of the base line in the region between 1170 and 1250 $cm^{-1}$ as shown by the red curve in the inset of Figure 6. In this region there

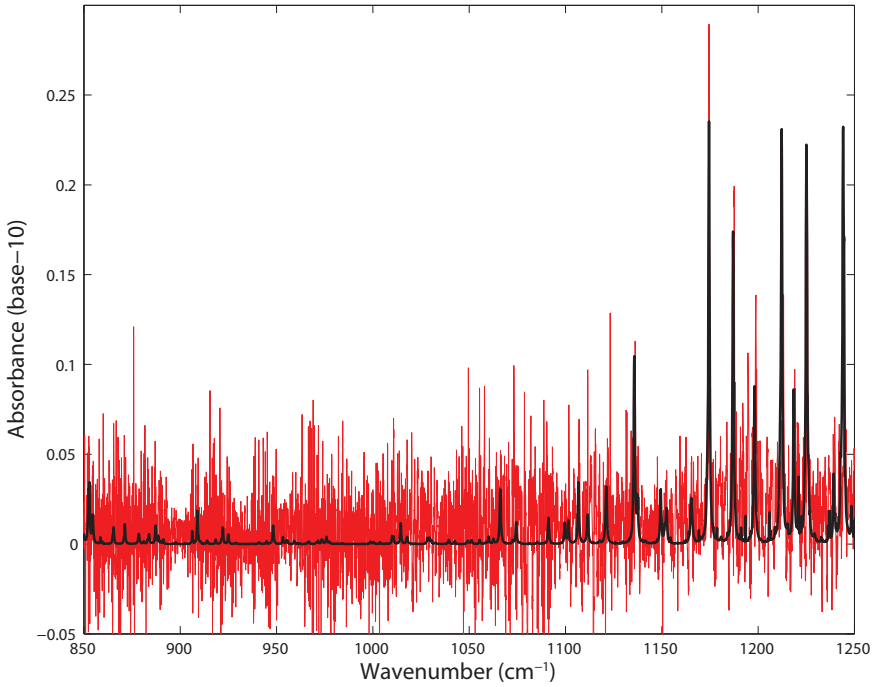


**Figure 5.** Lab air sample at 1001.0 mbar, at a temperature of 23.0 °C and a relative humidity of 31.0%. In black is depicted the spectrum of water from HITRAN database with a concentration of 1 %.

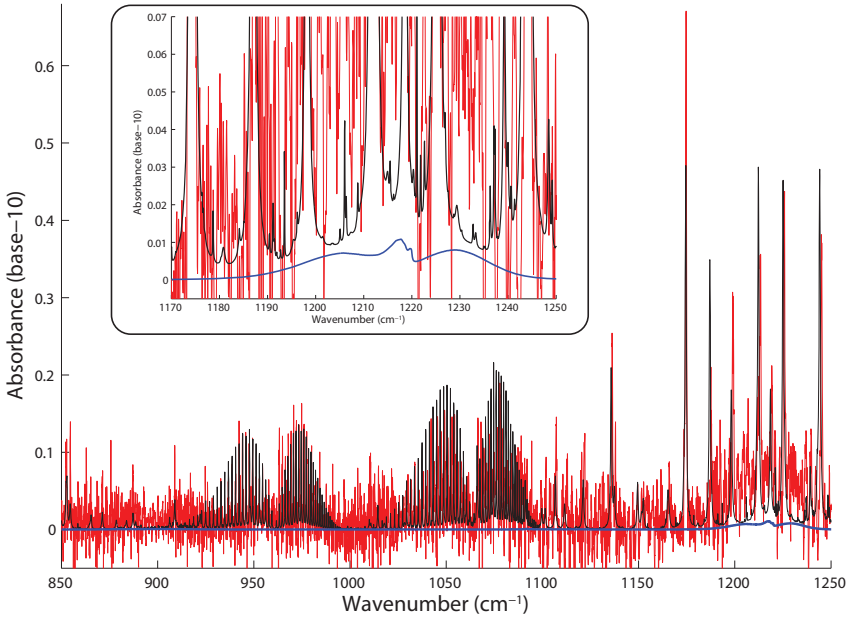


**Figure 6.** Breath sample at 900 mbar and flowing at 500 $ml_n$/min. In black is depicted the theoretical spectrum with 3% of $CO_2$ and 2% of water from HITRAN database and 500 ppbv of acetone from the PNNL database. In blue is the spectrum of acetone with a concentration of 500 ppbv taken from PNNL database.

is absorption from water and acetone. The black line in the inset of Figure 6 shows the water absorption and the 500 ppbv of acetone, giving a better match to the red curve corresponding to the measured spectrum. This concentration of acetone is in the normal range for a healthy person [23].

This measurement demonstrates the capability of the system to detect the presence of several molecules in a single sample. Using the overall profile allows to make an accurate and fast initial molecular identification of molecules with high concentrations. By examining closely the baseline of the spectrum we can identify and observe molecules with broad and smooth absorption profile. Their presence shifts the spectrum baseline and allows us to detect sub-ppmv concentrations.



## CONCLUSION

In this work we present a broadband spectroscopic gas analysis device which covers the molecular fingerprint region between 850 and 1250 $cm^{-1}$. It complements the direct absorption spectroscopic systems available in the 1250 - 3000 $cm^{-1}$ region. At the same time broadens the spectral bandwidth covered by one single setup. This device enables the study of complex gas mixtures because the robustness of the multipass cavity guarantees a stable interaction length over the full spectral scan. For applications requiring sensitivities in the ppmv and hundreds of ppbv levels this system is ideal to quickly estimate the composition of a gas sample without any pre-treatment. The sensitivity characterization gave us a *NEAS* of $2.99 \times 10^{-7} cm^{-1} Hz^{-1/2}$. Furthermore, the sensitivity can be increased by a factor of 10 by incrementing the delay introduced by the multipass cavity and measuring the absorption and the normalizing signal in one single detector [21].

The most important feature of the system is the fast identification of molecules with a broad and smooth spectral profile as shown with the acetone signature in the breath sample measurement. This shows that our system is reliable for applications such as the detection of molecular markers in complex gas mixtures like breath. Especially for cases where the concentration levels are in the order of hundreds of ppbv or higher.

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# 4 Exhaled breath profiling using broadband quantum cascade laser-based spectroscopy in healthy children and children with asthma and cystic fibrosis

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## ABSTRACT

### Background

Exhaled breath analysis is a potential noninvasive tool for diagnosing and monitoring airway diseases. Gas chromatography-mass spectrometry and electrochemical sensor arrays are the main techniques to detect volatile organic compounds (VOCs) in exhaled breath. We developed a broadband quantum cascade laser spectroscopy technique for VOC detection and -identification.

### Objectives

To assess the repeatability of exhaled breath profiling with broadband quantum cascade laser-based spectroscopy and to explore the clinical applicability by comparing exhaled breath samples from healthy children with those from children with asthma or cystic fibrosis (CF).

### Methods

Healthy children and children with stable asthma or stable CF, aged 6-18 years, were included. Two to four exhaled breath samples were collected in Tedlar bags and analyzed by quantum cascade laser spectroscopy to detect VOCs with an absorption profile in the wavenumber region between 832 and 1262.55 $cm^{-1}$.

### Results

We included 35 healthy children, 39 children with asthma and 15 with CF. Exhaled breath VOC profiles showed poor repeatability (Spearman's rho = 0.36 to 0.46) and agreement of the complete profiles. However, we were able to discriminate healthy children from children with stable asthma or stable CF and identified VOCs that were responsible for this discrimination.

### Conclusion

Broadband quantum cascade laser-based spectroscopy detected differences in VOC profiles in exhaled breath samples between healthy children and children with asthma or CF. The combination of a relatively easy and fast method and the possibility of molecule identification makes broadband quantum cascade laser-based spectroscopy attractive to investigate the diagnostic and prognostic potential of volatiles in exhaled breath.

## INTRODUCTION

Over the last decades, the measurement of volatile organic compounds (VOCs) in exhaled breath has raised great interest as a potential tool for diagnosis and management of respiratory diseases. Especially in pediatric respiratory medicine exhaled breath profiling is attractive to assess the presence and degree of airway inflammation, to distinguish disease phenotype and enable targeted and individualized treatment. Hydrocarbons are potential biomarkers for airway inflammation, as they derive from lipid peroxidation induced by e.g. reactive oxygen species during inflammatory reactions in the airways. Previous studies on VOC profiling in children with asthma and cystic fibrosis (CF) have identified hydrocarbons as the most discriminating VOCs between patients with and without asthma or CF. Hydrocarbons were helpful in the diagnosis of asthma and CF, and predicted clinical deterioration [1-5].

Currently, gas chromatography coupled to mass spectrometry (GC-MS) is the gold standard for the identification of VOCs. Disadvantages of this technique include the need for sample preconcentration, the fragmentation of molecules and the necessity for expensive equipment and highly trained personnel. An alternative is the electronic nose (eNose), an easy and fast sensor-based technique which allows online real-time analysis of the complete spectrum of VOCs, but cannot identify separate molecular components [6]. We developed a new broadband quantum cascade laser-based spectroscopy setup to detect VOC profiles in exhaled breath [7]. This technique enables the fast detection and identification of whole molecules with the potential for miniaturization, thereby combining the advantages of both previously mentioned techniques. In the present study, we explored the clinical applicability of this technique by determining the repeatability and comparing exhaled breath samples from healthy children with samples from children with either stable asthma or stable CF lung disease, two conditions that have different types of chronic airway inflammation as a prominent feature.

## MATERIALS AND METHODS

### Study subjects

We included children aged 6-18 years. Healthy controls were recruited at a primary school in Rotterdam. Healthy controls had a negative ISAAC questionnaire for asthma and allergy and a normal forced expiratory volume in 1 s ($FEV_1$) [8]. Children with stable asthma or stable CF were recruited at the outpatient clinic KinderHaven (Harbor Hospital, Rotterdam) and Sophia Children's Hospital (Erasmus Medical Center, Rotterdam), between September 2013 and February 2014. Inclusion criteria for stable asthma were a doctor's diagnosis of asthma, and well controlled asthma at the time of the study de-

fined as a stable dose of inhaled corticosteroids, need for short acting ß2 agonists less than 3 times per week, minimal daytime symptoms (<3 times per week), no limitations of activity, a normal lung function ($FEV_1$ >80% predicted or >80% of personal best), and no exacerbations in the previous 3 months. Atopy was defined as a positive radioallergosorbent test (RAST) or a positive skin-prick test ever, for at least one aeroallergen [9]. Children with stable CF had a positive sweat test or 2 CF-specific DNA mutations, and were not treated for exacerbations with oral or intravenous antibiotics in the 6 weeks before the study visit. Written informed consent was obtained from parents or caretakers, and from children themselves if 12 years or older. The study was approved by the Medical Ethical Committees of Erasmus MC-Sophia Children's Hospital and of the Harbor Hospital, Rotterdam.

**Study design**

In healthy children, exhaled breath samples were collected twice with 30 min intervals and again 24 hours and 1 week later in order to assess short- and long-term repeatability of exhaled breath profiling. In asthmatic children and children with CF we collected 2 exhaled breath samples 30 min apart during a routine clinical visit. Spirometry (Masterscreen, CareFusion, Würzburg, Germany) and measurements of fractional exhaled nitric oxide (FeNO, NIOX MINO NO analyzer, Aerocrine, Solna, Sweden) were performed according to ATS/ERS guidelines during the first visit only in the healthy children and the children with asthma [10,11].

Exhaled breath samples were collected during a single, slow exhalation from total lung capacity following 5 s of breath holding. Children exhaled via a disposable mouthpiece and one-way valve into a 3 L re-usable Tedlar bag (Tedlar bag 231-03, SKC Inc., Pennsylvania, USA) (Figure 1). Eating and exercise were not allowed within 1 h before sampling. The bags were transported the same day towards the laboratory at Delft University of Technology, where samples were analyzed immediately. Tedlar bags were cleaned by flushing 3 times with synthetic air (Purity grade 5.5, Linde, The Netherlands) and nitrogen (Purity grade 6, Linde, The Netherlands) after which they were heated overnight at 60°C, and again flushed with both synthetic air and nitrogen, according to the recommendations of the Environmental Protection Agency of USA (EPA 1991 Method 422) [12].

**Sample analysis**

Exhaled breath samples were analyzed using broadband quantum cascade laser-based spectroscopy [7]. For this purpose, we developed a quantum cascade laser spectroscopy setup, to detect volatile molecules with an absorption profile in the wavenumber region between 832 and 1262.55 $cm^{-1}$. The detection limit of this system is given by the noise equivalent absorption sensitivity (NEAS), 2.99 x $10^{-7} cm^{-1} Hz^{-1/27}$. The setup consisted of a Laser Scope unit (Block Engineering Inc., Massachusetts, USA), a home-built multipass

**Figure 1.** Collection of an exhaled breath sample in a healthy child.



cell of 0.6 liters and an infrared mercury cadmium telluride detector (PVI-4TE-10.6-0.3x0.3-TO8-BaF2, VIGO System S. A., Poland) (Figure 2). The Laser Scope unit contained a quantum cascade laser which scans wavenumber region between 832 and 1262.55 $cm^{-1}$ with a wavenumber resolution of 0.05 $cm^{-1}$. The laser was directed to the multipass cell, in which two astigmatic mirrors (Aerodyne Research Inc., Massachusetts, USA) ensured multiple reflections confining the laser to travel for 54.36 meters inside the volume of the multipass cell. When the laser exited the multipass cell its intensity profile was measured using an infrared Mercury Cadmium Telluride (MCT) detector. To measure the absorption profile of the breath samples we first recorded the laser signal with the multipass cell emptied, by reducing the pressure inside the cell to less than 0.1 mbar using both a pressure controller (P-702CV-1K1A-RAD-22-V, Bronkhorst High-Tech BV, Ruurlo, The Netherlands) and a diaphragm vacuum pump (MD 1, Vacuumbrand GMBH + CO KG, Wertheim, Germany). Subsequently, a breath sample was injected in the multipass cell using a set of mass flow controllers (F-201CV-1K0-RAD-22-V, Bronkhorst High-Tech BV, Ruurlo, The Netherlands) ensuring a continuous flow of 500 $mL.min^{-1}$ until the internal pressure reached 900 mbar, this uses around 600 mL of the breath sample. At this point the flow was reduced to 50 $mL.min^{-1}$ and the internal pressure stabilized during 6 min, the time needed for the quantum cascade laser scan. In total, we used at least 900 mL of each breath sample. Before injection, the breath sample passed through a Nafion dryer (MD-070-24S-4, Perma Pure LLC, New Jersey, USA) to improve the signal-to-background ratio. The absorption profile of the breath sample was calculated as the ratio of the signal of the breath sample over that of the empty cavity. This way, we removed the influence of any residues that may have been in the multipass cell before introducing the breath sample. Furthermore, after each measurement the multipass cell and the tubing were cleaned by flushing once with synthetic air and twice with nitrogen. This was done to

remove all gases from the cell. The empty cell was measured after this procedure to verify that no trace of detectable molecules were present in the cell. The total procedure for measuring one exhaled breath sample, including scanning, obtaining the complete absorption spectrum and cleaning the setup for the next sample, takes less than 15 min.

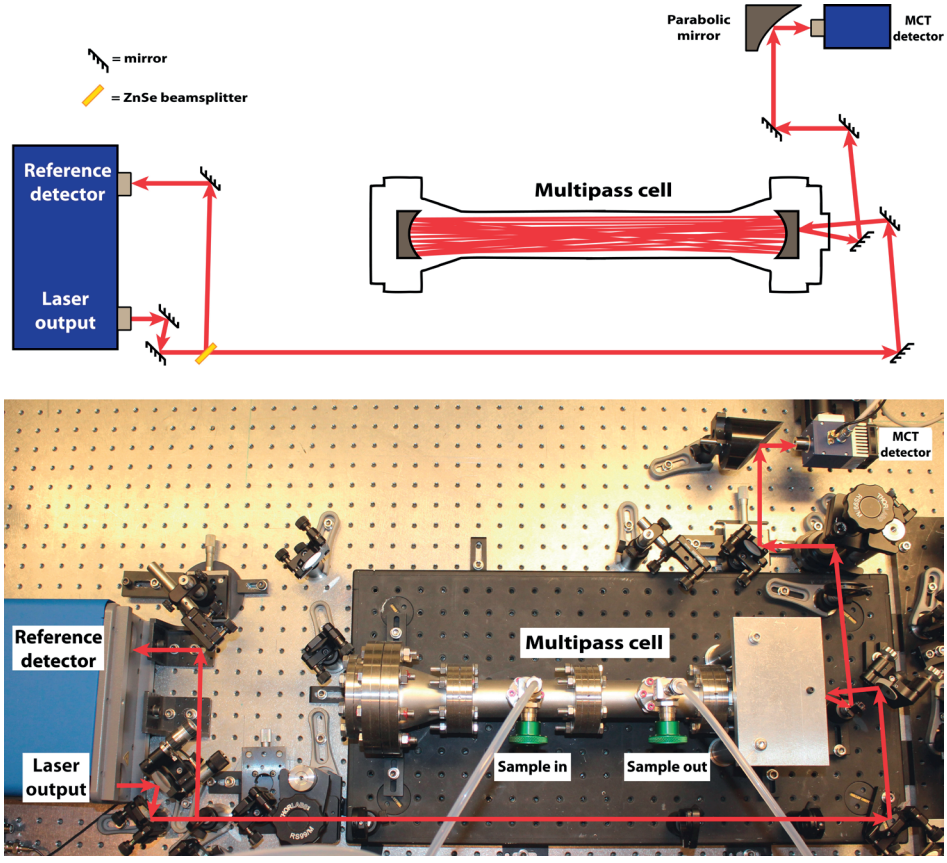


**Figure 2.** The spectroscopic setup consist of the quantum cascade laser, a multipass cell and a Mercury Cadmium Telluride (MCT) detector. The multipass cell has a volume of 0.6 L with a total length of 50 cm and a diameter of 4 cm in the middle of the tube. The housing for the astigmatic mirrors has a conical shape with a maximum diameter of 12 cm.

## Data analysis

Subject characteristics of the study groups were presented as median (interquartile range, IQR). The distribution of wavenumbers in all samples was approximately Gaussian with a similar median and variance. To increase the sensitivity of subsequent analyses for each spectroscopic wavenumber we subtracted the median of all wavenumbers, divided by the median absolute deviation and added the global median of all sample spectra. For all analyses, we separated the complete spectral data into 0.05 $cm^{-1}$ bins,

yielding 8612 bins in total from 832 $cm^{-1}$ through 1262.55 $cm^{-1}$. Each bin, referred to as a wavenumber point, was treated as a discrete measurement in all subsequent analyses. Spearman's correlation coefficient was used to compare the 1st and 2nd exhaled breath samples in each patient group and the 1st with the 2nd, 3rd and 4th exhaled breath sample in the healthy children. Agreement between the first 2 exhaled breath samples was assessed for each group with a Bland and Altman plot to assess the mean and 95% limits of agreement. Principal component analysis was performed on the mean of the first two exhaled breath profiles of each individual, in order to reduce the influence of intra-individual variability on the discriminative ability of laser spectroscopy to separate exhaled breath profiles of healthy children from exhaled breath profiles of children with stable asthma or CF. We employed the BioConductor package limma (version 3.22.1) to identify differences in spectroscopic wavenumbers between the 3 study groups [13]. Briefly, limma uses linear models combined with methods for variance stabilization and post-hoc multiple correction (Benjamini-Hochberg at false discovery rate < 0.05) [14]. As the absorption feature of molecules is given by a wavenumber region rather than a single wavenumber point, we considered wavenumber regions as significantly different when at least 10 neighboring wavenumber points had a p-value of less than 0.05. The width of these wavenumber regions is 0.5-1 $cm^{-1}$. The choice of this width was based on the fact that the narrowest absorption features of small molecules like $CO_2$ and $H_2O$ have a full width at half maximum (FWHM) of around 0.1 $cm^{-1}$, but their full spectral line width is about 0.5 $cm^{-1}$. All statistical calculations were performed using R version 3.1.2.



## RESULTS

### Subject characteristics

We included 35 healthy children, 39 children with asthma and 15 children with CF. We successfully collected exhaled breath samples in all children. The clinical characteristics of the three groups are presented in Table 1 [15].

### Repeatability of exhaled breath profiles

We collected a 2nd exhaled breath sample 30 min after the 1st exhaled breath sample in 35 healthy children, 31 children with asthma and 15 children with CF. In addition, we collected a 3rd exhaled breath sample after 24 hours and a 4th exhaled breath sample after 1 week in 34 healthy children. Each exhaled breath profile consists of 8612 individual wavenumber points with each a width of 0.05 $cm^{-1}$, in total covering a broadband spectral region from 832 $cm^{-1}$ until 1262.55 $cm^{-1}$. We found a poor overall correlation between the 1st and the 2nd exhaled breath profile in each group: Spearman's rho = 0.46 in healthy children, 0.43 in children with asthma and 0.43 in children with CF. Similar

**Table 1.** Subject characteristics

| | Healthy (n=35) | Asthma (n=39) | CF (n=15) |
|---|---|---|---|
| **Age (years)** | 9.9 (9.0-10.7) | 10.9 (8.7-14.0) | 11.0 (7.7-14.5) |
| **Sex (M:F)** | 15:20 | 26:13 | 4:11 |
| **Height (cm)** | 141.5 (133.0-149.0) | 150.0 (133.4-160.5) (n=38) | 145.2 (129.3-164.9) |
| **Weight (kg)** | 33.0 (27.5-37.0) | 42.9 (35.8-52.8) (n=38) | 36.4 (27.4-59.1) |
| **$FEV_1$ (% predicted)[a]** | 94.6 (83.0-106.0) (n=32) | 90.2 (84.7-99.8) (n=33) | 95.7 (83.9-105.6) |
| **FeNO** | 15.0 (11.0-18.0) (n=33) | 25.0 (12.0-39.0) (n=35) | n.a. |
| **Atopy** | 0/35 | 31/39 | n.a. |
| **ACT score** | n.a. | 22.0 (18.5-23.0) (n=29) | n.a. |

Definition of abbreviations: M: male; F: female; $FEV_1$: forced expiratory volume in 1 s; FeNO: fractional exhaled nitric oxide; ACT: asthma control test; n.a.: not applicable.
Data are presented as median (interquartile range).
[a] Percentage predicted according to the GLI-2012 equations [15].

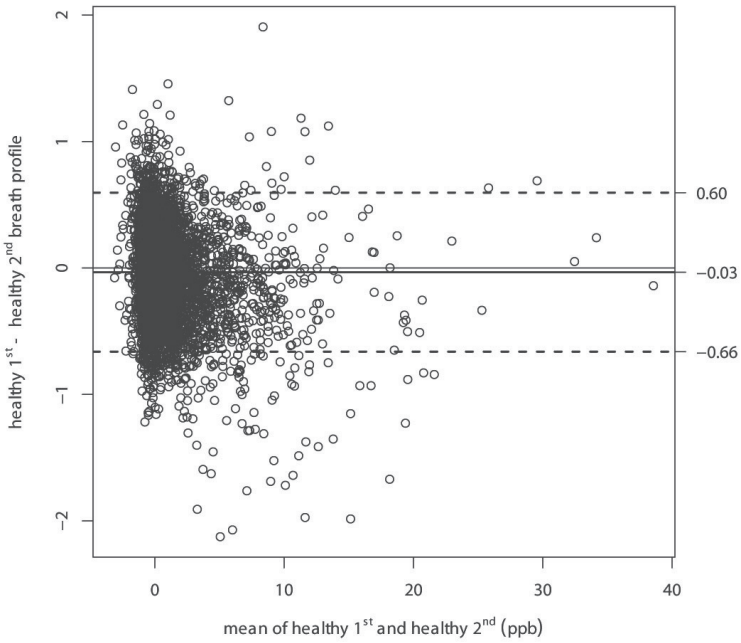


**Figure 3.** Bland and Altman plot showing moderate agreement between the 1st and the 2nd exhaled breath sample profile in 35 healthy children. Each dot represents the mean plotted against the difference of a single wavenumber point with a width of 0.05 $cm^{-1}$ in all healthy children. The vertical axis depicts the difference between the 1st and the 2nd exhaled breath profile, the horizontal axis the mean of the 1st and the 2nd exhaled breath profiles (parts per million volume, ppmv). The lines represents the mean and the 95% agreement limits.

overall correlations were shown in healthy children between the 1st and the 3rd measurement (Spearman's rho = 0.39) and the 1st and the 4th measurement (Spearman's rho = 0.36). Bland and Altman plots for each group showed relatively good agreement when individual wavenumber points were assessed with a mean difference of -0.03 and limits of agreement between 0.60 and -0.65 in the healthy children (Figure 3).

## Absorption spectra

Scatterplots depicting the separation between healthy children and children with asthma and between healthy children and children with CF by two principal components are shown in Figure 4. We found significantly different wavenumber regions when we compared the mean of the first two exhaled breath profiles of asthmatic children with those of healthy controls in the wavenumber regions 1181.80–1182.55 $cm^{-1}$ and 1261.40–1262.05 $cm^{-1}$. Between healthy children and CF patients we observed one significantly different wavenumber region between 1260.70 and 1261.65 $cm^{-1}$.



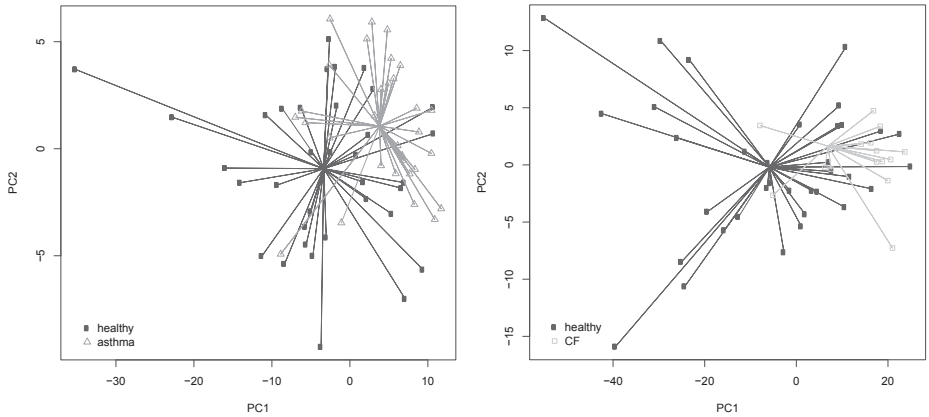


**Figure 4. (a)** 2D principal component plot showing the discrimination of the mean of the first two breath prints for each individual between healthy controls (circles) and children with asthma (triangles) and **(b)** between healthy controls (circles) and children with CF (squares) along two principal components with the greatest variance in spectroscopic data.

## Chemical identification of VOCs

Using the Pacific Northwest National Laboratory (PNNL) database [16], we located molecules with their absorption signature in the wavenumber regions which differed significantly between healthy children and the children with asthma or CF. We selected the molecules with an absorption signature corresponding to a minimum concentration of 10 parts per million volume (ppmv) and compared these with VOCs previously reported to be present in human exhaled breath [17]. In table 2 and 3 we summarize the VOCs which have their absorption spectra within the wavenumber regions that differentiated healthy

**Table 2.** VOCs which have been identified earlier in exhaled breath, with absorption signatures in the wavenumber regions that differentiated healthy children from children with stable asthma

| Compounds | | Wavenumber region ($cm^{-1}$) | |
|---|---|---|---|
| **CAS number** | **Name** | **1181.80 - 1182.55** | **1261.40 - 1262.05** |
| **64-19-7** | Acetic acid | ✓ | |
| **123-86-4** | Acetic acid, butyl ester | | ✓ |
| **141-78-6** | Acetic acid, ethyl ester / ethyl acetate | | ✓ |
| **110-19-0** | Acetic acid, isobutyl ester / isobutyl acetate | | ✓ |
| **108-21-4** | Acetic acid, isopropyl ester / isopropylacetate | | ✓ |
| **79-20-9** | Acetic acid, methyl acetate | | ✓ |
| **111-15-9** | 1-acetoxy-2-ethoxyethane | | ✓ |
| **105-54-4** | Butanoic acid, ethyl ester | ✓ | ✓ |
| **78-93-3** | 2-butanone | ✓ | |
| **107-92-6** | Butyric acid | ✓ | |
| **123-91-1** | 1,4-diethylene dioxide | | ✓ |
| **616-38-6** | Dimethyl carbonate | | ✓ |
| **115-10-6** | Dimethyl ether | ✓ | |
| **108-39-4** | 1-hydroxy-3-methylbenzene | ✓ | |
| **109-94-4** | Methanoic acid, ethyl ester/ ethyl formate | ✓ | |
| **107-31-3** | Methanoic acid, methyl ester/ methyl formate | ✓ | |
| **34590-94-8** | 2-methoxymethylethoxy/ Propanol | ✓ | ✓ |
| **75-09-2** | Methylene chloride / dichloromethane | | ✓ |
| **80-62-6** | 2-methylpropenoic acid, methyl ester / methyl methacrylate | ✓ | |
| **554-12-1** | Propanoic acid, methyl ester | ✓ | |
| **79-09-4** | Propanoic acid / propionic acid | ✓ | |
| **140-88-5** | 2-proponic acid, ethyl ester | ✓ | ✓ |
| **79-01-6** | 1,1,2-trichloroethene / algylen | ✓ | |

children from children with stable asthma and stable CF, and that have been identified previously in exhaled breath. The complete results are given in the online supplement.

## DISCUSSION

We used broadband quantum cascade laser-based spectroscopy to detect VOC profiles in exhaled breath samples and tested this in healthy children and children with asthma or CF. We found an overall poor repeatability and limited agreement on the short and long term, of the complete exhaled breath profiles. We identified VOC profiles that discriminated healthy children from children with stable asthma or stable CF. Our results

**Table 3.** VOCs which have been identified earlier in exhaled breath, with absorption signatures in the wavenumber region which differentiated healthy children from children with stable CF

| Compound | Wavenumber region ($cm^{-1}$) | |
|---|---|---|
| **CAS number** | **Name** | **1260.70 - 1261.65** |
| **123-86-4** | Acetic acid, butyl ester | ✓ |
| **141-78-6** | Acetic acid, ethyl ester / ethyl acetate | ✓ |
| **110-19-0** | Acetic acid, isobutyl ester / isobutyl acetate | ✓ |
| **108-21-4** | Acetic acid, isopropyl ester / isopropylacetate | ✓ |
| **79-20-9** | Acetic acid, methyl acetate | ✓ |
| **111-15-9** | 1-acetoxy-2-ethoxyethane | ✓ |
| **105-54-4** | Butanoic acid, ethyl ester | ✓ |
| **123-91-1** | 1,4-diethylene dioxide | ✓ |
| **616-38-6** | Dimethyl carbonate | ✓ |
| **34590-94-8** | 2-methoxymethylethoxy/ Propanol | ✓ |
| **75-09-2** | Methylene chloride / dichloromethane | ✓ |
| **140-88-5** | 2-proponic acid, ethyl ester | ✓ |



suggest that laser-based spectroscopy might be a useful technique for exhaled breath profiling.

This is the first study that investigates a laser-based spectroscopic method to detect VOC profiles in exhaled breath samples. Our method covered a broad wavenumber region [7]. Previous studies, investigating laser-based spectroscopy as a method to analyze VOCs in exhaled breath, focused on narrow wavenumber regions (10-20 $cm^{-1}$) representing absorption of only one or two specific molecules [18-20]. With our technique, we were able to detect VOC profiles within a broader wavenumber range. In addition, VOC identification was possible with the use of molecular databases such as the PNNL database. Identification of molecules is important to understand underlying inflammatory processes, which may be useful to develop new and/or personalized treatments. The advantages of detecting VOC profiles with our technique over GC-MS are the ability to detect whole molecules instead of fragmented molecules, the relatively fast and easy analysis and the potential for miniaturization.

We showed poor repeatability of complete exhaled breath profiles both on the short term and on the long term. However, we also showed a relatively good agreement of individual wavenumber points in the first and second breath sample. A previous study, in which 3 breath samples were collected with a 2 min interval, also showed high coefficients of variation for VOC profiles when compared to individual molecules [21]. The limited repeatability might be explained by the fact that we investigated a large spectroscopic range comprising 8612 individual wavenumber points. Furthermore, we consider it plausible that a high variability of exhaled breath content could be the result

of multiple daily life factors, like eating, drinking, exercise and the variable composition of ambient air. All children in this study refrained from eating, drinking and exercise one hour prior to exhaled breath sampling. Surprisingly, the long term repeatability in the healthy children was similar to the short term repeatability. This suggested that VOCs reflecting the pulmonary condition may be relatively stable over time but comprise only a small amount of the complete profile.

Previous studies have shown that exhaled breath VOC profiles measured with GC-MS and the eNose could discriminate healthy controls from asthmatic children and from children with CF with a relatively high sensitivity and specificity and limited intra-individual variability [2,3,22-24]. We were also able to discriminate healthy children from children with stable asthma or stable CF, however, the limited separation between the groups in the PCA suggests that the majority of wavenumber points and spectra representing VOCs did not differ between healthy and diseased children. Previous studies all reported different sets of best-discriminative VOCs for specific diagnoses, and there clearly is a need for external validation [2-5,22-25]. Since hydrocarbons have been found to be discriminative VOCs in several different studies regarding respiratory inflammatory diseases, we focused our setup on the detection of hydrocarbons and set the wavenumber range of our laser between 832 and 1262.55 $cm^{-1}$ covering the complete absorption spectra of this group of molecules. Surprisingly, molecules with absorption spectra in the wavenumber regions that differed between healthy and diseased children in the present study included carboxylic acids, esters and ethers, which can derive from hydrocarbons. A recent review showed that these molecules represent around 20% of all molecules found in exhaled breath samples [17]. When we focused on molecules which have been described before in exhaled breath, we noticed overlap between those in asthma and CF. This is not unexpected, as CF is characterized by chronic neutrophilic airway inflammation, and varying degrees of neutrophilic inflammation have been observed in childhood asthma as well [26]. Based on the current data it is not possible to quantify the amount of airway inflammation or the intensity of the disease.

The main limitations of our study and earlier breath profiling studies include lack of standardization of sample collection and analysis between studies, thereby hampering the overall comparability. For instance, the effect of expiratory flow remains unclear although current data do not suggest that flow control is needed [24,27-29]. Therefore, we did not standardize expiratory flow making our sampling technique technically easy and applicable for children of all ages. We cannot rule out contamination of exhaled breath samples by disposable plastic components (mouthpieces, tubes and valves). As we used the same type of disposables in all children, this would not affect between-group differences. Storage time in Tedlar bags was kept less than 12 h for all exhaled breath samples [30]. In order to avoid contamination we cleaned the multipass cell after each measurement, and used the laser signal of the empty cell immediately after performing

the cleaning procedure as a reference for the following measurement, thereby we ruled out the influence of water, methane, acetone, ethanol and $CO_2$ on our exhaled breath profiles. Another possible source of measurement variability is the fluctuations in the intensity of the laser. We have addressed this issue by monitoring the laser emission using a reference detector [7]. The electronic noise is kept minimum by using thermally cooled detectors. Finally, our spectroscopic setup has a sensitivity in the order of parts per million volume (ppmv), which is less than the parts per trillion volume (pptv) sensitivity of other laser-based techniques such as cavity ring down spectroscopy [31]. To what extent higher sensitivity would result in better results remains to be shown.

In conclusion, we developed a broadband quantum cascade laser-based spectroscopy technique for the detection of VOC profiles in exhaled breath samples. We showed overall poor short- and long term repeatability of this technique, but found different exhaled breath profiles in healthy children versus children with airway inflammation due to asthma or CF. Additional validation and attempts to improve the repeatability are needed before further clinical studies can be undertaken. The combination of a relatively easy and fast method and the possibility of molecule identification makes broadband quantum cascade laser-based spectroscopy attractive to investigate the diagnostic and prognostic potential of volatiles in exhaled breath.

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## SUPPLEMENTAL TABLES

**Table 1.** VOCs with absorption signature in the wavenumber regions which differentiated healthy children from children with stable asthma

| Compounds | | Wavenumber region ($cm^{-1}$) | |
|---|---|---|---|
| **CAS number** | **Name** | **1181.80 - 1182.55** | **1261.40 - 1262.05** |
| **431-89-0** | 1,1,1,2,3,3,3-Heptafluoropropane (HFC227EA) | | ✓ |
| **75-68-3** | 1,1,1-Chlorodifluoroethane (Freon-142B) | ✓ | |
| **421-50-1** | 1,1,1-Trifluoroacetone | ✓ | |
| **420-46-2** | 1,1,1-Trifluoroethane (R143A) | | ✓ |
| **79-01-6 *** | 1,1,2-trichloroethene / algylen | ✓ | |
| **124-73-2** | 1,2-Dibomotetrafluoroethane (Freon-114B2) | ✓ | |
| **106-93-4** | 1,2-Dibromoethane (EDB) | ✓ | |
| **354-23-4** | 1,2-Dichloro-1,1,2-trifluoroethane (F132A) | ✓ | |
| **123-91-1 *** | 1,4-diethylene dioxide | | ✓ |
| **111-15-9 *** | 1-acetoxy-2-ethoxyethane | | ✓ |
| **354-25-6** | 1-Chloro-1,1,2,2-tetrafluoroethane (R124A) | ✓ | ✓ |
| **108-39-4 *** | 1-hydroxy-3-methylbenzene | ✓ | |
| **75-89-8** | 2,2,2-Trifluoroethanol | ✓ | ✓ |
| **25256-77-4** | 2,2,4-Trimethyl-1,3-pentanediol isobutyrate (Texanol) | ✓ | ✓ |
| **306-83-2** | 2,2-Dichloro-1,1,1-trifluoroethane (Freon-123) | ✓ | |
| **76-11-9** | 2,2-Difluorotetrachloroethane (F112A) | ✓ | |
| **78-93-3 *** | 2-butanone | ✓ | |
| **75-88-7** | 2-Chloro-1,1,1-trifluoroethane (R133A) | | ✓ |
| **2837-89-0** | 2-Chlroro-1,1,1,2-tetrafluoroethane (HCFC-124) | ✓ | |
| **138495-42-8** | 2H, 3H-Perfluoropentane | ✓ | ✓ |
| **34590-94-8 *** | 2-methoxymethylethoxy / Propanol | ✓ | ✓ |
| **80-62-6 *** | 2-methylpropenoic acid, methyl ester / methyl methacrylate | ✓ | |
| **140-88-5 *** | 2-proponic acid, ethyl ester | ✓ | ✓ |
| **64-19-7 *** | Acetic acid | ✓ | |
| **123-86-4 *** | Acetic acid, butyl ester | | ✓ |
| **141-78-6 *** | Acetic acid, ethyl ester / ethyl acetate | | ✓ |
| **110-19-0 *** | Acetic acid, isobutyl ester / isobutyl acetate | | ✓ |
| **108-21-4 *** | Acetic acid, isopropyl ester / isopropylacetate | | ✓ |
| **79-20-9 *** | Acetic acid, methyl acetate | | ✓ |
| **75-86-5** | Acetone cyanohydrin | ✓ | |
| **105-54-4 *** | Butanoic acid, ethyl ester | ✓ | ✓ |
| **107-92-6 *** | Butyric acid | ✓ | |

**Table 1.** (continued)

| Compounds | | Wavenumber region ($cm^{-1}$) | |
|---|---|---|---|
| **CAS number** | **Name** | **1181.80 - 1182.55** | **1261.40 - 1262.05** |
| **353-50-4** | Carbonyl fluoride | | ✓ |
| **107-30-2** | Chloromethyl methyl ether | ✓ | |
| **76-15-3** | Chloropentafluoroethane (R115) | ✓ | |
| **1189-71-5** | Chlorosulfonyl isocyanate (CSI) | ✓ | |
| **108-20-3** | Diisopropyl ether | ✓ | |
| **108-18-9** | Diisopropylamine | ✓ | |
| **616-38-6 *** | Dimethyl carbonate | | ✓ |
| **115-10-6 *** | Dimethyl ether | ✓ | |
| **79-44-7** | Dimethylcarbamoyl chloride | | ✓ |
| **541-41-3** | Ethyl chloroformate | ✓ | |
| **383-63-1** | Ethyl trifluoroacetate | ✓ | |
| **76-13-1** | Freon-113 (1,1,2-Trichlorortrifluoroethane) | ✓ | |
| **76-14-2** | Freon-114 (1,2-dichlorortetrafluoroethane) | ✓ | ✓ |
| **811-97-2** | Freon-134a (1,1,1,2-tetrafluoroethane) | ✓ | |
| **76-19-7** | Freon-218 (octafluoropropane) | | ✓ |
| **115-25-3** | Freon-C318 (octafluorocyclobutane) | | ✓ |
| **684-16-2** | Hexafluoroacetone | | ✓ |
| **76-16-4** | Hexafluoroethane (Freon-116) | | ✓ |
| **381-10-5** | Hexafluoroisobutylene | ✓ | ✓ |
| **116-15-4** | Hexafluoropropene | ✓ | |
| **124-63-0** | Methanesulfonyl chloride | ✓ | |
| **109-94-4 *** | Methanoic acid, ethyl ester/ ethyl formate | ✓ | |
| **107-31-3 *** | Methanoic acid, methyl ester/ methyl formate | ✓ | |
| **96-33-3** | Methyl acrylate | ✓ | ✓ |
| **93-58-3** | Methyl benzoate | ✓ | ✓ |
| **547-63-7** | Methyl isobutyrate | ✓ | ✓ |
| **598-98-1** | Methyl pivalate | ✓ | |
| **119-36-8** | Methyl salicylate | ✓ | ✓ |
| **79-22-1** | Methylchloroformate (MCF) | ✓ | |
| | Methyldichlorodisilanes (mixed isomers) | | ✓ |
| **75-09-2 *** | Methylene chloride / dichloromethane | | ✓ |
| **97-66-7** | N,N-Diethylaniline | | ✓ |
| **628-63-7** | n-Amyl acetate | | ✓ |
| **123-63-7** | Paraldehyde | ✓ | |
| **355-25-9** | Perfluorobutane | ✓ | ✓ |
| **382-21-8** | Perfluoroisobutylene (PFIB) | ✓ | |
| **79-09-4 *** | Propanoic acid / propionic acid | ✓ | |

**Table 1.** (continued)

| Compounds | | Wavenumber region ($cm^{-1}$) | |
|---|---|---|---|
| **CAS number** | **Name** | **1181.80 - 1182.55** | **1261.40 - 1262.05** |
| **554-12-1 *** | Propanoic acid, methyl ester | ✓ | |
| **624-65-7** | Propargyl chloride | | ✓ |
| **109-06-4** | Propyl acetate | | ✓ |
| **7791-25-5** | Sulfuryl chloride | ✓ | |
| **2699-79-8** | Sulfuryl fluoride | | ✓ |
| **994-05-8** | tert-Amyl methyl ether | ✓ | |
| **75-73-0** | Tetrafluoromethane | | ✓ |
| **75-05-1** | Trifluoroacetic acid | ✓ | ✓ |
| **407-25-0** | Trifluoroacetic anhydride | ✓ | ✓ |
| **354-32-5** | Trifluoroacetyl chloride | ✓ | ✓ |
| **75-46-7** | Trifluoromethane (Freon-23) | ✓ | |
| **373-80-8** | Trifluoromethylsulfur pentafluoride | | ✓ |
| **334-99-6** | Trifluoronitrosomethane | ✓ | ✓ |

All VOCs identified with PNNL database, with absorption spectra within the significantly different wavenumber regions between healthy children and children with stable asthma.
* VOCs that have been identified before in exhaled breath and included in Table 2 of the main article.

**Table 2.** VOCs with absorption signature in the wavenumber regions which differentiated healthy children from children with stable CF

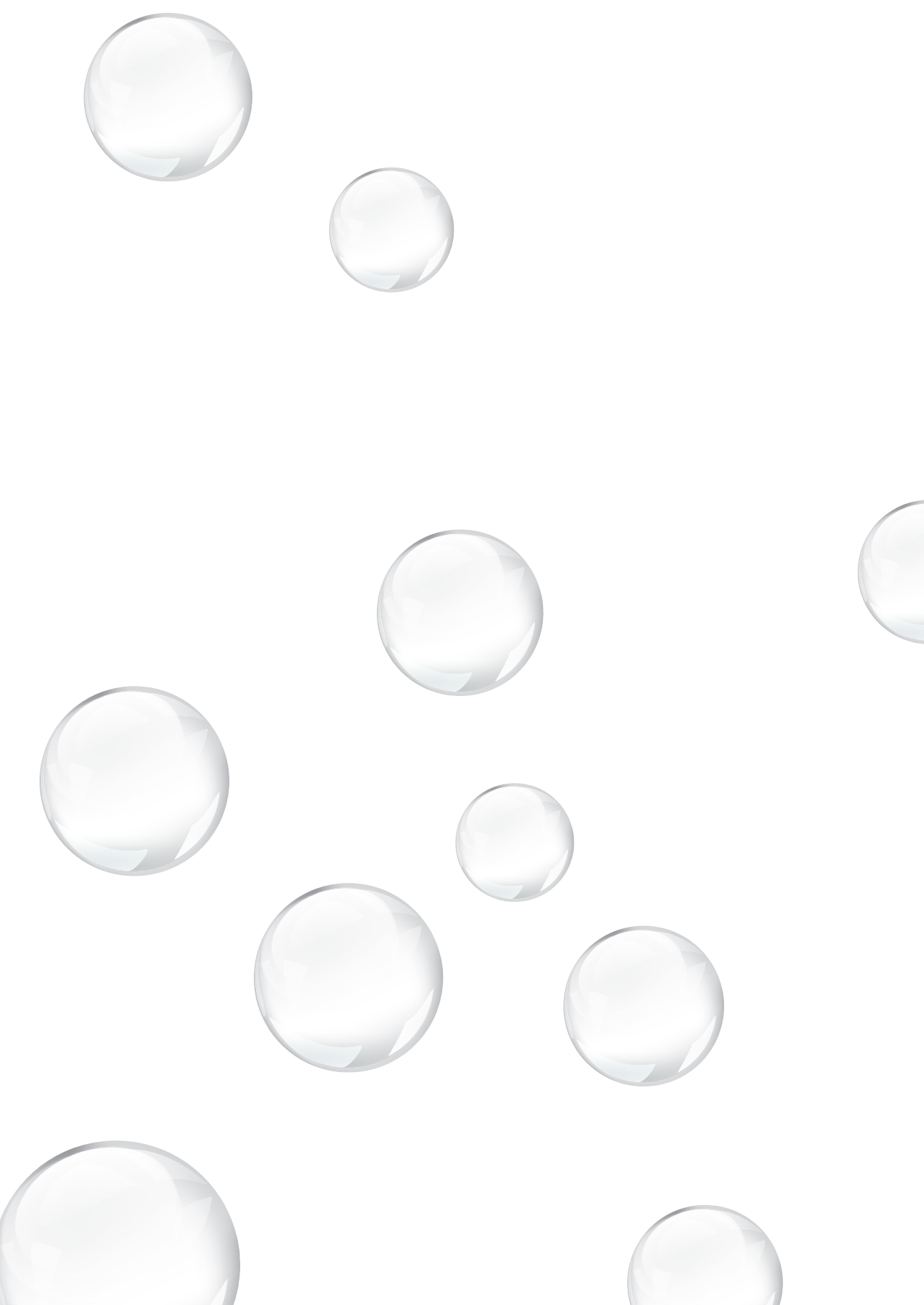
| Compound | | Wavenumber region ($cm^{-1}$) |
|---|---|---|
| **CAS number** | **Name** | **1260.70 - 1261.65** |
| **431-89-0** | 1,1,1,2,3,3,3-Heptafluoropropane (HFC227EA) | ✓ |
| **421-50-1** | 1,1,1-Trifluoroacetone | |
| **420-46-2** | 1,1,1-Trifluoroethane (R143A) | ✓ |
| **123-91-1 *** | 1,4-diethylene dioxide | ✓ |
| **111-15-9 *** | 1-acetoxy-2-ethoxyethane | ✓ |
| **354-25-6** | 1-Chloro-1,1,2,2-tetrafluoroethane (R124A) | ✓ |
| **75-89-8** | 2,2,2-Trifluoroethanol | ✓ |
| **25256-77-4** | 2,2,4-Trimethyl-1,3-pentanediol isobutyrate (Texanol) | ✓ |
| **75-88-7** | 2-Chloro-1,1,1-trifluoroethane (R133A) | ✓ |
| **138495-42-8** | 2H, 3H-Perfluoropentane | ✓ |
| **34590-94-8 *** | 2-methoxymethylethoxy / Propanol | ✓ |
| **140-88-5 *** | 2-proponic acid, ethyl ester | ✓ |
| **123-86-4 *** | Acetic acid, butyl ester | ✓ |
| **141-78-6 *** | Acetic acid, ethyl ester / ethyl acetate | ✓ |
| **110-19-0 *** | Acetic acid, isobutyl ester / isobutyl acetate | ✓ |

**Table 2.** (continued)

| Compound | | Wavenumber region ($cm^{-1}$) |
|---|---|---|
| **CAS number** | **Name** | **1260.70 - 1261.65** |
| **108-21-4 *** | Acetic acid, isopropyl ester / isopropylacetate | ✓ |
| **79-20-9 *** | Acetic acid, methyl acetate | ✓ |
| **105-54-4 *** | Butanoic acid, ethyl ester | ✓ |
| **353-50-4** | Carbonyl fluoride | ✓ |
| **616-38-6 *** | Dimethyl carbonate | ✓ |
| **79-44-7** | Dimethylcarbamoyl chloride | ✓ |
| **76-19-7** | Freon-218 (octafluoropropane) | ✓ |
| **115-25-3** | Freon-C318 (octafluorocyclobutane) | ✓ |
| **684-16-2** | Hexafluoroacetone | ✓ |
| **76-16-4** | Hexafluoroethane (Freon-116) | ✓ |
| **381-10-5** | Hexafluoroisobutylene | ✓ |
| **93-58-3** | Methyl benzoate | ✓ |
| **547-63-7** | Methyl isobutyrate | ✓ |
| **119-36-8** | Methyl salicylate | ✓ |
| | Methyldichlorodisilanes (mixed isomers) | ✓ |
| **75-09-2 *** | Methylene chloride / dichloromethane | ✓ |
| **97-66-7** | N,N-Diethylaniline | ✓ |
| **628-63-7** | n-Amyl acetate | ✓ |
| **355-25-9** | Perfluorobutane | ✓ |
| **624-65-7** | Propargyl chloride | ✓ |
| **109-06-4** | Propyl acetate | ✓ |
| **2699-79-8** | Sulfuryl fluoride | ✓ |
| **75-73-0** | Tetrafluoromethane | ✓ |
| **75-05-1** | Trifluoroacetic acid | ✓ |
| **407-25-0** | Trifluoroacetic anhydride | ✓ |
| **354-32-5** | Trifluoroacetyl chloride | ✓ |
| **373-80-8** | Trifluoromethylsulfur pentafluoride | ✓ |
| **334-99-6** | Trifluoronitrosomethane | ✓ |

All VOCs identified with PNNL database, with absorption spectra within the significantly different wavenumber regions between healthy children and children with stable CF.

* VOCs that have been identified before in exhaled breath and are included in Table 3 of the main article.



# Part III

## Bronchopulmonary dysplasia: early markers in tracheal aspirates and clinical follow up

# 5 Angiogenic growth factors in serial tracheal aspirates and the development of bronchopulmonary dysplasia in preterm infants

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Paper in progress.

## ABSTRACT

### Background

The mechanisms responsible for dysangiogenesis and alveolar simplification in bronchopulmonary dysplasia (BPD) are poorly understood. Subpopulations of macrophages may well contribute to dysangiogenesis in BPD.

Aim: The aim of this study was to investigate whether macrophages and their products in serial tracheal aspirates (TAs) of ventilated preterm children correlate with BPD development.

### Methods

We tested serial TAs of preterm infants who did or did not develop severe BPD on the presence of angiogenesis-related proteins and macrophages by proteome profiling, multiplex assay, cytological examination and RT-PCR. Second, we examined the overall angiogenic activity of TAs using pericyte human endothelial cell co-cultures.

### Results

TAs contained a broad variety of alternative angiogenesis-related factors. We observed a wide range angiogenic activity on pericyte-human endothelial cell co-cultures by TAs. Tubule growth did not correlate with VEGF, but did correlate with some alternative angiogenesis-related factors. The proangiogenic factors EMMPRIN, GDF15 and HGF, the pro-inflammatory cytokines TNF-α, IL-1α and IL-1β, and the M2-type macrophage-related proteins CCL17 and CCL24 were present at significantly higher levels in TAs of preterm infants without BPD compared to those with severe BPD. Additionally, we found significant higher gene expression of the M2-related genes MRC1 and CD206 in infants without BPD.

### Conclusion

We observed higher levels of pro-angiogenic growth factors, pro-inflammatory cytokines and M2-type macrophage-related proteins in serial TAs of preterm infants who did not develop BPD. Our findings support a role for M2-like macrophages in angiogenesis and tissue homeostasis during alveolarisation. We speculate that M2 macrophages and their products may be potential therapeutic targets for prevention and treatment of BPD.

## INTRODUCTION

Bronchopulmonary dysplasia (BPD) is a chronic lung disease that occurs in extremely preterm infants and is characterized by impaired alveolarisation [1-4]. Currently, the exact pathogenesis of BPD is unclear and treatment is limited and symptomatic. Histology of lungs from patients who died from BPD show remarkable abnormalities in the pulmonary vascular network with a reduction in small arteries, abnormal distribution of capillaries in the distal lung, and intrapulmonary arteriovenous connections [5-7]. Disruption of microvascular development in immature lungs has been postulated as a critical factor in the abnormal alveolar development in BPD [6, 8, 9]. The signals linking alveolar and vascular growth in the developing lung are not well understood. Decreased vascular endothelial growth factor (VEGF) concentrations have been found in bronchoalveolar lavage fluid (BALF) of infants developing BPD [10], although data are conflicting [11-13]. More recent studies show an association between BPD and alternative regulators of angiogenesis, such as endoglin, endothelial monocyte activating polypeptide II (EMAP-II) and endostatin [14-16]. Also chemokines with angiogenic effects such as monocyte chemoattractant proteins (MCP)-1, MCP-2 and MCP-3, and macrophage inflammatory proteins (MIP)-1α and MIP-1β were found to be associated with the development of BPD [16, 17]. Since many of these angiogenic growth factors are products of inflammatory cells (e.g., monocytes/macrophages, neutrophils), they may provide a link between inflammation and dysangiogenesis, independent of VEGF [6,17,18]. Macrophages and their precursor monocytes are able to produce a wide array of angiogenic growth factors including endoglin, endostatin and EMAP-II. They belong to a heterogeneous group of myeloid cells with a high degree of plasticity in response to local micro-environmental stimuli and are important for tissue homeostasis [19-22]. Monocytes/macrophages can be subdivided in different subsets (M1, M2a-d). The most polarized subsets express either M1 markers like tumor necrosis factor (TNF)-α and interleukin (IL)-1 or M2 markers like YM1 as well as multiple pro-angiogenic genes, but mixed phenotypes have also been described. M2-polarized macrophages have been demonstrated in neonatal lungs during the alveolarisation stage of lung development. Their localization to sites of branching morphogenesis suggests a role in normal lung growth and development [23].

The aim of the present study was to investigate the presence of angiogenic factors in serial tracheal aspirates (TAs) of preterm infants with a focus on macrophage-derived factors and to compare these between infants who did and did not develop severe BPD. We hypothesized that perinatal lung macrophages display an M2 phenotype and that changes in the M2-related protein expression profile correlates with BPD development.

## METHODS

### Patient inclusion

Preterm infants (≤ 32 weeks gestational age, GA) in need of mechanical ventilation in the first week of life were recruited at the neonatal intensive care unit of Erasmus MC-Sophia Children's Hospital. Children with a congenital disorder affecting lung structure and/or function and children who were intubated only to administer surfactant were excluded. BPD was defined as need for oxygen supplementation for ≥ 28 days [24]. BPD severity was assessed by means of an oxygen reduction test at 36 weeks postmenstrual age (PMA) [25]. Relevant maternal and neonatal characteristics were prospectively collected from patient records. Two groups of infants were formed in this study: preterm infants who developed severe BPD at 36 weeks PMA (n=22), and preterm infants who did not develop BPD (n=23). Written informed consent was obtained from the parents. The study was approved by the medical ethical committee of Erasmus MC, Rotterdam (MEC-2013-062, NL43229.078.13).

### Tracheal aspirate collection and processing

We obtained TAs directly after intubation in the first week of life, and at consecutive days (1, 3, 5, and 7), during routine clinical suctioning procedures where 0.5 mL of sterile isotonic saline was instilled into the endotracheal tube, and after 5 s was suctioned with a 5F catheter positioned at the end of the endotracheal tube. After centrifugation for 5 min at 300×g, the supernatant was collected, aliquoted in extra low binding Eppendorf tubes (BiozymTC, Landgraaf, the Netherlands), and stored at -80°C until analysis. Cytospin preparations for morphological analysis were made with a cytocentrifuge (Cytospin 4, Thermo Scientific, Breda, the Netherlands) at 680 rpm for 7 min with low acceleration. Cytospins with 50,000 cells per slide were stained with May-Grünwald Giemsa staining. A differential cell count was performed by two observers (EM and IK).

### Proteome profiling, multiplexed fluorescent bead-based immunoassay (Luminex) and ELISA

A set of 102 predefined growth factors and cytokines was analyzed with the proteome profiler array from R&D systems (Human XL Cytokine Array Kit, ARY022). Undiluted pooled TA samples (in total 1.5 ml) from the first 3 days of 4 patients with severe BPD were compared with undiluted pooled samples from the first 3 days of 6 patients without BPD. This assay was performed a second time with pooled samples from 6 different severe BPD patients and 8 patients without BPD.

Next, we used a customized magnetic bead-based immunoassay (Luminex, custom made screening assay, R&D systems) in which we included angiogenic factors that showed high expression in the proteome analysis. These included: VEGF, angiopoietin

(Ang)1, Ang2, platelet-derived growth factor (PDGF)α, PDGFβ, extracellular matrix metalloproteinase inducer (EMMPRIN)/CD147, growth differentiation factor (GDF)15, hepatocyte growth factor (HGF), epidermal growth factor (EGF), C-X-C motif chemokine (CXCL)1/GROα, CXCL4/PF4, CXCL5/ENA78, CXCL8/ IL-8, CXCL9/MIG, CXCL10/IL-10, CXCL13/BLCBCA, CXCL14/BRAK, C-C motif chemokine (CCL)2/MCP-1, CCL21/6Ckine, MIF, TNF-α, IL-1α, IL-1β and IL-6. We quantified this large set of growth factors, chemokines and cytokines in 139 serial TAs from 45 infants. CCL17 and CCL24 were additionally measured by ELISA (R&D systems, Quantikine ELISA). All assays were used according to the manufacturer's protocol.

**mRNA isolation and qPCR**

Sorted cells from TAs were stored at -80˚C in RLT lysis buffer (QIAGENE, US) and mRNA was extracted using RNeasy Micro Kit from QIAGEN (QIAGENE, US) according to the manufacturer's instructions. The mRNA concentration was measured on NanoDrop (Thermofisher, US). mRNA was amplified using Ovation® PART NO. 3312 PicoSL WTA System V2 (NuGENE, US) and purified with MinElute Reaction Cleanup Kit (QIAGENE, US). The following human primers were used: MRC1 forward 5' -CCTCTGGTGAACGGAATGAT- '3 and MRC1 reverse 5'-AGGCCAGCACCCGTTAAAAT-'3, and CD68 forward 5'-GCTACATGGCGGTGGAGTACAA-'3. CD68 reverse 5'-ATGATGAGAGGCAGCAAGATGG-'3, CD206 forward 5'-TTGGACGGATAGATGGAGGG, CD206 reverse 5'-CCAGGCAGTTGAGGAGGTTC, CD163 forward 5'-CCAGTCCCAAACACTGTCCT-3, reverse 5'-ATGCCAGTGAGCTTCCCGTTCAGC-3', and β-actin forward 5'-GCA CCA CAC CTT CTA CAA TG and reverse 5'- TGC TTG CTG ATC CAC ATC TG. Quantitative real-time PCR was carried out by Lightcycler 480 (Roche) with the Universal Probe Library system (Roche, West Sussex, UK). Gene expression was analyzed using the comparative Ct method with target gene mRNA levels being normalized to β-actin.

**Pericyte HUVEC co-culture**

Human umbilical vein endothelial cells (HUVEC) were cultured in EGM-2 (EBM-2 basal medium supplemented with EGM-2 bullit kit; Lonza) till passage 6 under standard culture conditions (37˚C and 5% $CO_2$). Human brain derived pericytes were cultured in DMEM with 10% FCS till passage 6 under standard culture conditions. We used HUVEC that express green fluorescent protein (GFR) and pericytes that express red fluorescent protein (RFP). For aspirate exposure, HUVEC GFP and pericytes RFP were diluted at a 5:1 ratio ($1.0*10^6$ : $0.20*10^6$/ml) in co-culture medium (EBM-2 basal medium with 2% FCS, ascorbic acid and FGF from the EGM-2 bullit kit; Lonza). For every condition, 0.2 µg/ml of feline/human/rhesus macaque CXCL12/SDF1α, human SCF/c-kit ligand and human IL-3 was added. Collagen type 1 was diluted in co-culture medium to a final concentration of 2 mg/ml with the cell mix. This co-culture system constitutes a bioassay to

quantify angiogenesis, as angiogenic factors will cause outgrowth of tubules that can be measured. In this co-culture bioassay, we tested whether TAs influences angiogenesis *in vitro*. Aspirates from severe BPD (n=6), no BPD (n=6) patients or PBS (n=6) were added as 10% of the total mixture. From this mix, 50 µl was transferred to a single well of a 96 wells flat bottom plate. After 1 hour incubation at 37˚C in 5% $CO_2$, co-culture medium supplemented 1:1 with severe BPD, no BPD or PBS was added on top of the collagen co-culture gel. Samples were incubated for 4 days at 37˚C in 5% $CO_2$. On day 2 images were taken using Olympus IX51 fluorescence microscope with CellSenseDimension software. Images were analyzed using AngioSys.

### Statistical analysis

Continuous variables were expressed as mean ± standard deviation (SD) for normally distributed data or median (interquartile range, IQR) for not normally distributed data, and categorical variables were expressed as n/N (%). We compared preterm infants who developed severe BPD with infants who did not develop BPD, using Fisher's exact test for categorical variables and the Mann-Whitney U test for continuous variables. Cell counts between these two groups and between different time points were compared using the Kruskal-Wallis test. Concentrations of growth factors, chemokines and cytokines in TAs were compared between the two groups for each time point separately using the Mann-Whitney U test. Concentrations below the detection limit were imputed as the lower detection limit as published by R&D [26]. Concentrations were log transformed in order to obtain a near normal distribution. Linear mixed effects models with unstructured covariance matrix were used to examine the change over time of growth factors, chemokines and cytokines concentrations in TAs, thereby providing estimates of the average change in the samples over time and compared between groups. Independent variables in the linear mixed effect models were birth weight corrected for GA, time between birth and intubation and time at which TAs were obtained (post intubation day 0, 1, 3, 5 and 7, as categorical variable with day 0 as reference level). Correlations between tubule growth and concentrations of growth factors, cytokines and chemokines were assessed with Spearman correlation coefficient. Two-sided significance level was 0.05 without correction for multiple testing. GraphPad Prism software (version 4.0, GraphPad) and SPSS version 21.0 software (SPSS, Inc., Chicago, IL) were used for statistical analyses.

## RESULTS

### Subject characteristics

We included serial TAs from 23 preterm infants who did not develop BPD and 22 who developed severe BPD. Infants with BPD were born at significantly lower GA, with lower

birth weight, received more postnatal surfactant, experienced more complications like persistent ductus arteriosus, and had a significantly longer duration of invasive mechanical ventilation and oxygen supplementation. Patient characteristics are given in Table 1.

**Table 1.** Patient characteristics

| | No BPD | Severe BPD | p value |
|---|---|---|---|
| **Number of patients** | 23 | 22 | |
| **Number of TAs** | Day 0: 15 | Day 0: 12 | |
| | Day 1: 17 | Day 1: 17 | |
| | Day 3: 15 | Day 3: 13 | |
| | Day 5: 11 | Day 5: 19 | |
| | Day 7: 6 | Day 7: 14 | |
| **Gestational age (weeks)** | 29.1 (26.7-30.4) | 25.9 (24.9-26.3) | < 0.001 |
| **Birth weight (gram)** | 1080 ± 374 | 762 ± 217 | 0.001 |
| **Sex (male : female)** | 12 : 11 | 11 : 11 | 1.000 |
| **Antenatal corticosteroids** | No: 5 (21.7%)<br>Yes, not complete: 6 (26.1%)<br>Yes, complete: 12 (52.2%) | No: 2 (9.1%)<br>Yes, not complete: 8 (36.4%)<br>Yes, complete: 12 (54.5%) | 0.516 |
| **Maternal preeclampsia** | 4 (17.4%) | 5 (22.7%) | 0.722 |
| **Chorioamnionitis** | 2 (8.7%) | 8 (36.4%) | 0.072 |
| **Postnatal surfactant** | No: 6 (26.1%)<br>1 gift: 9 (39.1%)<br>≥ 2 gifts: 8 (34.8%) | No: 1 (4.5%)<br>1 gift: 6 (27.3%)<br>≥ 2 gifts: 15 (68.2%) | 0.043 |
| **Early onset sepsis** | 0 (0.0%) | 1 (4.5%) | 0.489 |
| **Late onset sepsis** | 7 (30.4%) | 13 (59.1%) | 0.075 |
| **PDA** | 10 (43.5%) | 20 (90.9%) | 0.001 |
| **Total days invasive ventilation** | 6 (4-13) | 22 (17-35) | < 0.001 |
| **Total days oxygen exposure** | 10 (0-18) | 62 (46-69) | < 0.001 |

Definition of abbreviations: BPD: bronchopulmonary dysplasia; TAs: tracheal aspirates; PDA: persistent ductus arteriosus. Continuous variables are expressed as mean ± standard deviation (SD) for normally distributed data or median (interquartile range, IQR) for data that were not normally distributed. Categorical data are expressed as n (%).

## Angiogenesis-related factors in tracheal aspirates

We explored which angiogenesis related factors are present in TAs, using an antibody mediated proteome-profiling assay. The proteome profiler detected all classical growth factors involved in angiogenesis in TAs of preterm infants, such as VEGF, Ang1 and -2, PDGFα, PDGFβ and fibroblast growth factor (FGF) (Figure 1A). Additionally TAs contained a variety of alternative angiogenesis-related proteins (Figure 1B). Amongst these were CXCR-chemokines and CC-chemokines and other angiogenic proteins like EGF, HGF, GDF15 and EMMPRIN. The TAs contained both products of M1 macrophages (TNF-α,

IL1-β, IL-6, CCL3/MIP-1alpha, CCL4/MIP-3α, CCL5/RANTES) and of M2 macrophages (chitinase-3-like-1, IL-10, IL-Ra, CCL17/TARC). TAs also contained cytokines known to be involved in the induction of M1 (interferon (IFN)-γ and granulocyte-macrophage colony-stimulating factor (GM-CSF)), and M2 macrophages (IL-4, IL-6, IL-13, IL-33 and leukemia inhibitory factor (LIF)) (Figure 1C). Secretory IgA levels showed little variation between the samples, indicative of a low variance in dilution between different patient samples (Figure S1). To judge variance we repeated the experiment on comparable samples of different patients. Complete data of the two experiments are shown in Figure S2. We conclude that TAs of preterm infants, besides classical growth factors involved in angiogenesis, contained a variety of alternative angiogenesis related factors, representatives of both M1 and M2-type macrophages.

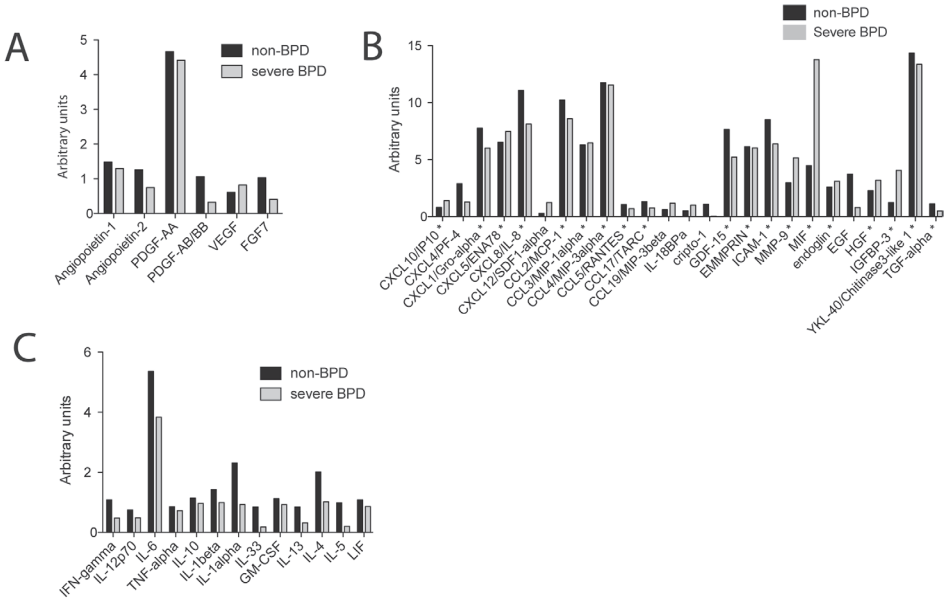


**Figure 1.** Proteomic analysis of angiogenesis-related factors in TAs of preterm born infants who did or did not develop severe BPD. **(A)** Results of a semi-quantitative antibody mediated proteome-profiling assay (R&D systems) on classical angiogenic growth factors, **(B)** other angiogenesis related growth factors and **(C)** proinflammatory cytokines in TAs of premature children. The data represents one of two experiments. To obtain enough material to perform the assay (1.5 ml per condition) we pooled TA samples obtained within the first 3 days of life of 4 patients who later developed severe BPD (grey bars) and 6 matched patients who did not develop BPD (black bars). Due to pooling no SD values or indications of significance could be given. Numbers on the Y-axis represent mean pixel density of the blot. Alternative growth factors that are either chemoattractants or products of monocytes/macrophages were marked with an asterix.

## Macrophage numbers and M2 gene expression in tracheal aspirates of preterm infants

Total cell numbers were lowest in the aspirates taken directly after intubation, and increased at later time points (Figure 2B). Differential cell counts showed that epithelial cells were highest on the day of intubation (day 0), while the percentage of neutrophils and macrophages increased later (Figure 2C-E). We found no differences in the cellular distribution patterns between infants who did and did not develop severe BPD (Figure 2C-E).

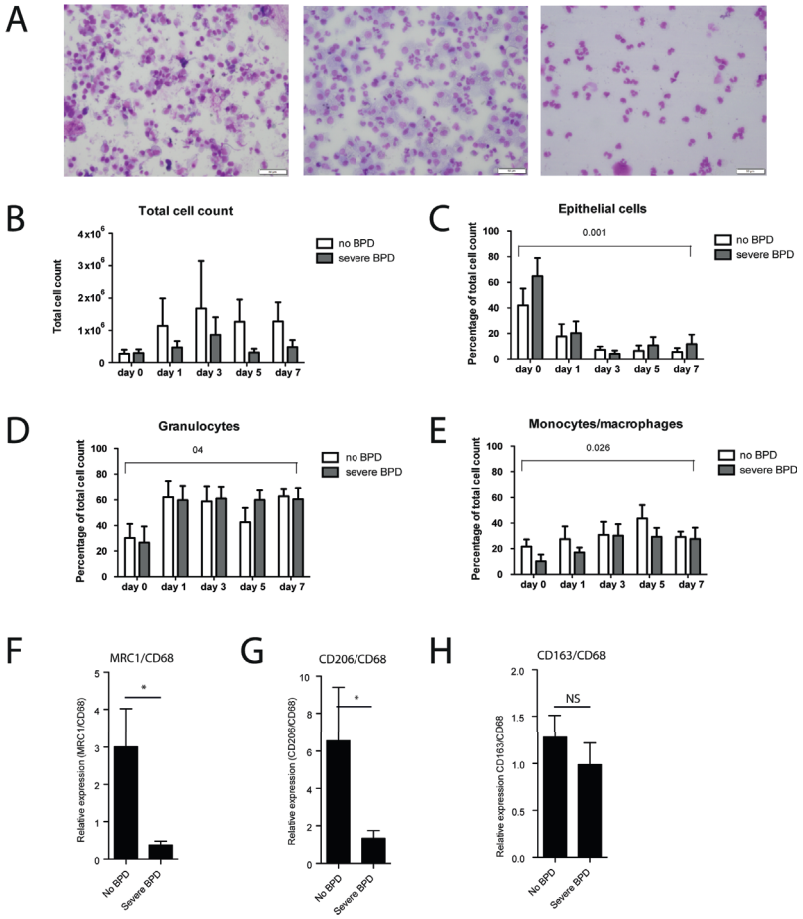


**Figure 2.** Presence of M2-like macrophages in TAs of preterm infants without BPD. **(A)** May-Grünwald Giemsa staining of three cytospin preparations (40X) from TAs of preterm born infants showing the predominant presence of epithelial cells (left panel), monocytes/macrophages (middle panel) and granulocytes (right panel). **(B)** Total cell counts and percentages of epithelial cells **(C)**, granulocytes **(D)** and monocytes/macrophages in TAs of preterm infants who developed severe BPD or did not develop BPD. **(F)** Gene expression of the M2 markers MRC1, CD206 **(G)** and CD163 **(H)** relative to CD68 in total cell lysates of TAs from children with and without BPD.

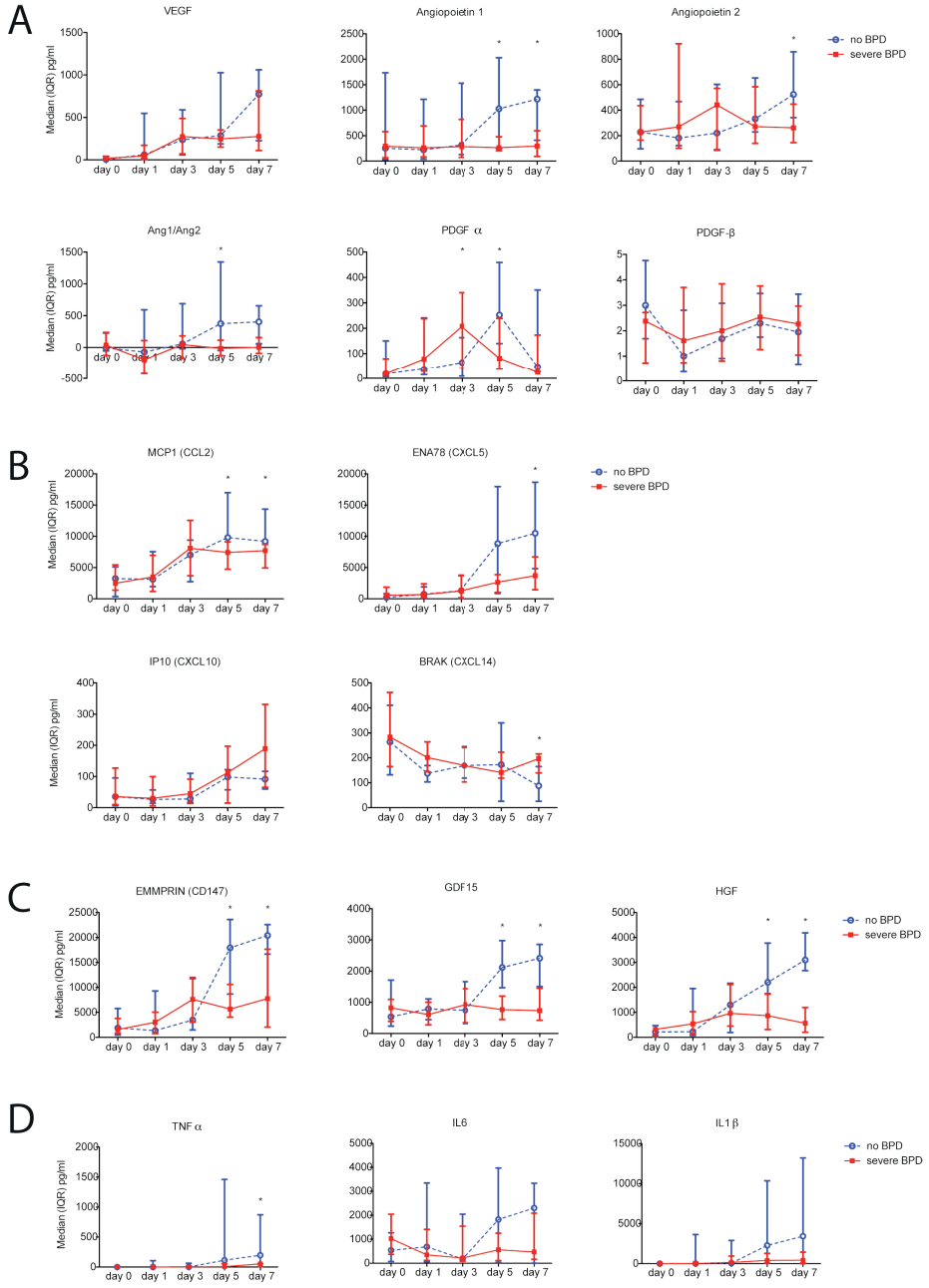


**Figure 3.** Macrophage-derived growth factors and chemokines over time. **(A)** Classical growth factors, **(B)** chemokines, **(C)** pro-inflammatory cytokines and **(D)** other alternative growth factors (ng/ml) in TAs of infants who developed bronchopulmonary dysplasia (BPD; •, continuous red line) and infants who did not develop BPD (no BPD; ○, dashed blue line), analyzed by Luminex assay. Dots represent median values, whiskers 25$^{th}$ and 75$^{th}$ percentiles, and group comparison by means of univariate analysis per time point, * = $p < 0.05$. Numbers of observations at different time points are given in Table 1.

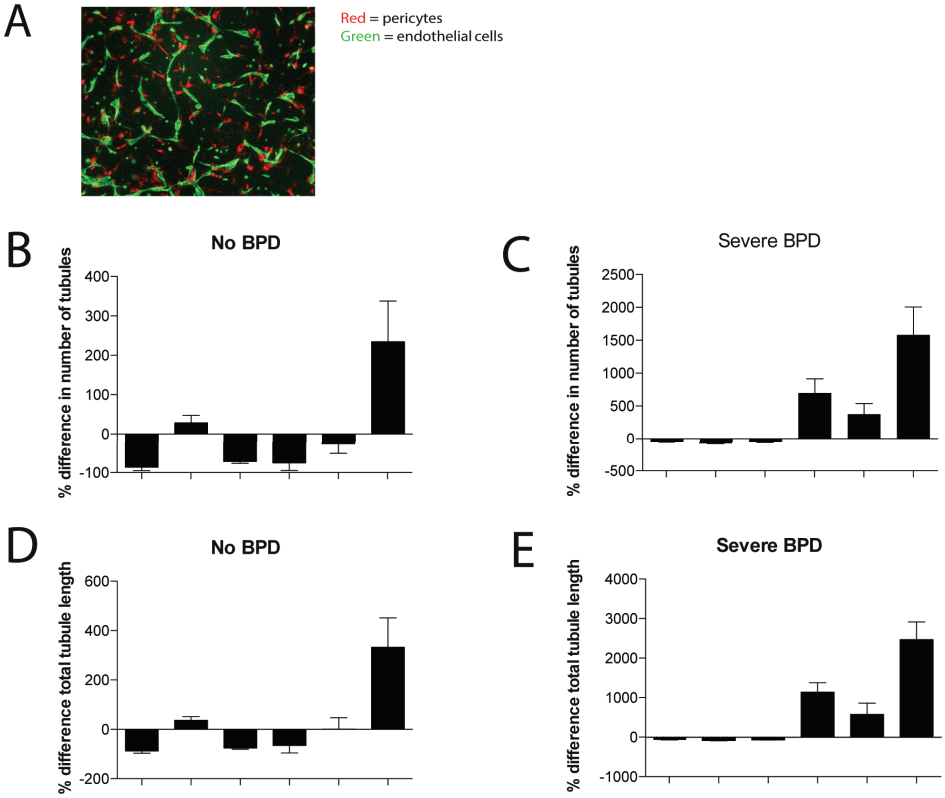


**Figure 4.** Monocyte/macrophage derived angiogenesis related factors in TAs of preterm born infants and effect on angiogenesis in vitro. **(A)** Representative pericyte-HUVEC co-culture showing endothelial cells in green and pericytes in red. Percent difference in number of tubules **(B and C)** and total tubule length **(D and E)** between pericyte-HUVEC co-cultures grown in the presence of medium diluted with PBS (1:1) or diluted with TAs (1:1) from preterm infants without BPD(n=6) or with severe BPD (n=6). Each bar represent the median of and individual patient, error bars are IQR of 6 measurements of a given TAs sample. No significant differences were found between TAs of children with and without BPD.

To investigate whether macrophage profiles are different between preterm infants with and without BPD we measured gene expression of the M2-macrophage specific markers MRC-1, CD206 and CD163 in TA total cell lysates by RT-PCR. We used CD68 as a macrophage specific housekeeping gene. We found significantly higher MRC1/CD68 and CD206/CD68 ratio's in preterm children who did not develop BPD (Figure 2F-G), indicating the presence of higher numbers of M2-like macrophages in this group. CD163 gene expression was not different between the two groups (Figure 2H).

### Difference in growth factors, cytokines and chemokines in serial TAs of preterm infants with and without BPD development

Concentrations of growth factors and cytokines in the Luminex assay and ELISA were above the detection limit in most TAs. Median concentrations (pg/mL) over time with 25$^{th}$ and 75$^{th}$ percentiles in infants who did not develop BPD and those who developed severe BPD are presented in Figure 3 and S3. Linear mixed models with each factor as dependent variable found an overall significant difference over time for HGF, EMMPRIN, GDF15, IL-1α, IL-1β and TNF-α, the M2-chemokines CCL17/TARC and CCL22/MDC and the classical growth factors Ang1, Ang2 and PDGFα, with lower concentrations in TAs of patients who developed severe BPD and higher concentrations in TAs of patients who did not develop BPD. For none of the CXCL chemokines significant differences were found.

### Effect of tracheal aspirates on angiogenesis *in vitro*

Average effects of TAs from infants with and without severe BPD on tubule growth, namely number and length of tubules, were not significantly different, respectively $p$=0.11 and $p$=0.15 (Figure 4). There was marked intersubject variation between samples: TAs from children without BPD (n=6) either inhibited (n=4) or enhanced (n=2) tubule formation, and TAs from infants with severe BPD (n=5) inhibited (n=2) or strongly enhanced (n=3) tubule formation. Tubule formation did not correlate with VEGF concentrations, but did correlate with several alternative growth factors. In infants with severe BPD the number and length of tubules correlated negatively with concentrations of endoglin ($r_s$=-0.841, $p$=0.04 and $r_s$=-0.841, $p$=0.04, respectively). In infants without BPD tubule growth correlated negatively with CXCL1/GROα, TNF-α and Ang1 (data not shown) and we found a positive correlation with GDF15 ($r_s$=0.886, $p$=0.02 and $r_s$=0.928, $p$=0.01, respectively).

## DISCUSSION

We showed that TAs of preterm infants contained classical angiogenic growth factors and a broad array of alternative angiogenesis-related proteins in relatively high concentrations. In a co-culture bioassay, the angiogenic effect of TAs was overall not different between infants who did or did not develop BPD, with marked interindividual variation. Tubule growth in these assays did not correlate with the classical angiogenic growth factor VEGF, but did correlate with several alternative growth factors. A selection of alternative proangiogenic factors including HGF, EMMPRIN and GDF15, M1/pro-inflammatory cytokines IL-1α, IL-1β and TNF-α, and M2-type factors CCL17/TARC and CCL22/MDC were higher in serial TAs of preterm infants who did not develop BPD compared to children who developed severe BPD. In addition, we found higher expression of the M2-

macrophage specific genes MRC1 and CD206 in those who did not develop BPD. These data support an effect of M2-type macrophage-derived angiogenic growth factors in the pathogenesis of BPD.

Previous studies focusing on the classical vascular growth factors VEGF and the Ang/Tie-2 system in lungs of patients developing BPD have reported conflicting results [11, 12, 27]. More recent studies postulated that inflammatory mediators like endoglin-1 and chemokines like MIP-1α and MCP-1 might cause a surge in inappropriate angiogenesis leading to aberrant alveolar capillaries in the developing lung [14-17, 28]. Besides previously identified alternative factors, such as endoglin, HGF and MMP-9, we also found factors not described in BPD before, but with well-known effects on angiogenesis such as EMMPRIN and GDF15. EMMPRIN is known to promote angiogenesis through its protease-inducing function and by affecting paracrine regulation of the VEGF/VEGF-receptor (VEGFR) system in endothelial cells [29]. GDF15, also known as MIC-1 (macrophage inhibitory cytokine 1) has anti-inflammatory functions [30] and a pro-survival and anti-oxidant role in hyperoxia [31, 32]. We found EMMPRIN and GDF15 in significantly higher concentrations in preterm children who did not develop BPD compared to those developing severe BPD.

We also noticed strong signals of CXCL5/ENA78, CXCL1/GROα and CXCL8/IL-8. These chemokines are well known for their effects on angiogenesis via ligation to the CXC chemokine receptor 2 (CXCR2), which is expressed on endothelial cells and epithelial cells, including the alveolar type II cell. The absence or inhibition of CXCR2 has been shown to reduce neutrophil accumulation, radical formation and lung injury caused by hyperoxia in adult mice and newborn rats [33]. Antibodies to CXCR2 or gene knockout also preserved alveolarization in murine models of BPD [34]. It has therefore been suggested that the CXCR2/CXCR2 ligand axis may be relevant in the pathogenesis of BPD. Similar data are present for the angiogenic CC-chemokines CCL2/MCP1, CCL3/MIP1α and CCL4/MIP2α, which also gave strong signals in our proteome assay. However, quantitative analysis of serial TAs only showed differences in infants with or without later BPD in ENA78/CXCL5 and MCP1/CCL2 and in none of the other chemokines, questioning the relevance of the above findings in mice for the human situation.

Previous studies have described higher levels of pro-inflammatory cytokines in TAs of preterm children who developed BPD, as recently reviewed elsewhere [35], and this seems contradictory to the present findings of higher levels of IL-1α, IL-1β and TNF-α and a clear trend towards higher levels of IL-6 in preterm infants who did not develop BPD. Compared to our findings, most samples in previous studies were collected within 24 hours after intubation and we observed differences at 5-7 days after intubation. Furthermore, some studies investigated BALF, while we studied TAs. Although the origin of most factors expressed in the proteome-profiling assay can be heterogeneous, it is likely that most factors originate from epithelial cells or from the inflammatory cells present in TAs, i.e. neutrophils and macrophages. Since TAs were taken from the proximal air-

ways, TA macrophages cannot be classified as alveolar macrophages. They most likely represent monocytes that migrated from the pulmonary interstitium into the tracheal lumen in response to chemoattractants, and differentiated into macrophages locally. This difference in cellular content between BALF and TAs may also explain the variance in concentrations of pro-inflammatory cytokines reported in different studies. Furthermore, evidence is also rising that coordinated expression of these cytokines during early immune responses plays a protective role as well. Fetal IL-6 is a regulatory cytokine of pulmonary surfactant proteins and plays an important role in lung maturity decreasing the incidence of respiratory distress syndrome in preterm neonates [36-39]. Intra-amniotic administration of IL-1α or IL-1β also induces lung maturation in preterm animals [40]. A recent study showed that absence of TNF-α strongly enhances inflammatory responses in newborn mouse lungs undergoing mechanical ventilation. Absence of TNF-α was associated with excess activation of TGF-β signaling, resulting in increased inflammation and apoptosis, and reduced NF-κB activity [41].

The relatively late increase in CCL17, CCL22 and pro-inflammatory cytokine concentrations paralleled a late increase of monocytes/macrophages in TAs. The low cell count in TAs did not allow for cell sorting and precise characterization of macrophages. Although total numbers of monocytes/macrophages did not differ between patients who did or did not develop BPD, we did find higher gene expression of the M2-macrophage markers MRC1 and CD206 in TAs cells of preterm infants who did not develop BPD. Together with the higher soluble M2 markers (CCL17 and CCL24) and higher concentrations of cytokines important for the induction of M2 macrophages (IL-4, IL-5, IL-13 and IL-33) this suggests high M2-macrophage activity in children who did not develop BPD.

The combination of pro-angiogenic factors, pro-inflammatory cytokines and M2-related factors reflects a similar expression profile as embryogenic macrophages and tumor-associated macrophages (TAMs), subsets of macrophages well-known for their function in angiogenesis. These factors were consistently higher in TAs of infants without BPD compared with TAs of infants with severe BPD. In addition, M2 macrophages have been found in neonatal lungs at the tips of alveolar septa where they seem to guide branching morphogenesis suggesting a role in ensuring normal lung growth and development [23]. An improved understanding of the role of macrophages under homeostatic and inflammatory conditions is important, as modulation of monocytes and macrophages and their products in the context of lung development may provide new future prevention or treatment modalities for BPD.

Some limitations of our study should be considered. The infants who did and did not develop severe BPD differed with respect to GA at birth, birth weight, postnatal surfactant, persistent ductus arteriosus and total days of invasive mechanical ventilation and oxygen supplementation, and the number of TA samples decreased over time, as many infants could be extubated within 7 days. This inevitably introduced selection bias. We

did not obtain TAs from preterm infants who did not need mechanical ventilation. Therefore, one can argue that a proper control group is missing. Few TAs were macroscopically contaminated with blood. Growth factors, chemokines and cytokines are also present in plasma, and this might have influenced our results. We consider it unlikely that such contamination differed systematically between groups, and affected the comparisons. Finally, no formal correction for multiple testing of different growth factors, chemokines and cytokines was performed.

In conclusion, we found a wide spectrum of angiogenic factors in TAs of ventilated preterm children during the first week of life which influenced tubular growth *in vitro*. Some pro-angiogenic factors, pro-inflammatory cytokines and M2-related factors were higher in infants who did not develop BPD. Further studies to better understand macrophage-related angiogenesis under inflammatory conditions in the newborn lung are warranted as they may lead to new prevention or treatment modalities for BPD.

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## SUPPLEMENTAL FIGURES

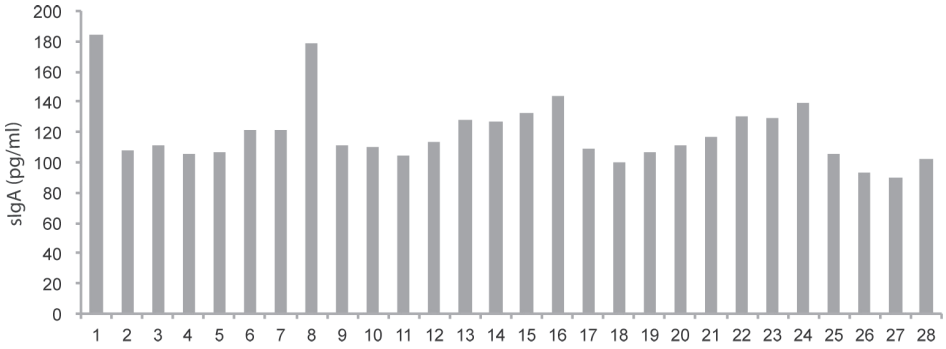


**Figure 1.** Secretory IgA in 28 randomly selected tracheal aspirates of preterm infants.

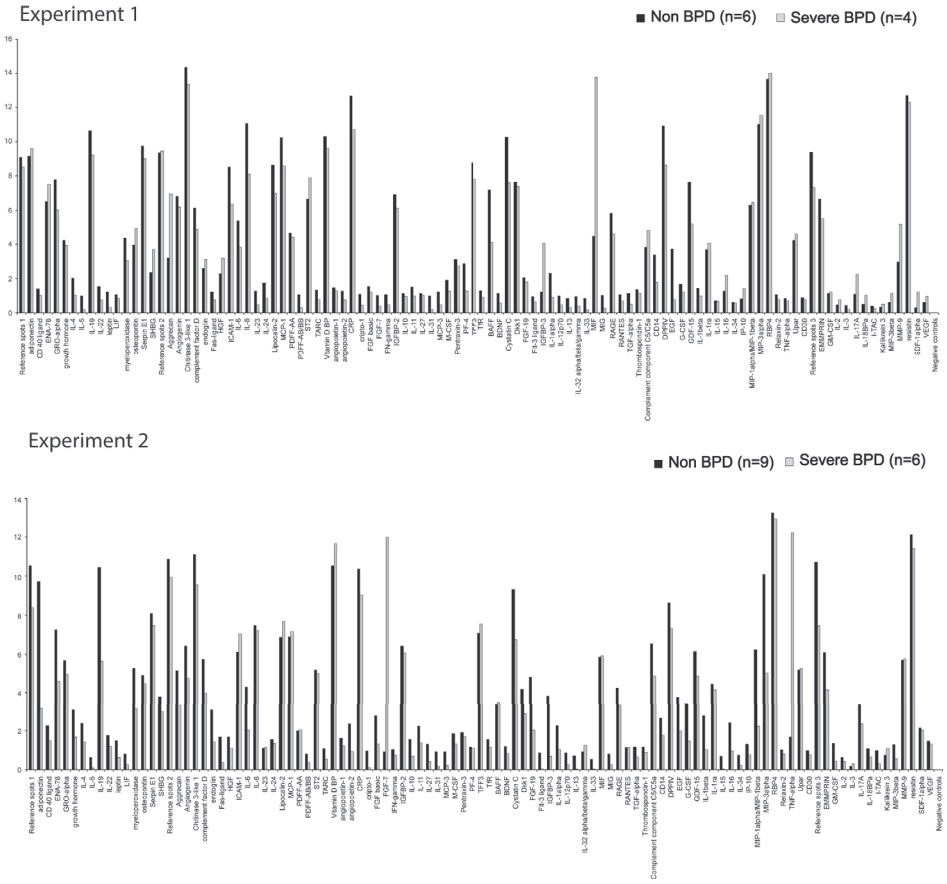


**Figure 2.** Complete proteome profiling data of two separate experiments on different TA samples of preterm infants who later developed severe BPD (grey bars) or did not develop BPD (black bars). The legend indicates the number of patients per group. The experiments illustrate the variability between patient samples. Note that overall both experiments showed the same expression pattern.

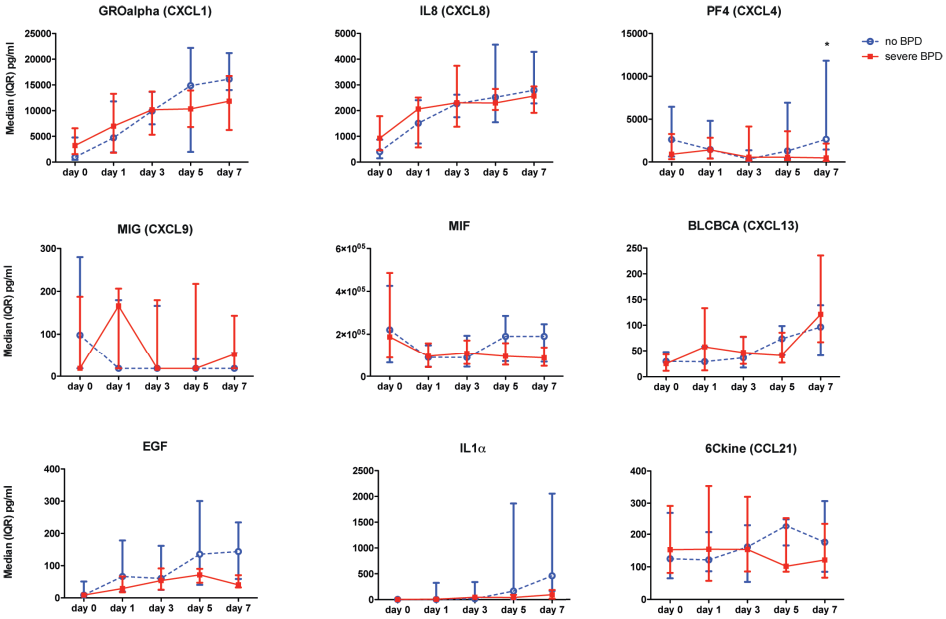


**Figure 3.** Additional alternative growth factors (ng/ml) in TAs of infants who developed bronchopulmonary dysplasia (BPD; •, continuous red line) and infants who did not develop BPD (no BPD; ○, dashed blue line) analyzed by Luminex. Dots represent median values, whiskers 25th and 75th percentiles, and group comparison by means of univariate analysis per time point, * = $p < 0.05$.

# 6 Ceramides in tracheal aspirates of preterm infants: marker for bronchopulmonary dysplasia

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**Submitted**

## ABSTRACT

### Background

In an experimental mouse model we showed that ceramides play a role in the pathogenesis of bronchopulmonary dysplasia (BPD) and are a potential target for therapeutic intervention. We investigated whether ceramides are detectable in tracheal aspirates (TAs) of preterm infants and differ between infants with or without BPD.

### Methods

Infants born ≤ 32 weeks of gestational age in need for mechanical ventilation in the first week of life were included. TAs were obtained directly after intubation and at day 1, 3, 5, 7, and 14. Ceramide concentrations were measured by tandem mass spectrometry. At 36 weeks postmenstrual age BPD was defined as having had ≥ 28 days supplemental oxygen.

### Results

122 infants were included, of which 14 died and 41 developed BPD. All infants showed an increase in ceramides after the first day of intubation. The ceramide profile differed significantly between preterm infants who did and did not develop BPD. However, the ceramide profile had no additional predictive value for BPD development over GA at birth, birth weight and total days of mechanical ventilation.

### Conclusions

Ceramides in TAs may be an early marker for BPD development and the ceramide pathway is a potential new target for future therapeutic interventions.

## INTRODUCTION

Bronchopulmonary dysplasia (BPD) is a serious complication affecting preterm born infants, with an incidence ranging from 4% in infants born at a gestational age (GA) of 31 weeks to 56% in infants born at a GA of < 26 weeks [1]. BPD is defined as need for oxygen supplementation for ≥ 28 days at 36 weeks postmenstrual age [2]. Genetic predisposition and exposure to environmental factors, such as chorio-amnionitis, preeclampsia, GA at birth, birth weight, mechanical ventilation, supplemental oxygen exposure, postnatal infections, persistent ductus arteriosus (PDA) and malnutrition, have been implicated in the etiology of BPD. To date, treatment is symptomatic and children with BPD are at increased risk of long term respiratory sequelae [3].

Sphingolipids are important structure-bearing constituents of the cell membrane that also function as regulatory molecules in cell proliferation and cell death, endothelial barrier function, angiogenesis, and immune response [4-6]. Altered sphingolipid levels have been shown to play a role in asthma [7-9], cystic fibrosis [10, 11], and cigarette-smoke or radiation induced lung injury [12-14]. This stresses the biological importance of the sphingolipid metabolism in lung disease [5]. Two important sphingolipids are ceramides and sphingosine-1-phosphate (S1P). Ceramides act as a precursor for all other sphingolipids; S1P is generated from ceramide via sphingosine [15]. Ceramides and S1P play an important role in apoptosis, with ceramides stimulating apoptosis and cell cycle arrest and S1P stimulating cell survival and proliferation [16]. Apoptosis may be critical in BPD as increased apoptosis has been found in pulmonary epithelial cells of BPD patients and in animal models of BPD [17, 18]. Recently we observed a transient increase in ceramide concentrations in bronchoalveolar lavage (BAL) fluid of a mouse model of BPD during the first 2-4 weeks of hyperoxia. Supplementation of D-sphingosine, a synthetic precursor of S1P, during normoxic recovery of hyperoxia-induced lung damage accelerated normalization of ceramide concentrations and improved the hyperoxia-induced alveolar arrest in this mouse model [19]. These findings suggest that ceramides are involved in the development of BPD, may be used as an early biomarker for BPD, and might be a target for new therapeutic interventions.

The aim of the present study was to translate these findings to humans, by analyzing ceramide profiles in tracheal aspirates (TAs) of preterm infants. We hypothesized that ceramide concentrations in TAs were higher in preterm born infants who developed BPD compared to those who did not, and may be an early biomarker for BPD.

## MATERIALS AND METHODS

### Study subjects and design

Preterm infants (≤ 32 weeks GA) in need of mechanical ventilation in the first week of life were recruited at the Neonatal Intensive Care Unit of Erasmus MC-Sophia Children's Hospital. Children with a congenital disorder affecting lung structure and/or function and children who were intubated only to administer surfactant were excluded. We obtained TAs directly after intubation, and as long as children were intubated at consecutive days 1, 3, 5, 7 and 14, during routine suctioning procedures. BPD was diagnosed according to criteria of Jobe and Bancalari [2]. BPD severity was assessed by means of an oxygen reduction test at 36 weeks postmenstrual age [20]. Relevant maternal and neonatal characteristics including maternal age, preeclampsia, chorio-amnionitis, antenatal steroids, mode of delivery, GA at birth, birth weight, sex, surfactant treatment, early or late onset sepsis, persistent ductus arteriosus (PDA), postnatal systemic steroids, days on mechanical ventilation and days on oxygen were prospectively collected from patients records. Written informed consent was obtained from the parents. The study was approved by the medical ethical committee of Erasmus MC, Rotterdam, The Netherlands (MEC-2013-062, NL43229.078.13).

### Tracheal aspirate collection and processing

TAs were collected directly after intubation, and at days 1, 3, 5, 7, and 14 as long as the patient was intubated. TAs were obtained at least 6 h after surfactant administration. During the procedure 0.5 ml of sterile isotonic saline was instilled into the endotracheal tube, and after 5 s suctioning was performed with a 5F catheter positioned at the end of the endotracheal tube. TAs were directly stored at 4°C. After centrifugation for 5 min at 300×g, the supernatant was collected, aliquoted in 1.5 ml extra low binding Eppendorf tubes (BiozymTC, Landgraaf, the Netherlands), and stored at -80°C until analysis. Subsequently, cell suspensions were stored in 100 µL 1x phosphate-buffered saline (PBS). Cells were counted and a maximum of 4 standardized cytospins with 50,000 cells per slide were prepared. Cytospin preparations were made with a cytocentrifuge (Cytospin 4, Thermo Scientific, Breda, the Netherlands) at 680 rpm for 7 min with low acceleration. The cytospins were air dried for at least 24 h and subsequently wrapped in aluminum foil and stored at -20°C. For each TA sample one cytospin was stained with May-Grünwald Giemsa staining. Per slide a differential cell count was performed by two observers (EM and IK) [21, 22].

### Liquid chromatography tandem mass spectrometry

Ceramide concentrations (ng/mL) in TAs were measured as previously described [19]. Briefly, lipids were extracted from TA samples (200 µL) and ceramides were separated using high performance liquid chromatography (LC) and quantified by tandem mass

spectrometry (MS/MS). The analyses were performed at the Analytical Facility for Bioactive Molecules of the Hospital for Sick Children, Toronto, ON, Canada.

**Statistical analysis**

Continuous variables were expressed as mean ± standard deviation (SD) for normally distributed data or median (interquartile range, IQR) for data that were not normally distributed, and categorical variables were expressed as n (%). We compared infants who did not develop BPD with those who did develop BPD or died because of respiratory failure before 36 weeks PMA using Fisher's exact test for categorical variables or the Mann-Whitney U test for continuous variables. Ceramide concentrations were compared for each time point separately using the Mann-Whitney U test. Ceramide concentrations below the detection limit were imputed as the lower detection limit (0.03 ng/ml). Ceramide concentrations were logarithmically transformed in order to obtain a near normal distribution. Spearman's rho was used to assess the correlation between individual ceramides and the correlation between ceramides and cell counts. Cell counts between different time points were compared using the Kruskal-Wallis test. Principal components analysis was applied to achieve a simpler structure combining ceramides that were highly correlated. Linear mixed effects models were used to examine the change over time of the mean logarithmically transformed ceramide concentrations, providing estimates of the average change in the samples. Independent variables in the linear mixed effect models were BPD outcome, GA at birth, birth weight SDS, time between birth and intubation and total days of invasive ventilation at 36 weeks PMA, and time at which TAs were obtained. Time at which TAs were obtained (post intubation day 0, 1, 3, 5, 7 and 14) was treated as categorical variable with day 0 as reference level. A random intercept was included in the linear mixed models to account for within-subject correlations.

To explore whether longitudinal observations of the ceramide profile or individual ceramides predicts the development of BPD we used a two-step approach. First, a linear regression of the log-transformed ceramides on time, coded as continuous variable, for each patient and ceramide was performed. The estimates of the intercept and the slope of the ceramide profile and individual ceramides were then included as independent variables in a logistic regression for the development of BPD. Patients for whom ceramide concentrations were not available at two or more time points were excluded from this analysis, because no linear regression could be performed in that case. GA at birth, birth weight SDS, time between birth and intubation and total days of invasive ventilation were included as independent variables in this logistic regression model. The calibration of the logistic regression model was assessed using the Hosmer-Lemeshow test. All statistical tests used a two-sided significance level of 0.05, without correction for multiple testing. SPSS version 21.0 software (SPSS, Inc., Chicago, IL) and Excel version 2010 software were used for statistical analyses.

## RESULTS

### Patient characteristics

We included 122 preterm born infants with a median GA (IQR) of 27.2 (25.6-29.6) weeks and a mean birth weight (SD) of 1003 (371) grams between September 2013 and December 2014 (Table 1). Fourteen infants died before 36 weeks PMA, of whom 3 due to respiratory failure. Forty-one infants (33.6%) developed BPD, 16 mild-moderate and 25 severe (Figure 1). Infants who developed BPD were born at significantly lower GA, with lower birth weight, received more antenatal corticosteroids and postnatal surfactant, experienced more complications like late onset sepsis and PDA, and had a significantly longer duration of mechanical ventilation and oxygen supplementation.

### Ceramides in tracheal aspirates of preterm born infants

We collected 341 TAs from 111 preterm infants included in our final analysis, of which 84 TAs were collected at day 0, 87 at day 1, 52 at day 3, 47 at day 5, 39 at day 7, and 32 at day 14. All ceramides were highly correlated with each other (data not shown). The concentrations of each individual ceramide were above the detection limit in the majority of TAs, except for CerDiHy 18:1, of which 92.1% of the samples had concentrations below

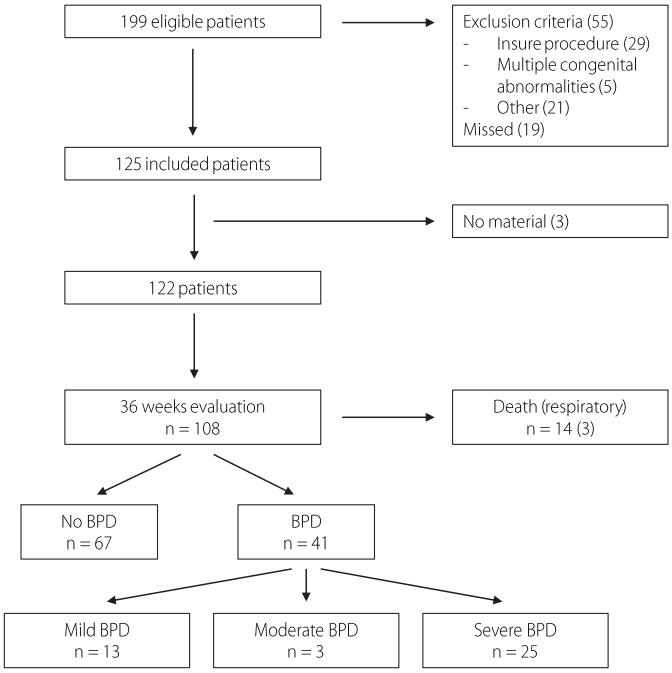


**Figure 1.** Flow diagram study population. Overview of the eligible and included patients between September 2013 and December 2014.

**Table 1.** Patient characteristics

| | No BPD | BPD or respiratory death | *p* value |
|---|---|---|---|
| **Number of patients** | 67 | 44 | |
| **Number of TAs** | Day 0: 51 | Day 0: 34 | |
| | Day 1: 48 | Day 1: 39 | |
| | Day 3: 18 | Day 3: 34 | |
| | Day 5: 12 | Day 5: 35 | |
| | Day 7: 8 | Day 7: 31 | |
| | Day 14: 3 | Day 14: 29 | |
| **Gestational age (weeks)** | 29.4 (27.9-30.4) | 25.9 (24.5-26.3) | < 0.001 |
| **Birth weight (gram)** | 1205 ± 346 | 768 ± 230 | < 0.001 |
| **Sex (male : female)** | 33 : 34 | 25 : 19 | 0.446 |
| **Antenatal corticosteroids** | No: 17 (25.4%)<br>Not complete: 17 (25.4%)<br>Yes, complete: 33 (49.2%) | No: 3 (6.8%)<br>Not complete: 12 (17.9%)<br>Yes, complete: 29 (65.9%) | < 0.001 |
| **Maternal preeclampsia** | 14 (20.9%) | 11 (25.0%) | 0.647 |
| **Chorioamnionitis** | 14 (20.9%) | 15 (34.1%) | 0.203 |
| **Postnatal surfactant** | No: 12 (17.9%) | No: 4 (9.1%) | < 0.001 |
| | 1 dose: 36 (53.7%) | 1 dose: 12 (27.3%) | |
| | ≥ 2 doses: 19 (28.4%) | ≥ 2 doses: 28 (63.6%) | |
| **Early onset sepsis** | 2 (3.0%) | 2 (4.5%) | 0.648 |
| **Late onset sepsis** | 14 (20.9%) | 23 (52.3%) | 0.001 |
| **PDA** | 24 (35.8%) | 38 (86.4%) | < 0.001 |
| **Total days mechanical ventilation** | 2 (1-6) | 22 (11-33) | < 0.001 |
| **Total days supplemental oxygen exposure** | 3 (0-13) | 55 (40-67) | < 0.001 |

Definition of abbreviations: BPD: bronchopulmonary dysplasia; PDA: persistent ductus arteriosus. Maternal preeclampsia was defined as newly developed hypertension (systolic ≥ 140 mmHg or diastolic ≥ 90 mmHg) after 20 weeks of pregnancy and proteinuria (protein-creatinine ratio ≥ 30 mg/mmol or ≥ 300 mg/24u). Chorioamnionitis was determined based on placental pathology findings. Treatment with prenatal corticosteroids was considered adequate when infants were born at least 12 hours after the second antenatal corticosteroid administration. Early onset sepsis was defined as a positive blood culture in the first 72 hours after birth, late onset sepsis as a positive blood culture after the first 72 hours after birth and in the first 3 months of life. Continuous variables are expressed as mean ± standard deviation (SD) for normally distributed data or median (interquartile range, IQR) for data that were not normally distributed. Categorical data are expressed as n (%).

the detection limit. Therefore, this ceramide was excluded from further analysis. Secretory IgA levels showed little variation between the samples, indicative of a low variance in dilution between different patient samples (data not shown). All infants showed an increase in ceramides after the first day of intubation. Ceramides in infants who did not develop BPD generally showed a late rise in ceramides until day 14, and this was not seen in those who developed BPD. Median ceramide concentrations (ng/ml) over time with

25th and 75th percentiles in infants who did and did not develop BPD are presented in Figure 2. Principal component analysis revealed that a single component was sufficient to represent the data of all ceramides, explaining 84.2% of all variance. We therefore computed a new variable representing the mean of the logarithmically transformed ceramides per patient and per time point, as this mean was similar to the single principal component. The linear mixed model performed with this new variable as dependent variable found an overall significant difference in ceramide profile between patients who did and did not develop BPD ($p$= 0.026) and a significant change in ceramide profile over time compared to the day of intubation (Table 2). Multivariable logistic regression analysis showed that the ceramide profile over time had no additional predictive value over known predictors of BPD outcome (GA at birth, birth weight, total days invasive ventilation) (Table 3). When we performed separate multivariable logistic regression analysis for all the individual ceramides for each time point, individual ceramides had no significant predictive value over the known predictors (Supplemental data).

**Table 2.** The change in overall ceramide profile

| | Ceramide profile level | |
|---|---|---|
| **Predictor** | **Coefficient (95% CI)** | ***p* value** |
| GA at birth | -0.044 (-0.200 – 0.112) | 0.575 |
| Birth weight (SDS score) | 0.177 (-0.049 – 0.403) | 0.123 |
| Total days invasive ventilation | 0.014 (-0.009 – 0.037) | 0.221 |
| Time between birth and intubation (days) | 0.002 (-0.003 – 0.007) | 0.442 |
| **BPD diagnosis** | | |
| No BPD | 0[a] | |
| BPD or respiratory death | -0.797 (-1.497 – 0.098) | 0.026[b] |
| **Post intubation day** | | |
| Day 0 | 0[a] | |
| Day 1 | 1.019 (0.565 – 1.472) | <0.001[b] |
| Day 3 | 0.458 (-0.083 – 1.000) | 0.097 |
| Day 5 | 0.871 (0.303 – 1.440) | 0.003[b] |
| Day 7 | 0.634 (0.023 – 1.244) | 0.042[b] |
| Day 14 | 0.410 (-0.257 – 1.076) | 0.227 |

This linear mixed model investigates the association between GA, birth weight (SDS score), total days invasive ventilation, time between birth and intubation, time of obtaining TA (post intubation day 0, 1, 3, 5, 7 or 14) and the occurrence of BPD and respiratory death on rates of change in ceramide profile. [a] No BPD and post intubation day 0 are used as reference category, [b] p<0.05.

## Cell count in tracheal aspirates

We found no difference in total cell count of TA samples between infants who did and who did not develop BPD or died because of respiratory failure. Overall, TAs acquired

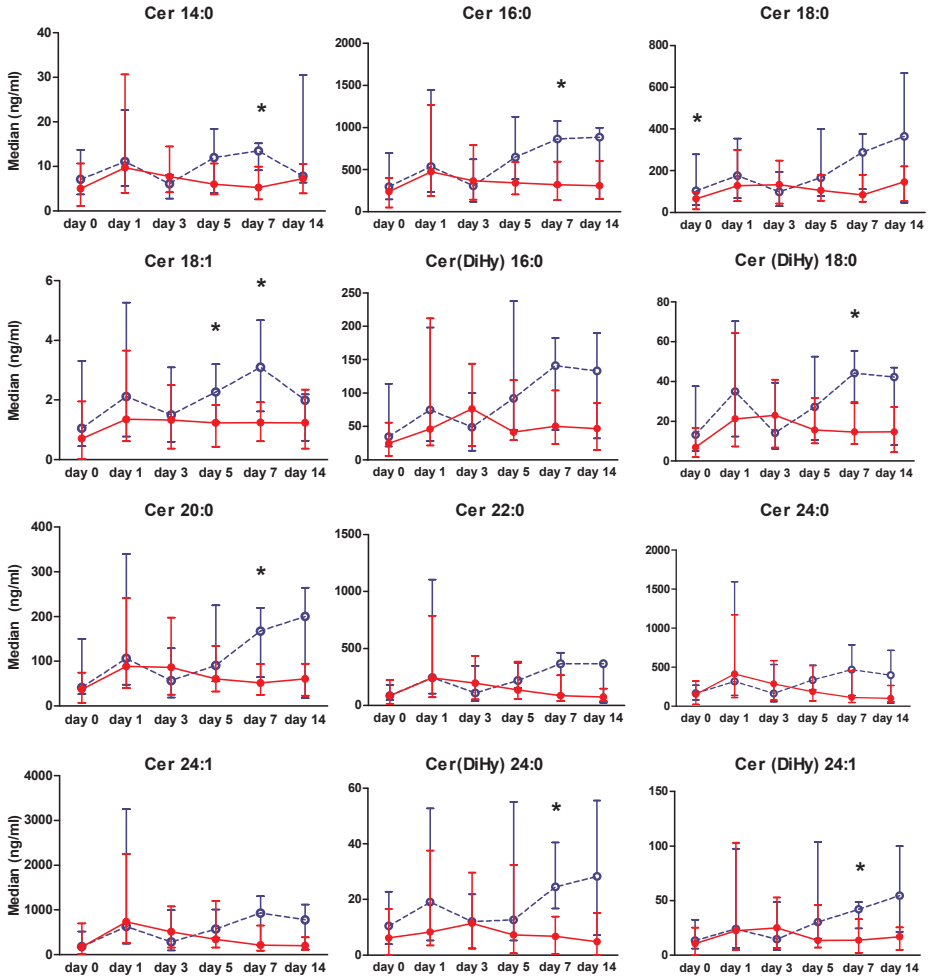


**Figure 2.** Ceramide patterns (ng/ml) in tracheal aspirates of preterm infants who developed bronchopulmonary dysplasia (BPD; •, continuous line) and infants who did not developed BPD (○, dashed line). Dots represent median values, whiskers 25$^{th}$ and 75$^{th}$ percentiles, and group comparison by means of univariate analysis per time point, * = $p < 0.05$.

at day 14 contained significantly more cells than TAs obtained at day 0, adjusted for multiple comparisons; 530,000 (207,000-1,044,000) versus 136,500 (45,000-354,750), respectively, p=0.003. Differential cell count showed a significant decrease of epithelial cells and a significant increase in granulocytes and macrophages and/or monocytes (Figure 3). Overall, there was a significant correlation between ceramide profile and total cell count, but no significant correlation between ceramide profile and percentage of granulocytes and macrophages or monocytes (data not shown).

**Table 3.** Multivariable logistic regression analysis of BPD and respiratory death

| Independent variable | OR | 95% CI | *p* value |
|---|---|---|---|
| **GA at birth** | 0.558[a] | 0.327-0.954 | 0.033 |
| **Birth weight (SDS score)** | 0.884 | 0.424-1.846 | 0.486 |
| **Total days mechanical ventilation** | 1.126[a] | 1.002-1.265 | 0.046 |
| **Time between birth and intubation** | 0.868 | 0.566-1.332 | 0.518 |
| **Intercept ceramide profile** | 0.836 | 0.493-1.420 | 0.508 |
| **Slope ceramide profile** | 0.604 | 0.205-1.776 | 0.359 |

The dependent variable in this analysis is BPD outcome (0 = did not develop BPD, 1= did develop BPD or died because of respiratory failure). n = 79, Hosmer-Lemeshow test (p= 0.468), [a] p< 0.05.

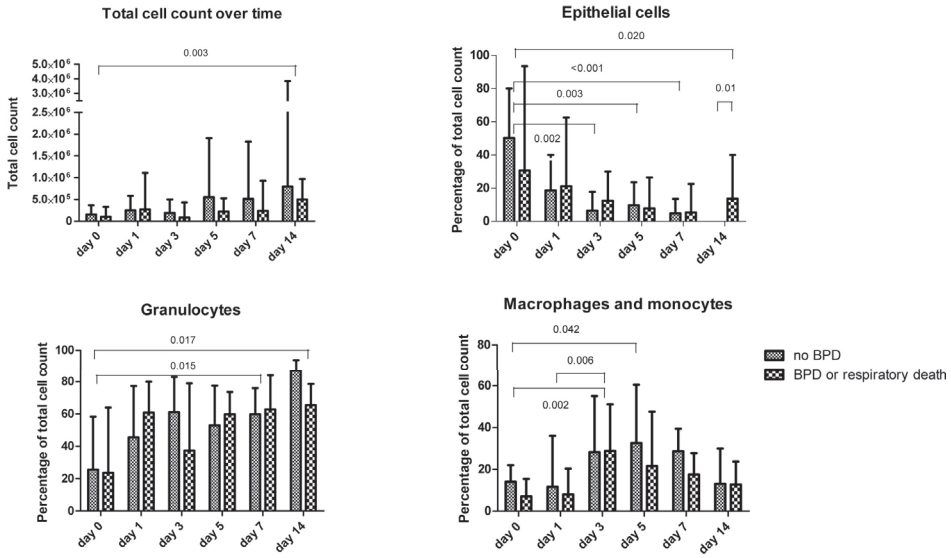


**Figure 3.** Cell distribution in TAs of preterm born infants. a) Total cell count for each time point between two groups (no BPD and BPD or respiratory death). b) Percentage of epithelial cells in TAs of infants who did not and who did develop BPD or died because of respiratory failure. c) Percentage of granulocytes in TAs of infants who did not and who did develop BPD or died because of respiratory failure. d) Percentage of macrophages or monocytes in TAs of infants who did not and who did develop BPD or died because of respiratory failure.

## DISCUSSION

We showed an overall increase in ceramides in TAs of preterm infants after the first day of mechanical ventilation. Infants who developed BPD had lower ceramide concentrations compared to those who did not, in particular at day 7 after intubation. Ceramide profiles changed over time, and were significantly different between infants who did or did not develop BPD. However, the combined ceramide profiles over time had no additional predictive value over known predictors of BPD.

This is the first study investigating ceramide profiles in TAs of preterm born infants. We recently showed in a mouse model of BPD that changes in ceramide levels may be a factor in hyperoxia- induced lung injury, and affect proper lung development and function [19]. With this study we aimed to reproduce these findings in preterm infants. We collected TAs at multiple time points, which enabled us to observe the development of ceramide concentrations over time. The prospective design allowed us to investigate the predictive value of ceramide concentrations for BPD development when compared to known clinical risk factors.

Ceramides play an important role in apoptosis and lung inflammation [23] and mediate acute lung injury by increasing alveolar permeability and pro-inflammatory cytokine production [24]. Long chain ceramides (Cer 16:0, Cer 18:0 and Cer 20:0) have anti-proliferative and pro-apoptotic effects, whereas very long chain ceramides (Cer 22:0, Cer 24:0 and Cer 24:1) promote cell proliferation [25]. In the present study, we observed an early increase in both long chain and very long chain ceramides in TAs of preterm born infants after 1 day of mechanical ventilation and oxygen supplementation. This is consistent with our results obtained in the mouse model showing an early increase of ceramides after start of exposure to hyperoxia. In infants, we found a late increase of both long chain and very long chain ceramides in those who did not develop BPD. These results at first seem to be in contrast with our earlier findings of increased ceramides in hyperoxia-exposed mice [19]. However, in our study all preterm infants were exposed to both mechanical ventilation and supplemental oxygen. Therefore, we speculate that infants in our study were comparable to hyperoxia-exposed mice, as they showed an increase in ceramides directly after intubation and exposure to oxygen supplementation. Due to medical ethical reasons it was not possible to include a control group of infants who were not intubated. When we combined the individual ceramides into a ceramide profile we indeed found a significant change in the profile over time and a significant different profile in those infants who did develop BPD compared to the infants who did not develop BPD. In a multivariable analysis the ceramide profile over time had no additional predictive value over GA at birth, birth weight, and total days mechanical ventilation. However, when we considered the individual ceramides we found borderline significant additional predictive value of the very long chain ceramides (Cer 22:0, Cer 24:0 and Cer 24:1) that are known to promote cell proliferation (supplemental data). Altogether, an increased initial ceramide-triggered apoptotic signaling is present in all preterm infants exposed to mechanical ventilation and hyperoxia. Apoptosis of alveolar epithelial cells has been found before in the lungs of preterm infants that were subjected to ventilation and oxygen treatment [17] and in mouse models of BPD [26]. Secondly, our results suggest that a reduced late increase in ceramides that may function as a proliferation signal, may predispose preterm infants to develop BPD. Given these findings, it is worthwhile to investigate the role of ventilation and hyperoxia-induced ceramide production in

epithelial apoptosis as a mechanism responsible for pulmonary apoptosis and inhibition of alveolar development in preterm infants with BPD.

Snoek *et al.* investigated ceramide concentrations in infants born > 32 weeks GA with a congenital diaphragmatic hernia (CDH) and did not find a difference in ceramide concentrations between infants with and without BPD [27]. This discrepancy may be explained by the fact that the pathophysiology of BPD development in neonates with CDH is different from that in preterm infants. Patients with CDH are not surfactant deficient [28] and inflammatory processes seem to play a less important role. It should be noted that the ceramide concentrations in TAs of infants with CDH were in the same range as in the present study. Therefore, lower ceramide concentrations might predispose for BPD both in infants with lung hypoplasia due to CDH and in ventilated preterm infants.

Ceramides can be increased by increased sphingomyelin metabolism via sphingomyelinases, by increased production via *de novo* synthesis or from sphingosine by ceramide synthase [5]. For this reason, it would be interesting to analyze the quantity of sphingomyelins in the TAs in our preterm infants. However, this was not feasible as most infants were treated with exogenous surfactant, which contains abundant sphingomyelins.

Pulmonary inflammation has always been considered a hallmark of BPD development. Previous studies showed an association between increased pro-inflammatory cytokine concentrations in blood and lung derived fluid and development of BPD [29]. In the current study, we found a significant increase in granulocytes and macrophages or monocytes in TAs over time; however, this increase did not significantly differ between infants with or without later BPD. Furthermore, the overall ceramide profile did not correlate with the percentage of granulocytes and macrophages or monocytes, suggesting that the concentrations of ceramides might not be influenced by local inflammation in the lungs.

A few limitations of our study should be considered. We analyzed ceramides in TAs obtained from routine suctioning procedures, and not in BAL fluid which might yield larger samples and better standardization, but is more invasive. However, there is evidence that TAs are suitable substitutes for BAL samples in newborns [30]. Second, it was impossible to obtain TAs from preterm infants who did not need mechanical ventilation. Therefore, one can argue that a proper control group is missing. Few TAs (18%) were macroscopically contaminated with blood. Ceramides are present in plasma, and this might have influenced our results [31]. However, sensitivity analysis after excluding these specimens, yielded similar results (data not shown). Patient characteristics between the infants who did and did not develop BPD differed with respect to GA at birth, birth weight, antenatal corticosteroids, postnatal surfactant, late onset sepsis, PDA and total days of mechanical ventilation. Based on a recent review regarding clinical predictors for BPD we chose only to correct for GA at birth, birth weight and total days of mechanical ventilation [32]. Correcting for all differentially expressed variables shown in Table 1 did not change the results, but yielded not enough statistical power due to the small

sample size. The number of TA samples decreased over time, as many infants could be extubated within 14 days. This inevitably introduced selection bias. However, again sensitivity analysis including only those infants with at least one late TA sample available (13 infants without and 36 infants with BPD), yielded similar results (data not shown). Also, one could argue about the fact that BPD development is a continuum and a binary division based on the 36 weeks definition will introduce loss of power. In the future, it will be interesting to relate ceramides to BPD severity based on for example structural lung abnormalities and lung function. Finally, no formal correction for multiple testing of different ceramides was performed.

In conclusion, a pattern with early increase and subsequent decrease in ceramides in preterm infants exposed to mechanical ventilation and supplemental oxygen seems to predispose for BPD development. Therefore, ceramide profiles in TAs may be a new early marker for BPD and the ceramide pathway in general might be a potential new target for future prevention or treatment of BPD.

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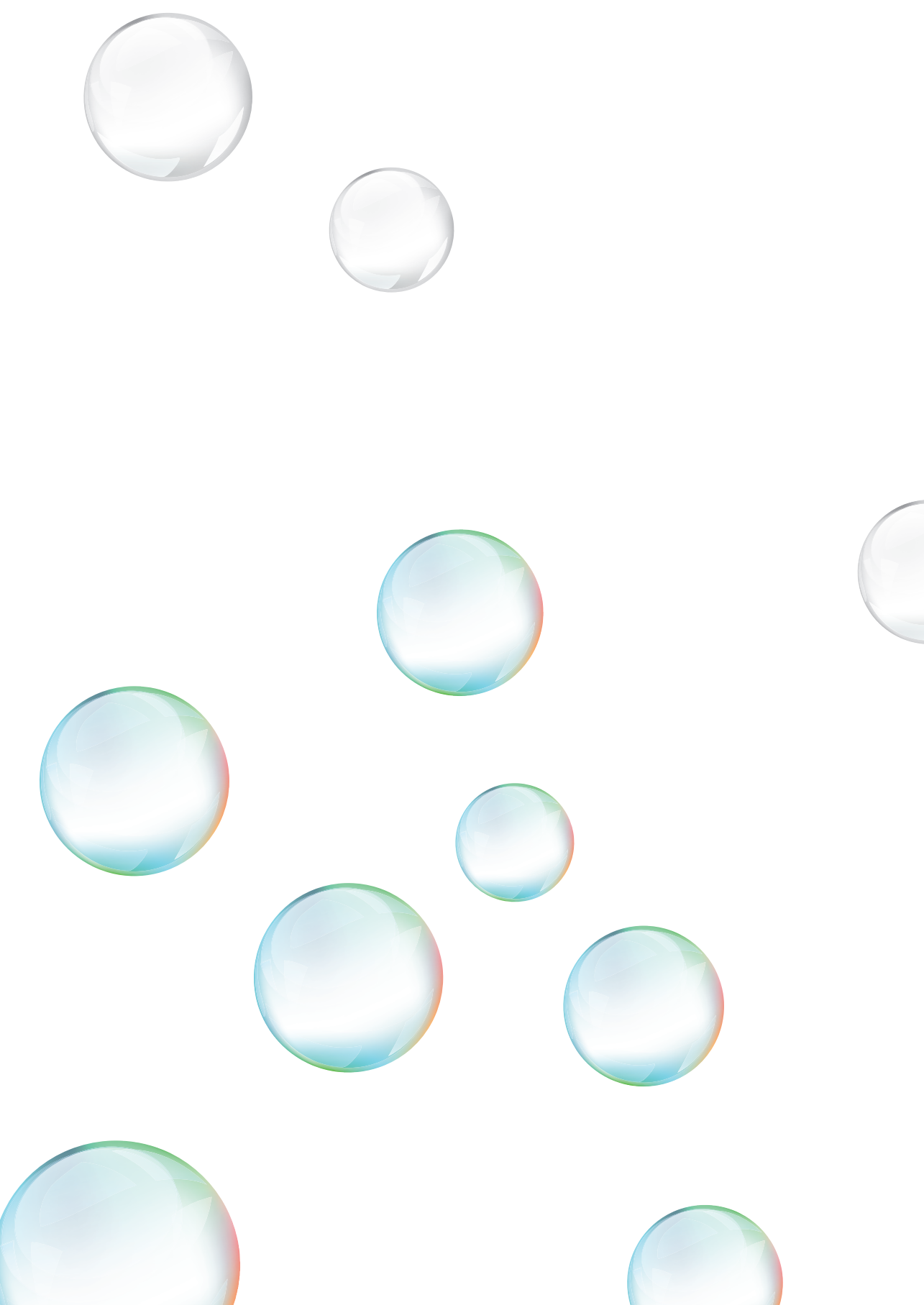

# 7 Lung CT imaging in patients with bronchopulmonary dysplasia: a systematic review

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## ABSTRACT

### Background

Bronchopulmonary dysplasia (BPD) is a common respiratory complication of preterm birth and associated with long term respiratory sequelae. Chest computed tomography (CT) is a sensitive tool to obtain insight in structural lung abnormalities and may be a predictor for later symptoms.

### Objectives

To give an overview of chest CT scoring methods that are used to evaluate chest CT scans of BPD patients. To review which structural lung abnormalities are described in children and adults with BPD and whether these are related to clinical outcomes.

### Methods

An extensive literature search was conducted for relevant studies on chest CT imaging in patients born preterm with BPD.

### Results

We retrieved 316 original papers of which 16 articles and 3 abstracts fulfilled our inclusion criteria. Overall, we identified nine different semi-quantitative scoring methods. Chest CT scans revealed structural abnormalities in > 85% of BPD patients. These abnormalities are decreased pulmonary attenuation, opacities, bronchial wall thickening, and consolidations. Some have been found to be negatively correlated with lung function and respiratory symptoms.

### Conclusions

None of the currently described scoring systems are appropriately validated or superior over another. Future studies are needed to generate a validated and universal chest CT quantitative scoring method for patients with BPD.

## INTRODUCTION

Lung damage is an important sequela of extreme preterm birth. In Europe, 4-56% of surviving preterm infants, depending on gestational age at birth, are suffering from bronchopulmonary dysplasia (BPD) [1]. BPD is a lifelong condition associated with long term respiratory sequelae like lower lung function, severe obstructive airways disease and pulmonary hypertension, but also neurological and cognitive impairment, which all have a major impact on the quality of life and on survival [2].

BPD was first described in 1967 by Northway *et al.* and defined as persistent respiratory symptoms and need for oxygen supplementation in the first 28 days of life [3]. BPD development was mostly related to the severity of respiratory distress syndrome and chest radiographs showed especially hyperinflation and cystic areas. Lung histology from autopsy specimens of patients diagnosed with BPD as a result of preterm birth showed extensive inflammatory and fibrotic changes in both airways and lung parenchyma. Since the initial description by Northway *et al.*, major changes in neonatal care have led to increased survival of more extremely preterm born infants (< 28 weeks of gestational age). Parallel to the changes in care and the younger gestational age, the spectrum of lung abnormalities in these surviving preterm infants has changed and is often referred to as 'new' BPD. Lung histology of infants who died from this new BPD show changes suggestive of impairment in normal lung development with reduced alveolar septation and abnormal vasculature and chest radiographs with particularly less cystic areas [4].

Chest computed tomography (CT) is considered the most sensitive imaging modality to detect structural abnormalities in patients with BPD [5]. Chest CT has the potential to play an important role as outcome measure for clinical studies aiming to reduce the incidence of BPD. In addition, chest CT could provide insight in the pathophysiology of BPD and might be a predictor for later symptoms and impairments. However, before chest CT can play a role as outcome measure in clinical studies, sensitive and reproducible image analysis methods need to be developed to quantify and characterize the morphological lung changes. Ideally such imaging analysis method should be automated. A considerable number of CT protocols and scoring methods have been described over the last 30 years to characterize and quantify the structural abnormalities on chest CT scans of preterm born patients with BPD. However, to date there are no validated and universally accepted CT protocols and scoring systems for quantifying structural changes.

The primary aim of this systematic review is to identify all different scoring systems which have been used to evaluate chest CT scans of BPD patients. We aimed to investigate which structural lung abnormalities on chest CT are observed in children and adults with BPD, and whether these structural abnormalities have been described to relate to clinical outcomes like lung function and other respiratory sequelae. Finally, based on the

results of this review, we propose a universal chest CT quantitative scoring method for patients diagnosed with BPD that has the potential to be automated.

## METHODS

A systematic literature search was conducted in cooperation with experienced medical librarians (WB and GJ) on the application of chest CT performed in patients with BPD. We searched Embase, Medline, Web-of-Science, Scopus, Cochrane, Google scholar, and PubMed databases for relevant publications. The last search was run on July 23, 2015. Key words included: chronic lung disease OR bronchopulmonary dysplasia OR BPD OR infant OR premature AND X-ray computed tomography AND scoring OR scale OR grading OR classification. Titles and abstracts were screened by two independent reviewers (EM and KL). We only included articles published in English that described original research, and addressed chest CT findings in patients born preterm with BPD. Reference lists of included articles were reviewed for additional references.

## RESULTS

In total 316 articles were retrieved. Figure 1 shows the flow chart for all harvested papers. Finally, 16 articles met the inclusion criteria, and three additional articles were retrieved from their reference lists. In total, we included 19 original articles, of which three were only published as abstracts. Table 1 summarizes the 19 included articles. Year of publication varied between 1994 and 2013. The year of birth of the included preterm born infants in the studies varied between 1974 and 2008 and subsequently different diagnostic criteria for BPD were used. The age at which the chest CT scan was made varied between 34.9 weeks postmenstrual age and 33 years of age. Table 2 outlines the structural components scored using semi-quantitative scoring systems. In eight studies both inspiratory and expiratory chest CT scans were made. In total, nine different scoring systems were identified. Intra-observer and inter-observer variability were described in, respectively, 5 and 12 of the 19 articles and showed kappa's for different components scored between 0.45 and 0.95 [6-10] and between 0.18 and 0.99 [6-17]. The most commonly scored structural components were hyperlucency, emphysema, opacities (both linear and subpleural), bronchial wall thickening and collapse, consolidation, or atelectasis (Table 2). Overall, emphysema scored the best intra-observer and inter-observer agreement. The scoring systems assessed the extent of structural lung damage based on either the number of affected segments or lobes or on the number of lesions present. None of the scoring systems were fully quantitative.

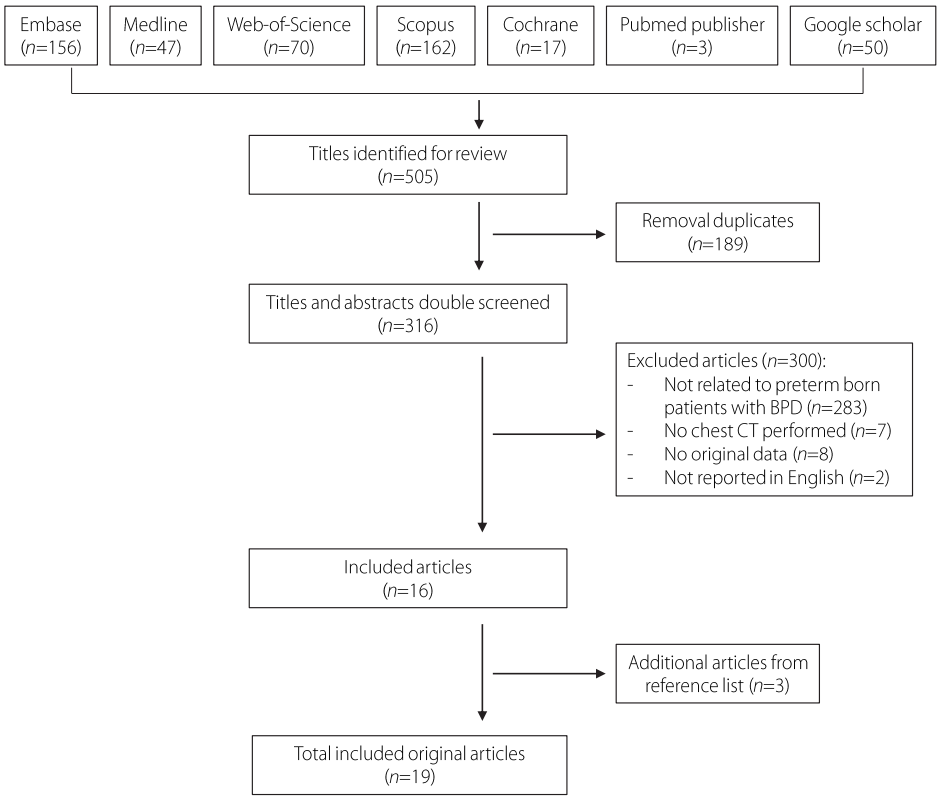


**Figure 1.** Flow chart of included articles.

All studies showed abnormal CT findings in patients with BPD, both in the neonatal period and later in life [5-23]. Furthermore, most studies which assessed the clinical severity of BPD (mild, moderate, severe) found an increase in CT abnormalities within the patients with more severe BPD [8, 10-12, 17, 19]. Long *et al.* defined a clinical severity score at 36 weeks (mean airway pressure x $FiO_2$ at 36 weeks) and also found significant positive correlation with architectural distortion, linear and triangular opacities and air trapping on CT scans [21]. In seven studies, CT findings of BPD patients were compared with control patients, which were either healthy term born [9, 16] or preterm born without BPD [6, 7, 15, 19, 23]. The studies in which patients > 1 year of age were investigated found higher CT scores in the BPD patients compared with control patients [9, 16, 19, 23]. In contrast, studies performing chest CT during the neonatal period found abnormal CT findings in almost all preterm infants exposed to mechanical ventilation and oxygen exposure irrespective of whether they had a clinical BPD diagnosis [6, 7, 15].

In 8 out of the 19 articles the correlation between scoring parameters and lung function parameters were described. All studies assessed different lung function parameters with

**Table 1.** Description of studies

| First author, year of publication | Study design | Study population (period, location) | Inclusion criteria | Exclusion criteria | Number eligible patients | Number included patients | Number BPD patients | BPD definition | Gestational age in weeks PMA median (range) | Birth weight in gram median (range) | Age in years at chest CT imaging median (range) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Caskey, 2013 (abstract)** | Case-control | Not reported | Not reported | Not reported | Not reported | 39 | 20 | Not reported | BPD: 27.8 (2.2)* Control: 30.6 (2.1)* | BPD: 901 (260)* Control: 1248 (188)* | BPD: 24.4 (3.5)* Control: 25.9 (3.7)* |
| **la Tour, 2013** | Retrospective | 1998-2007, Switzerland | < 37 weeks GA, diagnosis moderate/ severe BPD | Not reported | 88 | 19 | 19 | Oxygen suppl ≥ 28 days | 26.1 (24.3-33.3) | 740 (510-1370) | 14.6 (1.5-53.7) months |
| **Shin, 2013** | Prospective | 2006-2008, Korea | < 32 weeks GA, birthweight < 1500 gram, BPD | Congenital chest deformities, heart disease, past pulmonary surgery or serious infection | 59 | 42 | 42 | Oxygen suppl ≥ 28 days | 26.3 (24.0-31.3)* | 838 (490-1500)* | 39.1 (34.9-54.7) weeks PMA* |
| **Long, 2011 (abstract)** | Not reported | Not reported | Clinically refractory moderate/ severe BPD | Not reported | Not reported | 55 | 55 | Not reported | Not reported | 775 ± 256* | 45 ± 34 weeks* |
| **Sarria, 2011** | Case-control | Not reported, USA | 24-29 weeks GA, BPD | Congenital cardiorespiratory disease | Not reported | 39 | 39 | Oxygen suppl at 28 days post-partum or at 36 weeks PMA | 25.5 (23-29)* | 870 (490-1440)* | CLDI: 12 (5-18) months* Control: 17 (4-33) months* |
| **Wong, 2011** | Observational | 1980-1988, Australia | Birthweight < 1500 gram | Intellectual impairment | 138 | 51 | Not reported | Birthweight < 1500 gram | 27 (24-31) | 900 (565-1435) | 20 (18-33) |

**Table 1.** Description of studies (continued)

| First author, year of publication | Study design | Study population (period, location) | Inclusion criteria | Exclusion criteria | Number eligible patients | Number included patients | Number BPD patients | BPD definition | Gestational age in weeks PMA median (range) | Birth weight in gram median (range) | Age in years at chest CT imaging median (range) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Boechat, 2010** | Prospective | 1998-2000, Brazil | < 34 weeks GA or < 1500 gram | SGA, congenital malformations, infections, genetic syndromes | 97 | 86 | 24 | Oxygen suppl ≥ 28 days | 28 (23-33)* (n=179) | 1101 (610-1480)* (n =179) | 59 ± 26 days* |
| **Brostrom, 2010** | Case-control | 1992-1997, Sweden | < 32 weeks GA diagnosed with RDS or BPD | Not reported | 97 | 60 | 32 | Oxygen suppl at 28 days post-partum | mild: 27 (24-30); moderate: 27.5 (25-30); severe: 28 (25-29) | mild: 1495 (845-2094); moderate: 1133 (597-1252); severe: 905 (775-1210) | 6-8* |
| **Wilson, 2010 (abstract)** | Not reported | Not reported | Adult survivors BPD | Not reported | Not reported | 51 | 51 | Not reported | Not reported | Not reported | Not reported |
| **Aukland, 2009** | Prospective | 1982-1985 (1st cohort) or 1991-1992 (2nd cohort), Norway | ≤ 28 weeks GA or ≤ 1000 gram | Not reported | 86 | 74 | 56 | Oxygen suppl at postnatal age ≥ 28 days or at ≥ 36 weeks PMA | mild: 26.7 ± 1.4; moderate/ severe: 26.3 ± 1.5* | mild: 968 ± 201.3; moderate/ severe: 852.4 ± 169.7* | 1st cohort: 18* 2nd cohort: 10* |
| **Ochiai, 2008** | Prospective | 1998-2004, Japan | < 37 weeks GA, BPD | Congenital cardio-respiratory disease | 51 | 42 | 38 | Oxygen suppl at postnatal age ≥ 28 days or at ≥ 36 weeks PMA | 26+3 (22+2 - 31+2) | 829 (484 – 1430) | 41 weeks and 3 days (34-72) * |

**Table 1.** Description of studies (continued)

| First author, year of publication | Study design | Study population (period, location) | Inclusion criteria | Exclusion criteria | Number eligible patients | Number included patients | Number BPD patients | BPD definition | Gestational age in weeks PMA median (range) | Birth weight in gram median (range) | Age in years at chest CT imaging median (range) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Wong, 2008** | Retrospective | 1980-1987, Australia | Birthweight < 1500 gram, requiring suppl oxygen at ≥ 36 weeks PMA | Sensori-neural impairment | 47 | 20 | 20 | Oxygen suppl at ≥ 36 weeks PMA | 27 (24-30) | 895 (635-1355) | 19 (17-33) |
| **Mahut, 2007** | Retrospective | 1999-2001, France | BPD with uncontrolled respiratory symptoms | Not reported | Not reported | 41 | 41 | Oxygen suppl ≥ 28 days | 27.2 (23.5-30.8)* | 914 (540-1490)* | 16 (10.6-20.2)* |
| **Aukland, 2006** | Prospective | 1982-1985 (first cohort) or 1991-1992 (second cohort), Norway | ≤ 28 weeks GA or ≤ 1000 gram | Not reported | 86 | 74 | 56 | Clinical diagnosis | Not reported | Not reported | 1st cohort: 18* 2nd cohort: 10* |
| **de Mello, 2003** | Prospective | 1998-2000, Brazil | < 34 weeks GA and < 1500 gram | SGA, congenital malformations, infections, genetic syndromes | 97 | 86 | 24 | Oxygen suppl ≥ 28 days | 28 ± 2.3* | 1101 ± 235.8* | 36 ± 2 weeks* |
| **Howling, 2000** | Case-control | Not reported | Adult survivors of neonatal diagnosed BPD | Not reported | Not reported | 15 | 5 | Clinical diagnosis | 31 (28-35) weeks | 1860 (990-2268) | BPD: 25 (20-26)* Control: 26 (18-30)* |

**Table 1.** Description of studies (continued)

| First author, year of publication | Study design | Study population (period, location) | Inclusion criteria | Exclusion criteria | Number eligible patients | Number included patients | Number BPD patients | BPD definition | Gestational age in weeks PMA median (range) | Birth weight in gram median (range) | Age in years at chest CT imaging median (range) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Aquino, 1999** | Prospective | Not reported | Premature birth, oxygen suppl at 30 days, current age > 5 years | Smokers, respiratory infection within 2 weeks of evaluation | Not reported | 26 | 25 | Oxygen suppl at 30 days PMA | 28 (22-36) weeks | 900 (482-2350) | 10 (5-18) |
| **Kubota, 1998** | Observational | Not reported | Not reported | Not reported | Not reported | 22 | 22 | Oxygen suppl at 28 days with persistent respiratory distress and chest X ray abnormalities | 29 ± 4.4* | 1245 ± 638* | 11.6 ± 11.9 months |
| **Oppenheim, 1994** | Observational | 1974-1992, not reported | BPD diagnosis and still require regular pulmonary follow-up | Not reported | Not reported | 23 | 23 | Oxygen suppl at 28 days PMA, and associated chest radiograph abnormalities | 31 ± 4* | 1460 ± 760* | 4.1 ± 3.7* |

Definition of abbreviations: BPD: bronchopulmonary dysplasia; CLDI: chronic lung disease of infancy; GA: gestational age; PMA: postmenstrual age; SGA: small for gestational age; suppl: supplementation. * Data represented as mean, with either range or standard deviation between brackets, or standard error annotated by means of ±.

**Table 2.** Structural components scored on chest CT

| | | Hyperlucency | | Opacities | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| First author, year of publication | Technique chest CT I/ E/ TV | Inspiratory | Expiratory | Emphysema or bullae | Bronchiectasis | Subpleural (triangular) | Linear | Bronchial wall thickening | Collapse/consolidation or atelectasis | Thickening interlobar septa | Bronchus-vessel ratio | Architectural distortion | Other |
| **Caskey, 2013 (abstract)** | Not reported | x | x | x | ? | x | x | ? | ? | ? | ? | ? | ? |
| **la Tour, 2013** | I + E | | | x | x | x | x | x | | | | | |
| **Shin, 2013** | TV | x | | x | | x | x | | x | x | | x | |
| **Long, 2011 (abstract)** | I + E | x 'ground glass' | x | x | x | x | x | x | x | | | x | |
| **Sarria, 2011** | I + E | x 'mosaic pattern of lung attenuation' | x 'hyper-expansion' | x | | x | | x 'thickening broncho-vascular bundle' | x | | | | Intercostal bulging |
| **Wong, 2011** | I + E | X | x | x | x | x | x | x 'peri-bronchial thickening' | x | x | x | | Mucus plugging |
| **Boechat, 2010** | TV | x 'ground glass opacity' | x | x 'air bubbles' | | | | | x | | | | |
| **Brostrom, 2010** | TV | | | x | | | x | x | | | | | Fibrosis |
| **Wilson, 2010 (abstract)** | Not reported | x | x | x | ? | x | x | ? | ? | ? | ? | ? | ? |
| **Aukland, 2009** | I + E | x | x | x | x | x | x | x 'peri-bronchial thickening' | x | | x | | |
| **Ochiai, 2008** | Not reported | x | | x | | x | | x 'thickening broncho-vascular bundle' | x | | | | Intercostal bulging Subjective impression |
| **Wong, 2008** | I + E | | x | x | x | | | x | | | x | x | |

**Table 2.** Structural components scored on chest CT (continued)

| First author, year of publication | Technique chest CT I/ E/ TV | Hyperlucency | | Opacities | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Inspiratory | Expiratory | Emphysema or bullae | Bronchiectasis | Subpleural (triangular) | Linear | Bronchial wall thickening | Collapse/consolidation or atelectasis | Thickening interlobar septa | Bronchus-vessel ratio | Architectural distortion | Other |
| **Mahut, 2007** | Not reported | x 'hyper-lucent areas' | | x | x | x | x | | | | | | |
| **Aukland, 2006** | I + E | x | x | x | x | x | x | x 'peri-bronchial thickening' | x | x | x | | Mucus plugging |
| **de Mello, 2003** | TV | x 'aeration disturbance and ground glass opacity' | | x | | x | | x | x | x | | | Paren-chymal bands |
| **Howling, 2000** | Not reported | x 'reduced lung attenuation' | | x | | | x | x | | | x | | |
| **Aquino, 1999** | I + E | x | x | | x | | | | | | | x | Reticular opacities |
| **Kubota, 1998** | I | x 'hyper-aeration' | | | | x | x | x 'thickening broncho-vascular bundle' | | | | | |
| **Oppenheim, 1994** | I or TV | x | | x | x | | x | x | x | x 'interstitial thickening' | | | Pleural thickening |

Definition of abbreviations: I:inspiratory; E: expiratory; TV: tidal volume breathing; ?: not reported.

diverse techniques at various ages. However, all but one study found significant correlations between CT abnormalities and lung function parameters [9, 15, 17-22]. Before the age of 2 years both lower compliance and forced residual capacity (FRC) were found in those children with more CT abnormalities present [15, 17]. At a later age, the severity of hypo attenuation, air trapping and architectural distortion correlated most with obstructive lung function, especially low forced expiratory volume in 1 s ($FEV_1$) [18-22]. Only one study assessed the diffusion capacity and found a reduction in 84% of adults with moderate to severe BPD, but this was not associated with the presence of emphysema [22].

Multiple perinatal factors are known to play a role in BPD development and might influence type and extent of structural lung damage. However, imaging studies usually only describe gestational age, birth weight, days of oxygen and days of ventilation. Some studies investigated the correlation between these neonatal factors and CT scores [11, 12, 17, 19, 21, 22]. Most studies found a significant correlation between the duration of oxygen treatment and CT scores at a later age, and higher CT scores in those infants who were discharged home on oxygen [12, 17, 19]. Wong *et al.* found an association between birth weight and severity of emphysema in adulthood [22]. Some studies also investigated the correlation between CT scores and long term respiratory outcome. Both Shin and Boechat *et al.* showed higher CT scores in infants with later respiratory morbidity, including persistent wheezing, hospitalization, or pneumonia in the 1st year of life [7, 10]. In contrast, Mahut *et al.* found no difference in CT scores at a mean age of 16 months between infants with and without frequent respiratory symptoms [17].

In only two studies, a second CT scan was made during follow up of BPD patients. Le Tour *et al.* describes four patients in which a second CT scan was made between 10.6 and 43.2 months after the first scan, in which the different lesions remained fairly stable over time [11]. Also the study of Broström *et al.* shows persistent abnormalities at 6-8 years of age, mainly in the children who were diagnosed with moderate or severe BPD [20]. In addition, Aukland *et al.* who investigated two cohorts with BPD patients born respectively between 1982-1984 ('old' BPD) and between 1991 and 1992 ('new' BPD), found no significant differences in CT scores between both cohorts at a later age of, respectively, 18 and 10 years [19]. Indeed, despite the difference in pathology, most chest CT findings found in patients with 'old' BPD are similar to those observed in patients with 'new' BPD. Mahut *et al.* described a relative absence of bronchial involvement in their BPD population at a mean age of 16 months [17]. However, others did find bronchial wall thickening in patients with 'new' BPD [11, 20]. Besides the study of La Tour *et al.* who included moderate to severe BPD patients, the presence of emphysema and bronchial wall thickening has only been found in children above the age of 8 years [14, 16, 20, 22].

## DISCUSSION

In this review, we included 19 studies which evaluated chest CT imaging in infants, children and adults with BPD. Nine different semi-quantitative scoring methods were used in these 19 studies. Overall, chest CT scans revealed abnormalities in > 85% of the patients. We found many similarities in the components being scored, with almost all studies reporting patterns of hypo attenuation on inspiratory and/or expiratory scans, linear or subpleural opacities, bronchial wall thickening and collapse, consolidation or atelectasis. Furthermore, both lower lung function and increased respiratory symptoms have been found associated with chest CT abnormalities.

The most sensitive structural abnormality associated with BPD severity is low attenuation on inspiratory or tidal breathing CT scans. The terminology used in the various studies to describe this hypo attenuation varies from decreased pulmonary attenuation, mosaic perfusion, emphysema, and abnormal or decreased density. This component seems to make up a large proportion of the lungs in BPD patients. These regions of low attenuation at inspiratory scans represent either hypo perfusion and/or hypoventilation, both contributing to impaired gas exchange and therefore highly relevant. When these regions of low attenuation persist in expiration they are often described as trapped air. Accurate quantification of the volume fraction of the lung of these low attenuation regions on the in- and expiratory scan, as was done for cystic fibrosis (CF), may be a promising predictor for future impaired lung function and symptoms [24, 25]. Long-term follow up of these changes is required to understand whether there is reversibility of this low attenuation and whether these changes negatively affect the long-term lung structure and respiratory morbidity. Currently, in- and expiratory scans are not possible before the age of 4-5 years, unless anesthesia is used, which may limit the usefulness of this parameter in young children. However, third generation scanners have also overcome the limitation of anesthesia in infants, by providing high quality images in free breathing conditions [26, 27].

The second most frequent structural abnormality scored in 15 of the 19 studies is opacities, with strands of atelectasis extending to the pleura (linear opacities) and creating pleural grooves (triangular or subpleural opacities). These abnormalities probably reflect alveolar septal fibrosis. In the few longitudinal studies these opacities did not change over time and may be considered a marker of acute and irreversible damage in patients with BPD [11, 20]. Both the presence of hypo attenuation and opacities are consistent with the hypothesis that the predominant abnormality in BPD is in the peripheral lung.

The third most often scored structural component is bronchial wall thickening. Again various terms have been used, like peribronchial thickening and thickening of the bronchovascular bundle. These regions of bronchial wall thickening most likely reflect peribronchial and peribronchiolar fibrosis or inflammation, both are likely to have a negative

impact on the work of breathing. It is unclear if bronchial wall thickening, observed in CT scans from term age to adulthood, represents ongoing disease or whether it is the end stage of acute damage in the neonatal period. To resolve this question bronchoscopy studies examining inflammatory markers in lavage fluid, which may be correlated with CT abnormalities, are needed as was done for CF [28]. To monitor the reversibility of long-term changes a sensitive objective method to determine airway dimensions is needed. Recently, such a system has been developed in our group [29].

Other structural changes such as bronchiectasis and mucus plugging, often reported on CT images of patients with CF were reported less frequent in patients with BPD. Therefore, it is unlikely that these structural abnormalities play a role in the pathophysiology of long-term symptoms in BPD patients. Taken together, all studies indicate persistent abnormalities in the lungs of patients born preterm irrespective of when in the evolutionary path of preterm neonatal care these patients were born ('old' versus 'new' BPD) and regardless of age at the time of chest CT imaging.

A striking finding of our review was that in only few studies a detailed description of the CT protocol was given. CT protocol has a major impact on the image quality, especially factors such as control of lung volume during acquisition, slice thickness and reconstruction kernel. These parameters influence the sensitivity of scoring methods and of future semi-automated image analysis systems [27]. There is clearly a need for standardization of chest CT and image analysis protocols in BPD patients. Of key importance is to use a chest CT protocol using the lowest possible radiation dose to generate images that will be of diagnostic quality. To give detailed recommendations for such a protocol is beyond the scope of this review. Amongst other such a protocol should take into account patient's age and cooperation, technical qualities of the CT scanner, and image analysis techniques to be used. In Supplemental Table S1, we give some general recommendations that could be used as starting point for a consensus meeting or task force addressing this issue.

Furthermore, the clinical information reported in these imaging studies of preterm born patients with BPD is inconsistent. And because most studies were retrospective, data collection may have been incomplete. Collection of perinatal data will allow us to improve our understanding of the pathophysiology of today's BPD and may lead to new preventive measures or adaption of treatment protocols. Therefore, standardized prospective collection of key data at relevant time points should be aimed for, including maternal (e.g., age mother, preeclampsia, premature rupture of membranes, chorio-amnionitis, mode of delivery), fetal (e.g., growth) and neonatal data (e.g., gestational age at birth, birth weight, postnatal infection, persistent ductus arteriosus, duration of respiratory support modes, treatment with oxygen supplementation, treatment with surfactant, and antenatal steroids). Besides standardization of protocols and complete collection of perinatal data, it is also important to compare CT to other monitoring modalities like quality of life, lung function and survival, if CT is to be used as primary endpoint or pre-

dictor in clinical trials. Of the studies included in this review, eight studies investigated the relation between chest CT findings and lung function. These studies showed that an increase in CT abnormalities is associated with lower flow rates suggesting that radiographic findings indeed reflect some fundamental aspects of the pulmonary sequelae from preterm birth [9, 15, 17-22]. Until now no intervention studies included chest CT as an outcome measure in BPD patients.

In 8 of the 19 studies, chest CT was performed in the first 2 years of life and in three studies even at term age (Table 1) [7-12, 15, 21]. In these three studies chest CT abnormalities reflect the short-term effects of BPD, which are amongst others caused by abnormal lung development and acute damage due to high oxygen and exposure to high pressure or volume ventilation [10, 12, 15]. The structural abnormalities described on CT scans of older children and adults with 'old' BPD seem to be associated with the amount of oxygen exposure during neonatal life. This association between CT abnormalities and oxygen duration is not per se causal, the oxygen duration may be just an indicator of severe lung disease. Although 'new' BPD seems to have a different histopathological background, CT findings in infants with 'new' BPD show similar abnormalities and are still found to be associated with both mechanical ventilation and oxygen exposure. Only two studies performed longitudinal chest CT imaging in few patients born between 1992 and 2007 and revealed that structural changes remain fairly stable over time [11, 20]. How lung structure changes related to 'new' BPD in former extremely preterm born infants will affect the normal lung function decline related to aging in adult life remains unknown. Therefore, there is a need for longitudinal follow up studies to investigate how these early structural abnormalities on CT scans will evolve. For example, how they relate to neonatal treatment, what they can learn us on pathophysiology, whether regions of low density are a risk factor for progressive emphysema in the long term, and if these early structural abnormalities may be a predictor of respiratory morbidity, such as chronic obstructive pulmonary disease (COPD), in later life.

A disadvantage of chest CT is its ionizing radiation exposure, and therefore it is less appropriate for frequent longitudinal assessment. However, for the follow up of BPD it is likely that only few CT scans are needed throughout life. Therefore the risks related to this extra radiation burden will be low [30]. Chest Magnetic Resonance Imaging (MRI) is a potential radiation free alternative for chest CT. Currently, MRI proves technically challenging because of several factors, particularly low 1H density of the lung parenchyma, air-tissue interfaces that lead to rapid signal decay, and motion artefacts. Recently, Walkup *et al.* demonstrated a diagnostic-quality, quantitative pulmonary MRI in neonates and found increased signal, probably due to combinations of fibrosis, edema and atelectasis, in all preterm born infants, although more pronounced in infants diagnosed with BPD [26]. Decreased signal, presumably reflecting alveolar simplification, was only present in chest MRI of the most severe BPD cases. Standardized follow up that includes

chest imaging allows continuous evaluation of the effect of therapy changes in the neonatal period on the structural lung changes later in life. Therefore, efforts should also be directed towards further optimization of imaging quality of chest MRI.

The most striking observation of this review is the variable terminology used to describe the structural components scored. Hence, there is no gold standard to describe the structural abnormalities demonstrated in patients with BPD. Therefore it is not possible to reliably compare results between studies, between patients of different ages and between 'old' BPD and 'new' BPD populations. In addition, all scoring methods are semi-quantitative which currently makes it impossible to make direct comparisons of the extent of the BPD defects between studies. Therefore, based on the results of this systematic review, we suggest a new CT scoring method for BPD, the Perth-Rotterdam Annotated Grid Morphometric Analysis, PRAGMA-BPD scoring method, which is based upon the methodology of the recently developed PRAGMA-CF and which is both qualitative and quantitative (Table 3) [25, 31]. PRAGMA-BPD is in design and training strategy similar as the PRAGMA-CF score; however, the pulmonary features which are scored are specific for BPD. Based upon our literature search we have included in the PRAGMA-BPD score the three most distinguishing features for BPD, namely decreased pulmonary attenuation (including mosaic perfusion, emphysema and bullae), opacities (linear and/or subpleural triangular), consolidations (collapse and/or atelectasis), and bronchial wall thickening. Each feature represents non-functional lung tissue and is likely to influence the respiratory prognosis to some extent. Besides PRAGMA-BPD being a qualitative score it is also quantitative. The volume fraction of each of the four components is expressed as a percentage of total lung volume which is highly relevant for statistical analysis in clinical studies, but probably also for the clinical outcome of BPD patients (Figure 2). In addition, the amount of architectural distortion of the lung, meaning an abnormal displacement of bronchi, vessels, fissures and/or septa caused by diffuse or localized lung disease, is scored. We distinguish in four categories (normal, mild, moderate and severe) based on the amounts of affected segments (Table 3). Clearly, our PRAGMA-BPD scoring method requires validation before it can be used for future clinical trials, for example, assessing intra-observer and inter-observer reliability, cross validation to other outcomes, like quality of life and lung function, and standardization within and across different centers. The ultimate goal of such a quantitative scoring system is to improve clinical and pathophysiological phenotyping of individual BPD patients, enabling development of new preventive measures to optimize lung development in early life and minimize respiratory morbidity at later age.

In summary, chest CT scans of BPD patients revealed unique structural abnormalities and the extent of these abnormalities correlates with BPD severity and lung function. Additionally, there clearly is a need for a standardized CT protocol and quantitative scoring method before chest CT can be used as a clinical outcome measure and predictor

for long-term structural abnormalities and respiratory morbidity in BPD patients. We propose our newly developed PRAGMA-BPD method as such a scoring method.

**Table 3.** PRAGMA-BPD score

| Structural component | PRAGMA-BDP score | Scoring method |
|---|---|---|
| Normal lung | Normal lung (GREEN) | Percentage of total lung volume |
| Decreased pulmonary attenuation<br>- hypo attenuation (inspiratory/ expiratory)<br>- mosaic perfusion<br>- emphysema<br>- bullae | Hypo attenuation (BLUE) | Percentage of total lung volume |
| Opacities<br>- linear<br>- subpleural triangular | Hyper attenuation (RED) | Percentage of total lung volume |
| Consolidations<br>- collapse<br>- atelectasis | Hyper attenuation (RED) | Percentage of total lung volume |
| Bronchial wall thickening | Bronchial wall thickening (PURPLE) | Percentage of total lung volume |
| Architectural distortion | Scored according the number of segments presenting architectural distortion<br>- Normal: no architectural distortion<br>- Mild: less than 6 segments<br>- Moderate: between 6 and 13 segments<br>- Severe: more than 13 segments | Percentage of total lung segments |



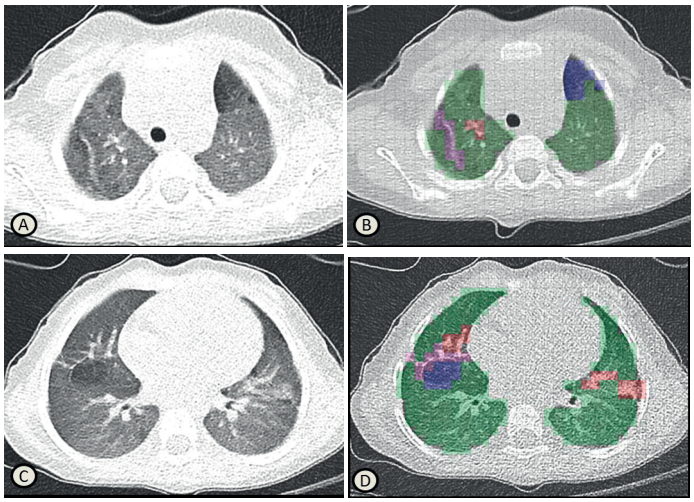


**Figure 2.** Apical **(A)** and basal **(C)** slices from inspiratory computed tomography scan in 1 years old female preterm born patient with severe BPD. **(B-D)** Slices annotated with PRAGMA-BPD: 1) red = opacities (linear an subpleural triangular) and consolidation; 2) purple = bronchial wall thickening; 3) blue = decreased pulmonary attenuation (bullae, emphysema, mosaic perfusion, trapped air); 4) green = no abnormality seen. In this patient, the assessment of the four components was as follow: 1) 5.81 ml (1.5%); 5.23 ml (1.3%), 3) 13.08 ml (3.4%) and 4) 364.97 ml (93.8%). Architectural distortion was deemed mild.

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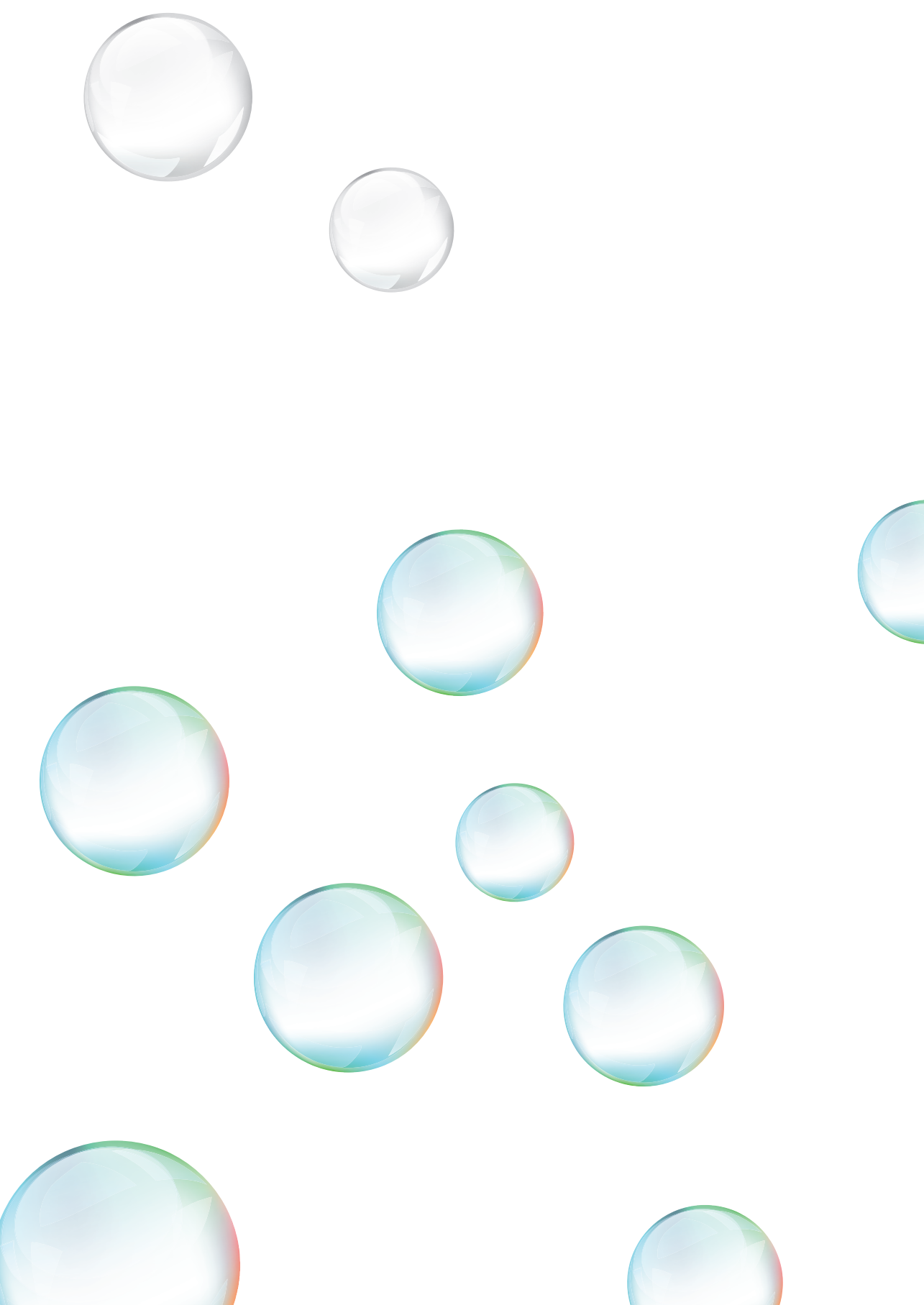
## SUPPLEMENTAL TABLE

**Table 1.** Concept proposal CT protocol for BPD patients

| Data acquisition mode | Volumetric, helical scan technique |
|---|---|
| Patient positioning | Supine, with arms above the head |
| Volume control < 3 years | Free breathing |
| Volume control 3-6 years | Technician guided to obtain a breath hold at maximum voluntary inspiration and expiration |
| Volume control ≥ 6 years | Spirometer controlled procedure to obtain an inspiratory chest CT at a volume level ≥ 95 inspiratory vital capacity and an expiratory chest CT at ≥ 95 vital capacity |
| $CTDI_{vol}$ (in 32 cm body phantom) for inspiratory scan<br>1 year old<br>5 years old<br>young adult | Optimal dose for BPD needs to be determined for each CT scanner to generate an image with sufficient parenchymal detail. Dose needed should probably be slightly higher than what has been recommended for cystic fibrosis. Doses below are the average doses used for clinical scan in 42 BPD patients in the ErasmusMC-Sophia (data on file).<br>≥ 0.6 mGy<br>≥ 1.0 mGy<br>≥ 2.2 mGy |
| Field of view | As close as possible to the entirety of the lungs, without cutting of the lung borders |
| $CTDI_{vol}$ for expiratory scan | 50% lower than inspiratory scan |
| Tube voltage | Low enough such that the recommended $CTDI_{vol}$ can be reached, e.g. 80 kV |
| Tube current | Adapt to recommended $CTDI_{vol}$ |
| Pitch | <1; lower limit determined by maximum scan time allowed |
| Slice thickness | Thinnest slice thickness e.g. 1 mm |
| Reconstruction increment | 50% overlap e.g. 0.5 mm with 1 mm slice thickness |
| Kernel for automated analysis | Sharp reconstruction filter without under- or overshoot at edges preferably a dedicated kernel for quantitative image analysis |
| Iterative reconstruction technique | If available, iterative reconstruction techniques can be applied in addition to the requested filtered back projection techniques |
| Shielding | Breast shielding by bismuth for example is discouraged |

This proposal is based upon guidelines for CT protocols for cystic fibrosis patients. These recommendations are based on the SCIFI CF project (W. Kuo, M. Kemner-van de Corput, A. Perez-Rovira, M. de Bruijne, I. Fajac, H. Tiddens, M. van straten. Multicenter chest CT standardization in children and adolescent with CF: The way forward. Submitted to ERJ 2016). The $CTDI_{vol}$ (CT dose index volume) represents the radiation dose for a 32 cm body phantom.



# 8 Structural and functional respiratory impairment in infants with severe bronchopulmonary dysplasia

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**Submitted.**

## ABSTRACT

### Background

Bronchopulmonary dysplasia (BPD) is the most frequent serious complication in preterm infants. We aimed to describe lung structure and ventilatory function of preterm infants with severe BPD and explored the association between early postnatal growth and these outcomes.

### Methods

We included preterm infants born ≤ 32 weeks gestational age (GA) with severe BPD. Lung structure was assessed on chest CT with the PRAGMA-BPD scoring system and ventilatory function by polysomnography (PSG) at 6 months corrected age. Postnatal growth was assessed by weight measured at birth, and at 2 and 6 months corrected age.

### Results

We included 49 infants (median (IQR) GA of 25.7 (24.6-26.3) weeks and mean (SD) birth weight of 760 (210) gram). 95.5% of the chest CT scans showed architectural distortion of the lung, and an oxygen desaturation index (ODI) > 5 was found in 74% of the infants. An increase in GA of 1 week was associated with higher total and normal lung volume (β coefficient (95% CI): 1.86 (0.15, 3.57) and 2.03 (0.41, 3.65) respectively), less hypoattenuation (-4.30 (-7.70, 0.90)%) and lower ODI (-36.70 (-64.20, -9.10)%). Higher weight at 6 months was independently associated with higher total and normal lung volume, and with less severe desaturations. Increased weight gain between 2 and 6 months of corrected age was associated with less severe desaturations during sleep (β coefficient (95% CI): 2.09 (0.49, 3.70)).

### Conclusion

Most preterm infants with severe BPD have structural lung abnormalities and impaired ventilatory function early in life, partly explained by birth characteristics and infant growth.

## INTRODUCTION

Over the last decades improved perinatal care has resulted in an increased survival of infants born extremely preterm. However, the incidence of severe bronchopulmonary dysplasia (BPD) did not decrease as more immature babies survive the neonatal period. In Europe, the incidence of BPD ranges from 4% in infants born at a gestational age (GA) of 31 weeks to 56% in infants born before 26 weeks GA [1]. Children with BPD are at increased risk of long-term respiratory morbidity. Early assessment of both lung structure and ventilatory function may help to detect those children who are more likely to have severe respiratory problems and are at risk for serious respiratory morbidity in later life.
Chest computed tomography (CT) studies revealed structural lung abnormalities in up to 85% of patients with BPD with worse CT abnormalities in patients with more severe BPD, both in the neonatal period and later in life [2]. Long-term outcome of children with BPD showed lower forced expiratory volume in 1 s ($FEV_1$) than age-matched children without BPD [3]. Data on lung function in preschool children with BPD are scarce given the low feasibility of most lung function tests in this age group. Moreover, most studies on long-term consequences of BPD included children born before 1990 and do not reflect current BPD population.
Polysomnography (PSG) has been used as a proxy marker for ventilatory reserve and can be performed early in life [4]. However, data on PSG findings in preterm infants with BPD are scarce [4-6]. Importantly, to date the relation between structural lung abnormalities and ventilatory function in the first year of life has not been established. In addition, preterm born infants with BPD are at risk of reduced postnatal growth, which might further contribute to impaired lung development, thereby influencing both lung structure and ventilatory function in later life [7-9].
Therefore, the aims of the present study were: 1) to assess lung structure on chest CT scans and ventilatory function by means of PSG in children with severe BPD at 6 months corrected age, 2) to examine how lung structure and function are related, and 3) to study the impact of GA at birth, birth weight and postnatal growth in the first months of life on these outcomes.



## METHODS

### Study design

Preterm infants (≤ 32 weeks GA) with severe BPD who were born at Erasmus MC-Sophia Children's Hospital or referred to us by other hospitals were included in our standardized multidisciplinary follow-up program from September 2013 onwards. Severe BPD was defined as oxygen supplementation for ≥ 28 days and need for either more than 30%

oxygen, more than 1L/min flow, continuous positive airway pressure (CPAP) or ventilator support at 36 weeks postmenstrual age (PMA) [10]. Children with congenital diseases that might affect lung structure or function were excluded. Our routine follow-up program includes consultation by a multidisciplinary team of a neonatologist, pediatric pulmonologist and pediatric cardiologist, physical examination and assessment of lung structure by chest CT scan, and ventilatory function by overnight PSG. In the current cross sectional study, we included all consecutive patients with a clinical follow up visit at 6 months corrected age. Written informed consent to use these data for research purposes was obtained from both parents of all infants. The study was approved by the medical ethical committee of Erasmus MC, Rotterdam, the Netherlands (MEC-2016-016).

### Chest CT imaging

Volumetric chest CT scans were acquired during free breathing in infants without sedation at 6 months of corrected age using a standardized protocol (online supplement). All CT scans were de-identified and analyzed in random order by an experienced thoracic radiologist (PC) using the quantitative Perth-Rotterdam Annotated Grid Morphometric Analysis (PRAGMA)-BPD scoring method [2]. This scoring system identifies lung tissue with a normal appearance, hypoattenuation, hyperattenuation and bronchial wall thickening and expresses their volume in ml and as percentage of total lung volume. In addition, the severity of architectural distortion of the lung, meaning an abnormal displacement of bronchi, vessels, fissures and/or septa caused by diffuse or localized lung disease, was scored. We distinguished four categories of architectural distortion (normal, mild, moderate, and severe) based on the number of affected segments (for details, see online supplemental data).

### Polysomnography measurements

Overnight PSG was performed at 6 months corrected age for at least 9 hours in a dark, quiet room. The following physiologic signals were recorded using BrainRT Shell+ (OSG BVBA, Rumst, Belgium): limited electroencephalogram (EEG), heart rate by electrocardiogram (ECG), chest and abdominal breathing movements by induction plethysmography, oronasal airflow by thermistor and oxygen saturation ($SaO_2$) by pulse oximetry. The recordings were analyzed by a trained researcher (EK). All PSG outcomes were scored according to the American Academy of Sleep Medicine (AASM) criteria [11]. We considered breathing rate, oxygen saturation and deepest desaturation, apnea hypopnea index (AHI), central apnea index (CAI) and oxygen desaturation index (ODI) as the most important representative measures for ventilatory function. Normal values were obtained from literature [12-15] and cutoff values defined as mean oxygen saturation ($SaO_2$) > 95%, deepest oxygen desaturation ($SaO_2$ nadir) > 92%, AHI ≤ 1, ODI < 5, and CAI ≤ 1.

### Postnatal growth

We assessed weight at birth, and at 2 and 6 months corrected age according to local standardized protocols. GA-adjusted birth weight SD scores (SDS) were constructed using reference growth standards [16]. Infant growth characteristics were converted into SDS using reference growth charts [17]. Infant weight gain was defined as the difference between weight at 6 months corrected age and weight at birth divided by the exact number of months between these two measurements. Similarly, weight gain between various other age intervals were calculated.

### Covariates

The following parameters were obtained from medical records: maternal age, pre-eclampsia, chorio-amnionitis, antenatal corticosteroids, mode of delivery, and child's sex, surfactant treatment, respiratory distress syndrome stage, early or late onset sepsis, persistent ductus arteriosus (PDA), days on mechanical ventilation (conventional or high frequency), days on supplemental oxygen, respiratory support at 36 weeks PMA, treatment at 6 months corrected age (e.g. (inhaled) corticosteroids, diuretics, supplemental oxygen, enteral tube feeding).

### Statistical analysis

For descriptive analyses of patient characteristics, lung structure and respiratory function, continuous variables were expressed as mean ± standard deviation (SD) for normally distributed data or median (interquartile range, IQR) for not normally distributed data, and categorical variables were expressed as n (%). We used multivariate linear regression models to examine the associations of lung structure with ventilatory function, and of birth and infant growth characteristics with parameters of lung structure and ventilatory function. Not normally distributed variables were transformed by log transformation for positively skewed data or arcsine transformation for negatively skewed data. Regression models were used to examine the association of weight gain as a change in SDS between various age intervals with lung structure and ventilatory function measures. To account for the correlation between birth and infant growth characteristics, we used conditional regression analyses to examine associations of weight and weight gain with lung structure and function. We constructed variables for sequential weight and weight gain, which are statistically independent from each other, allowing simultaneous inclusion in multiple regression models [18]. Thus, the associations of weight at a specific moment can be assessed in comparison with, and adjusted for, weight at other measuring moments. Selection of covariates was based on literature, if a covariate changed the effect estimate of the unadjusted analyses by ≥ 10% or if covariates were strongly related to the outcomes of interest. Based on this, sex, preeclampsia and age at time of CT scan and PSG were included as confounders in the models. All measures of association

are presented as beta coefficients or sympercent for log transformed outcomes with corresponding 95% confidence intervals. Analyses were performed using SPSS version 21.0 for Windows (IBM, Chicago, IL, USA).

**Table 1.** Patient characteristics

| | Severe BPD (n=49) |
|---|---|
| **Gestational age (weeks)** | 25.7 (24.6-26.3) |
| **Birth weight (gram)** | 760 ± 210 |
| **Sex (female)** | 20 (40.8%) |
| **Age mother (years)** | 31.5 (27.5-35.0) |
| **Antenatal corticosteroids** | None: 2 (4.3%)<br>Yes, not complete: 12 (25.5%)<br>Yes, complete: 33 (70.2%) |
| **Maternal preeclampsia** | 11 (23.4%) |
| **Chorioamnionitis** | 20 (43.5%) |
| **Mode of delivery** | Vaginal: 16 (32.7%)<br>Cesarean section: 33 (67.3%) |
| **Postnatal surfactant** | None: 6 (12.2%)<br>1 gift: 12 (24.5%)<br>≥ 2 gifts: 31 (63.3%) |
| **Early onset sepsis** | 4 (8.2%) |
| **Late onset sepsis** | 30 (61.2%) |
| **PDA** | 44 (89.8%) |
| **Total days invasive ventilation** | 30 ± 16 |
| **Total days oxygen exposure** | 60 ± 15 |
| **Oxygen supplementation at 6 months corrected age** | 12 (24.5%) |
| **Use of diuretics at 6 months corrected age** | 15 (30.6%) |
| **Use of ICS at 6 months corrected age** | 7 (14.3%) |
| **Use of enteral tube feeding at 6 months corrected age** | 16 (32.7%) |

Definition of abbreviations: BPD: bronchopulmonary dysplasia; PDA: persistent ductus arteriosus. Data were missing for antenatal corticosteroids (n=2), maternal preeclampsia (n = 2), chorio-amnionitis (n=3), total days invasive ventilation (n=2), total days oxygen exposure (n=8). Continuous variables are expressed as mean ± standard deviation (SD) for normally distributed data or median (interquartile range, IQR) for data that were not normally distributed. Categorical data are expressed as n (%).

## RESULTS

### Subject characteristics

49 infants were included with a median (IQR) GA of 25.7 (24.6-26.3) weeks with a mean (SD) birth weight of 760 (210) grams (Table 1). Chest CT was performed successfully in 45 infants at a corrected age of 6.2 (5.9-6.9) months. Architectural distortion was identified

**Table 2.** PRAGMA-BPD chest CT scores

| | Severe BPD (n=45) |
|---|---|
| **Total lung volume (ml)** | 264 (219-327) |
| **Normal lung volume (ml/ % total volume)** | 231 (197-307)/ 89.7 (85.6-93.2) |
| **Hypoattenuation (ml/ % total volume)** | 4 (1-10)/ 1.6 (0.5-3.8) |
| **Hyperattenuation (ml/ % total volume)** | 11 (5-27)/ 4.2 (2.0-9.1) |
| **Bronchial wall thickening (ml/ % total volume)** | 5 (2-9)/ 2.1 (0.8-2.9) |
| **Number of affected segments** | 6 ± 3 |
| **Percentage of affected segments of total number of segments** | 29.8 ± 16.8 |
| **Architectural distortion** | |
| **- Absent** | 2 (4.5%) |
| **- Mild** | 24 (53.3%) |
| **- Moderate** | 18 (40.0%) |
| **- Severe** | 1 (2.2%) |

Definition of abbreviations: CT: computed tomography; ml: millilitre. Continuous variables are expressed as mean ± standard deviation (SD) for normally distributed data or median (interquartile range, IQR) for data that were not normally distributed. Categorical data are expressed as n (%).

in 95.5% of all CT scans (Table 2). Median (IQR) total lung volume was 264 (219-327) ml, of which 1.6 (0.5-3.8) % showed hypoattenuation, 4.2 (2.0-9.1)% hyperattenuation and 2.1 (0.8-2.9) % bronchial wall thickening, while 89.7 (85.6-93.2)% had a normal appearance using the PRAGMA-BPD scoring method.

PSG was performed successfully in 47 infants at a corrected age of 6.2 (5.8-6.6) months (Table 3). The median total sleep time (TST) for overnight PSG was 534 (489-579) min with a mean (SD) sleep efficiency of 80.4 (14.7)% (n=45). The median (IQR) breathing rate was 30 (24-36) per min, the mean (SD) oxygen saturation (SaO2) was 96 (2) %, with a mean (SD) deepest oxygen desaturation (SaO2 nadir) of 85 (4)%. The median (IQR) AHI was 8.08 (4.59-11.60) (n=42) and the median (IQR) ODI was 10.30 (4.78-18.95) (n=45), with 74% of the infants having an abnormal ODI above 5.



### Relation lung structure and ventilatory function

Associations of chest CT scores and PSG parameters are presented in Table 4. We found per 10 ml increase of hypoattenuation a 3.3 (0.4, 6.3)% lower mean $SaO_2$, and an increase with one affected segment was associated with a 10.2 (0.9-19.5)% increase in ODI. However, when we adjusted for GA at birth, birth weight SDS and sex the associations became non-significant (data not shown). No other associations of lung structure measures with ventilatory function was observed.

**Table 3.** Polysomnography outcomes

| | Severe BPD (n=47) |
|---|---|
| **Corrected age at PSG (months)** | 6.2 (5.8-6.6) |
| **TST (min)** | 534 (489-579) |
| **TIB (min)** | 642 (579-703) |
| **SPT (min)** | 614 (559-650) |
| **Mean breathing rate (/min)** | 30 (24-36) |
| **Mean heart rate (/min)** | 113 (106-121) |
| **Bradycardia events** | 1 (0-14) |
| **Tachycardia events** | 1 (0-14) |
| **Mean $SaO_2$ (%)** | 96 ± 2 |
| **$SaO_2$-nadir (%)** | 85 ± 4 |
| **oAHI** | 1.51 (0.77-3.13) |
| **AHI** | 8.08 (4.59-11.60) |
| **ODI** | 10.30 (4.78-18.95) |
| **OAI** | 0.00 (0.00-0.11) |
| **CAI** | 2.82 (1.15-4.56) |
| **MAI** | 0.00 (0.00-0.00) |

Definition of abbreviations: TST: total sleep time; TIB: time in bed; SPT: sleep period time; mean SaO2: mean oxygen saturation; $SaO_2$-nadir: deepest oxygen desaturation. Obstructive apnea hypopnea index (oAHI) was defined as the sum of obstructive apneas (OAs); mixed apneas (MAs) and obstructive hypopneas (OHs) per hour of sleep. Apnea hypopnea index (AHI) was defined as the number of apneas and hypopneas per hour of sleep. Oxygen desaturation index (ODI) was defined as the sum of all desaturations ≥3% per hour of sleep. Obstructive apnea index (OAI) was calculated as the number of OAs per hour of sleep. Central apnea index (CAI) as the number of central apneas (CAs) per hour of sleep. Mixed apnea index (MAI) as the number of MAs per hour of sleep. oAHI≥1, AHI>1, ODI>5, OAI≥1, CAI≥1 and MAI≥1 were defined as abnormal. Data were missing for TIB (n=1), Mean breathing rate (n=2), Bradycardia events (n=1), Tachycardia events (n=1), oAHI (n=5), AHI (n=5), ODI (n=2), OAI (n=5), CAI (n=5), MAI (n=5), and AI (n=5). Continuous variables are expressed as mean ± standard deviation (SD) for normally distributed data or median (interquartile range, IQR) for data that were not normally distributed.

## Relation birth characteristics and infant growth with lung structure and ventilatory function

An increase in GA of 1 week and 100 grams of weight at birth was associated with higher total lung volume (β coefficient (95% CI): 1.86 (0.15, 3.57) and 0.99 (0.06, 1.92)), higher normal lung volume (β coefficient (95% CI): 2.03 (0.41, 3.65) and 1.05 (0.17, 1.94)), less hypoattenuation (-4.30 (-7.70, -0.90) and -2.50 (-4.40, -0.70) and lower ODI (-36.70 (-64.20, -9.10) and -19.10 (-35.00, -3.10) (Table 6a and b). The effect estimates of birth weight with lung structure and ventilatory function became non-significant when GA was taken into account.

Conditional analyses showed that higher weight at 6 months corrected age only, was associated with higher total and normal lung volume (β coefficient (95% CI): 2.36

**Table 4.** Associations between lung volumes and PSG outcomes

| | AHI | ODI | CAI | Breathing rate | Mean $SaO_2$ | $SaO_2$ nadir |
|---|---|---|---|---|---|---|
| | **Sym% (95% CI)** | **Sym% (95% CI)** | **Sym% (95% CI)** | **Sym% (95% CI)** | **Sym% (95% CI)** | **β (95% CI)** |
| **Total lung volume** | 0.30 (-3.30, 3.90) | -1.40 (-5.70, 3.00) | 3.40 (-0.70, 7.50) | -0.60 (-1.80, 0.60) | 0.00 (-0.30, 0.30) | 0.01 (-0.16, 0.18) |
| **Normal lung volume** | -0.10 (-3.70, 3.50) | -2.10 (-6.40, 2.20) | 3.00 (-1.10, 7.20) | -0.80 (-1.90, 0.40) | 0.10 (-0.20, 0.40) | 0.03 (-0.14, 0.20) |
| **Hypo-attenuated lung volume** | 32.90 (-6.20, 72.00) | 33.10 (-16.10, 82.40) | 32.30 (-14.50, 79.10) | 4.30 (-9.60, 18.10) | **-3.30 (-6.30, -0.40)*** | -1.14 (-3.07, 0.78) |
| **Hyper-attenuated lung volume** | 3.20 (-18.30, 24.70) | 13.60 (-11.90, 39.10) | 5.00 (-20.40, 30.40) | 4.70 (-2.40, 11.80) | -1.50 (-3.00, 0.10) | -0.40 (-1.39, 0.60) |
| **Bronchial wall thickening** | 15.80 (-61.00, 92.60) | 52.50 (-40.20, 145.20) | -15.10 (-106.00, 75.80) | -18.10 (-44.30, 8.20) | -1.70 (-7.50, 4.00) | -1.49 (-5.10, 2.12) |
| **Number of affected segments** | 3.30 (-5.00, 11.60) | **10.20 (0.90-19.50)*** | 4.70 (-5.10, 14.50) | 2.3 (-0.40, 5.00) | -0.50 (-1.10, 0.00) | -0.20 (-0.58, 0.17) |

Values are β coefficients (95% confidence interval, CI) for not transformed outcomes and sympercent (95% CI) for log or arcsine transformed outcomes and reflect the change or percent change in ventilatory function parameter per 10 ml increase in affected lung volume. AHI, ODI, CAI and breathing rate were log-transformed, mean $SaO_2$ was arcsine transformed to obtain a normal distribution. *P < 0.05. Models were not adjusted for covariates.

**Table 5a.** Conditional associations between birth characteristics, infant growth and lung structure

| | Total lung volume (per 10 ml) | Normal lung volume (per 10 ml) | Hypo-attenuated lung volume (per 10 ml) | Hyper-attenuated lung volume (per 10 ml) | Number affected segments |
|---|---|---|---|---|---|
| | **β (95% CI)** | **β (95% CI)** | **Sym% (95% CI)** | **Sym% (95% CI)** | **β (95% CI)** |
| **Birth weight (SDS)** | 1.82 (-0.46, 4.11) | 1.65 (-0.66, 3.96) | -3.00 (-7.40, 1.40) | 1.20 (-2.50, 5.00) | -0.31 (-1.46, 0.84) |
| **Weight at 2 months (SDS)** | 0.72 (-1.58, 3.01) | 0.62 (-1.70, 2.94) | 1.20 (-3.20, 5.60) | 0.60 (-3.10, 4.40) | 0.54 (-0.61, 1.69) |
| **Weight at 6 months (SDS)** | **2.36 (0.14, 4.59)*** | **2.53 (0.28, 4.78)*** | 3.30 (-1.00, 7.50) | -2.50 (-6.20, 1.10) | -0.85 (-1.97, 0.26) |

Conditional associations between birth characteristics and lung volumes. Values are β's (95% confidence interval, CI) for normal distributed outcomes and sympercent (95% CI) for log transformed outcomes and reflect the change in lung volume, and number of affected segments per 1 SDS increase of birth weight, independently from previous measurements. Hypo-attenuated and hyper-attenuated lung volumes (ml) were log-transformed to obtain a normal distribution. *P < 0.05. Models were adjusted for sex, preeclampsia and age at time of chest CT scan.

**Table 5b.** Conditional associations between birth characteristics, infant growth and PSG outcomes

| | AHI | ODI | CAI | Breathing rate | Mean $SaO_2$ | $SaO_2$ nadir |
|---|---|---|---|---|---|---|
| | **Sym% (95% CI)** | **Sym% (95% CI)** | **Sym% (95% CI)** | **Sym% (95% CI)** | **Sym% (95% CI)** | **β% (95% CI)** |
| **Birth weight (SDS)** | 10.4 (-19.0, 39.8) | -12.2 (-50.5, 26.2) | 17.6 (-19.7, 54.9) | -3.4 (-12.3, 5.5) | 1.6 (-0.7, 3.9) | -1.02 (-2.28, 0.24) |
| **Weight at 2 months (SDS)** | 11.3 (-18.8, 41.4) | -5.4 (-42.8, 32.1) | 1.8 (-36.4, 40.0) | -4.0 (-13.1, 5.1) | 0.5 (-1.8, 2.8) | -1.21 (-2.47, 0.06) |
| **Weight at 6 months (SDS)** | -13.7 (-39.5, 12.2) | -13.2 (-46.0, 19.7) | -1.2 (-33.9, 31.6) | 3.0 (-5.4, 11.5) | 1.7 (-0.5, 3.8) | **1.25 (0.08, 2.43)*** |

Conditional associations between birth characteristics and respiratory function. Values are β's (95% confidence interval, CI) for normally distributed outcomes and sympercent (95% CI) for either log or arcsine transformed outcomes and reflect the change in respiratory function per 1 SDS increase, independently from previous measurements. Apnea hyperpnoea index (AHI), oxygen desaturation index (ODI), central apnea index (CAI), breathing rate were log transformed and mean oxygen saturation ($SaO_2$) was arcsine transformed to obtain a normal distribution. *P < 0.05. Models were adjusted for sex, preeclampsia and age at time of PSG.

(0.14, 4.59) and 2.53 (0.28, 4.78), respectively, per SDS increase in weight) (Table 5a). Higher weight at 6 months corrected age was also independently associated with less severe oxygen desaturations (β coefficient (95% CI): 1.25 (0.08, 2.43), per SDS increase in weight) (Table 5b). Infant weight gain across all various time intervals did not affect lung structure at 6 months corrected age (Table 6a). Only infant weight gain between 2 and 6 months corrected age was associated with less severe oxygen desaturations during overnight PSG (β coefficient (95% CI): 2.09 (0.49, 3.70)) (Table 6b).

## DISCUSSION

Our findings show that among preterm infants with severe BPD almost all had lung architectural distortion and the majority had abnormal PSG at 6 months corrected age. The volume fraction of hypoattenuation was associated with mean $SaO_2$ measured during overnight PSG and the number of affected segments with ventilatory function as reflected by ODI, but results were explained by birth characteristics. A lower GA at birth was associated with a lower total and normal lung volume, more hypoattenuation and higher ODI. An increased weight gain in the first 6 months was associated with higher total and normal lung volume and with less ventilatory deficits.

It is well known that chest CT scans reveal structural abnormalities in most patients with BPD, with the most common abnormalities being pulmonary hypoattenuation, linear and pleural opacities, bronchial wall thickening and consolidations [2]. These findings derive from studies which have been performed in older children or adults and do not reflect current BPD population. Although in this study almost all infants with severe BPD showed architectural distortion, the lung volume which had a macroscopically normal appearance was high, namely 89.7 (85.6-93.2)%. The overall relatively high percentage of normal lung volume fits the reported high quality of life which is generally experienced by patients with BPD [19]. However, some children had less than 45% normal lung volume, which is likely to have a negative impact on long-term prognosis. In our opinion, it is important to recognize abnormal lung structure early in life in order to identify those children with the most severe disease, who are at risk for future severe respiratory morbidity and should be monitored more strictly.

Preterm birth affects later lung function. A systematic review showed lower mean $FEV_1$ in preterm born infants, with the lowest $FEV_1$ of 79.1 (76.9-81.3)% in infants with severe BPD defined as oxygen dependency at 36 weeks PMA [3]. As a proxy for ventilatory and respiratory function we performed overnight PSG, because with PSG the whole ventilatory system can be assessed. Studies with PSG results of preterm infants are scarce [4-6, 12, 20]. Infants with BPD may have normal oxygen saturation and respiratory rate during an outpatient clinic visit while awake, but still having desaturations during sleep along with

**Table 6a.** Associations between birth characteristics, infant growth and lung structure

| | Total lung volume (per 10 ml) | Normal lung volume (per 10 ml) | Hypoattenuated lung volume (per 10 ml) | Hyperattenuated lung volume (per 10 ml) | Number affected segments |
|---|---|---|---|---|---|
| | **β (95% CI)** | **β (95% CI)** | **Sym% (95% CI)** | **Sym% (95% CI)** | **β (95% CI)** |
| **Gestational age** | | | | | |
| Gestational age (weeks) | **1.86 (0.15, 3.57)*** | **2.03 (0.41, 3.65)*** | **-4.30 (-7.70, -0.90)*** | -0.30 (-3.30, 2.80) | -0.67 (-1.55, 0.20) |
| **Birth weight** | | | | | |
| Birth weight (100 g) | **0.99 (0.06, 1.92)*** | **1.05 (0.17, 1.94)*** | **-2.50 (-4.40, -0.70)**** | -0.30 (-1.90, 1.40) | -0.37 (-0.84, 0.10) |
| Gestational age adjusted birth weight (SDS) | 1.65 (-0.53, 3.83) | 1.55 (-0.56, 3.65) | -4.30 (-8.60, 0.10) | 0.60 (-3.20, 4.30) | -0.39 (-1.50, 0.71) |
| **Infant growth** | | | | | |
| Weight gain 0-2m (SDS) | 0.08 (-1.91, 2.07) | 0.24 (-1.63, 2.11) | 1.10 (-2.80, 5.00) | 0.00 (-3.40, 3.40) | 0.31 (-0.72, 1.34) |
| Weight gain 0-6m (SDS) | 0.90 (-0.94, 2.75) | 1.19 (-0.55, 2.93) | 2.00 (-1.60, 5.60) | -1.90 (-5.20, 1.40) | -0.27 (-1.24, 0.71) |
| Weight gain 2-6m (SDS) | 1.64 (-1.11, 4.39) | 1.87 (-0.72, 4.46) | 2.80 (-2.50, 8.10) | -3.30 (-8.00, 1.30) | -1.14 (-2.55, 0.26) |

Associations between birth characteristics and lung volumes. Values are β's (95% confidence interval, CI) and reflect the change in lung volume per ml, and number of affected per week increase of gestational age, per 100 grams or 1 SDS increase of birth weight, or per SDS increase in infant weight growth from birth until an corrected age of 6 months. Hypo-attenuated and hyper-attenuated lung volumes (ml) were log-transformed to obtain a normal distribution. *P < 0.05, **P < 0.01. All models were adjusted for sex, preeclampsia and age at time of chest CT scan. Infant growth variables were additionally adjusted for GA, birth weight and time between two measurements.

**Table 6b.** Associations between birth characteristics, infant growth and respiratory function

| | AHI | ODI | CAI | Breathing rate | Mean $SaO_2$ | $SaO_2$ nadir |
|---|---|---|---|---|---|---|
| | **Sym% (95% CI)** | **Sym% (95% CI)** | **Sym% (95% CI)** | **Sym% (95% CI)** | **Sym% (95% CI)** | **β (95% CI)** |
| **Gestational age** | | | | | | |
| Gestational age (weeks) | -16.70 (-37.70, 4.30) 0.38, 0.04) | **-36.70 (-64.20, -9.10)*** | 4.80 (-22.20, 31.90) | 2.50 (-5.30, 10.30) | 1.00 (-0.80, 2.80) | 0.12 (-1.03, 1.27) |
| **Birth weight** | | | | | | |
| Birth weight (100 g) | -4.80 (-16.90, 7.20) | **-19.10 (-35.00, -3.10)*** | 3.40 (-11.70, 18.50) | -0.70 (-5.00, 3.60) | 0.80 (-0.20, 1.70) | -0.13 (-0.76, 0.50) |
| Gestational age adjusted birth weight (SDS) | 3.50 (-24.40, 31.40) | -23.60 (-62.50, 15.30) | 13.80 (-20.70, 48.20) | -3.00 (-11.60, 5.60) | 1.50 (-0.70, 3.70) | -0.64 (-2.03, 0.76) |
| **Infant growth** | | | | | | |
| Weight gain 0-2m (SDS) | 11.80 (-15.80, 39.30) | -5.40 (-38.90, 28.20) | 2.90 (-31.70, 37.40) | -3.30 (-11.90, 5.20) | 0.30 (-1.90, 2.60) | -1.05 (-2.32, 0.22) |
| Weight gain 0-6m (SDS) | -4.70 (-30.70, 21.20) | -8.50 (-41.20, 24.20) | -9.30 (-42.50, 23.90) | -1.50 (-9.40, 6.50) | 1.20 (-0.80, 3.10) | 0.18 (-1.16, 1.51) |
| Weight gain 2-6m (SDS) | -18.70 (-53.60, 16.20) | -8.00 (-51.90, 35.90) | -5.40 (-49.90, 39.10) | 3.90(-7.70, 15.50) | 1.70 (-1.20, 4.60) | **2.09 (0.49, 3.70)*** |

Associations between birth characteristics and PSG outcomes. Values are β's (95% confidence interval, CI) and reflect the change in AHI, ODI, mean $SaO_2$ and $SaO_2$ nadir per week increase of gestational age, per 100 grams or 1 SDS increase of birth weight, or per SDS increase in infant weight growth from birth until an corrected age of 6 months. AHI and ODI were log-transformed and mean $SaO_2$ was arsine transformed to obtain a normal distribution. *P < 0.05. All models were adjusted for sex, pre-eclampsia and age at time of PSG. Infant growth variables were additionally adjusted for GA, birth weight and time between two measurements.

sleep-disordered breathing overnight [4, 6, 21]. Previously, Sekar *et al.* reported that preterm infants with BPD have more episodes of hypoxemia, central apneas and periodic breathing than preterm infants without BPD [20]. In the present study, we also found abnormal AHI and abnormal ODI in the majority of infants with severe BPD. Furthermore, while the mean $SaO_2$ during overnight PSG was within normal ranges, the deepest oxygen desaturation was below normal values with a mean of 85%. These results indicate that infants with severe BPD might be more vulnerable for desaturation during apneas, perhaps because of decreased ventilatory reserve, and overnight PSG registration before cessation of supplemental oxygen may be recommended.

Only few studies assessed both lung structure and ventilatory function in infants with BPD below 1 year of age [22, 23]. We found that hypoattenuation was associated with mean $SaO_2$ during overnight PSG and the extent of structural abnormalities showed an association with ventilatory function as reflected by ODI. In previous studies performed in the first year of life, chest CT scores correlated significantly with the clinical severity of BPD and with respiratory symptoms [22-26]. Only 2 studies performed both chest CT and respiratory function before the age of 2 years. In these studies a lower lung compliance and lower functional residual capacity (FRC) was reported for those children with most abnormal CT scans [22, 27]. The effect of these early abnormalities on respiratory health in later life is not clear. Most studies have examined lung structure and function at a later age and all but one study [28] reported significant correlations between CT abnormalities and lung function parameters. After infancy, specifically the volume of hypoattenuation and architectural distortion is correlated with obstructive lung function, mainly low $FEV_1$ [23, 29-32]. Studies are difficult to compare because lung function was assessed with diverse techniques at various ages.

Another important finding of our study was that both lower GA at birth and lower birthweight were associated with worse structural and functional respiratory outcomes at 6 months of corrected age, although only GA was independently associated. Previous research also showed that both lower GA at birth and lower birth weight were associated with lower lung function [33]. Preterm infants are born when lung development is still in the canalicular or saccular stage, resulting in vascular and alveolar simplification. This suggests that poor lung development especially at the time of preterm birth is crucial for the development of BPD. Our data indeed showed that GA at birth influences both lung structure and ventilatory function at later age.

Last, in our study we showed that early postnatal weight gain positively influenced both lung structure and ventilatory function in preterm infants with severe BPD. The association between infant growth and lung function in the first year of life has been assessed extensively in the context of early risk factors for asthma, and infant weight gain was consistently associated with higher $FEV_1$ and forced vital capacity (FVC), but lower $FEV_1$/FVC and forced expiratory flow after 75% of vital capacity ($FEF_{75}$) [34-36]. The underlying

mechanisms for the associations between infant weight gain and lung structure and function are unclear. The lower $FEV_1$/FVC ratio and $FEF_{75}$ might be explained by smaller airways in preterm born infants. In addition, animal studies showed that growth restriction might affect airway compliance and lead to impaired growth of bronchial walls, alterations in mucus producing tissues, a decrease in number of alveoli, thicker interalveolar septa and a greater volume density of lung tissue [37]. Another possible explanation for the observed associations between infant weight gain and lung structure and function is low grade systemic inflammation, associated with obesity. Both the increased amount of pro-inflammatory cytokines producing macrophages present in adipose tissue and the adipokines and leptin secreted by adipose tissue itself could lead to systemic low grade inflammation [38]. Recently, it was shown that a coordinated expression of pro-inflammatory cytokines like interleukin (IL)-6 and tumor necrosis factor (TNF)-α plays a protective role and might positively influence lung maturation [39-42]. In our study, we found an association between increased infant weight gain from 2 to 6 months corrected age and less severe oxygen desaturations. Furthermore, also weight at 6 months corrected age was independently associated with more healthy lung volume and less severe oxygen desaturations during sleep. We did not find an association between early growth and other parameters from PSG like breathing rate, mean oxygen saturation, AHI, CAI and ODI, suggesting that alveolar growth might be better reflected by oxygen desaturations than by other aspects of ventilation during sleep. This can be explained by the fact that AHI, CAI and ODI may also be influenced by central apneas and superficial breathing patterns which may occur in preterm infants. We can only speculate whether postnatal growth would have been better when prolonged supplemental oxygen was given to these preterm born infants and the influence on both total and normal lung volume and ventilatory function.

Our prospective cohort provides the opportunity to follow preterm born infants with severe BPD in a multidisciplinary team, with detailed measurements of both lung structure and function and detailed information on prenatal and postnatal exposures. There are some limitations to the present study. We perform chest CT scans during free breathing, acquiring CT scans at a volume level around FRC plus or minus tidal volume. The alternative would be to acquire chest CT scans at total lung capacity (TLC) level and at FRC level using a pressure-controlled protocol, but then general anesthesia is needed. Hence a free breathing protocol creates some noise in relation to the lack of volume standardization during CT acquisition.

PSG measurements reflect an important aspect of ventilatory and respiratory function. However, we lacked information on infant lung function as may be obtained by multiple breath washout (MBW) techniques. Recent studies suggest that the lung clearance index (LCI) as obtained with MBW is a sensitive marker of small airway disease [43]. Of the 49 infants, we had reproducible MBW measurements in only 18 (36.7%), mainly due

to standardization problems, and practical problems with using the equipment and software. We plan to repeat MBW tests at 12 months and spirometry from 5 years of age onwards, which will make it possible to correlate early structural abnormalities with later lung function.

Lastly, the clinical definition of BPD remains under debate. Previous research showed that preterm born infants who did not fulfill the criteria for BPD also showed structural and functional respiratory abnormalities [44, 45]. Therefore, it should be considered to screen all preterm infants born before 28 weeks GA in the first year of life for structural and functional respiratory impairment.

In conclusion, our results suggest that structural abnormalities of the lung and ventilatory impairment are present in most preterm born infants with severe BPD at a corrected age of 6 months. These outcomes are mainly affected by GA at birth and infant weight gain between 2 and 6 months corrected age. The long-term effect of these early impairments for respiratory morbidity in later life is not clear, stressing the importance of long-term follow up of all preterm born infants.

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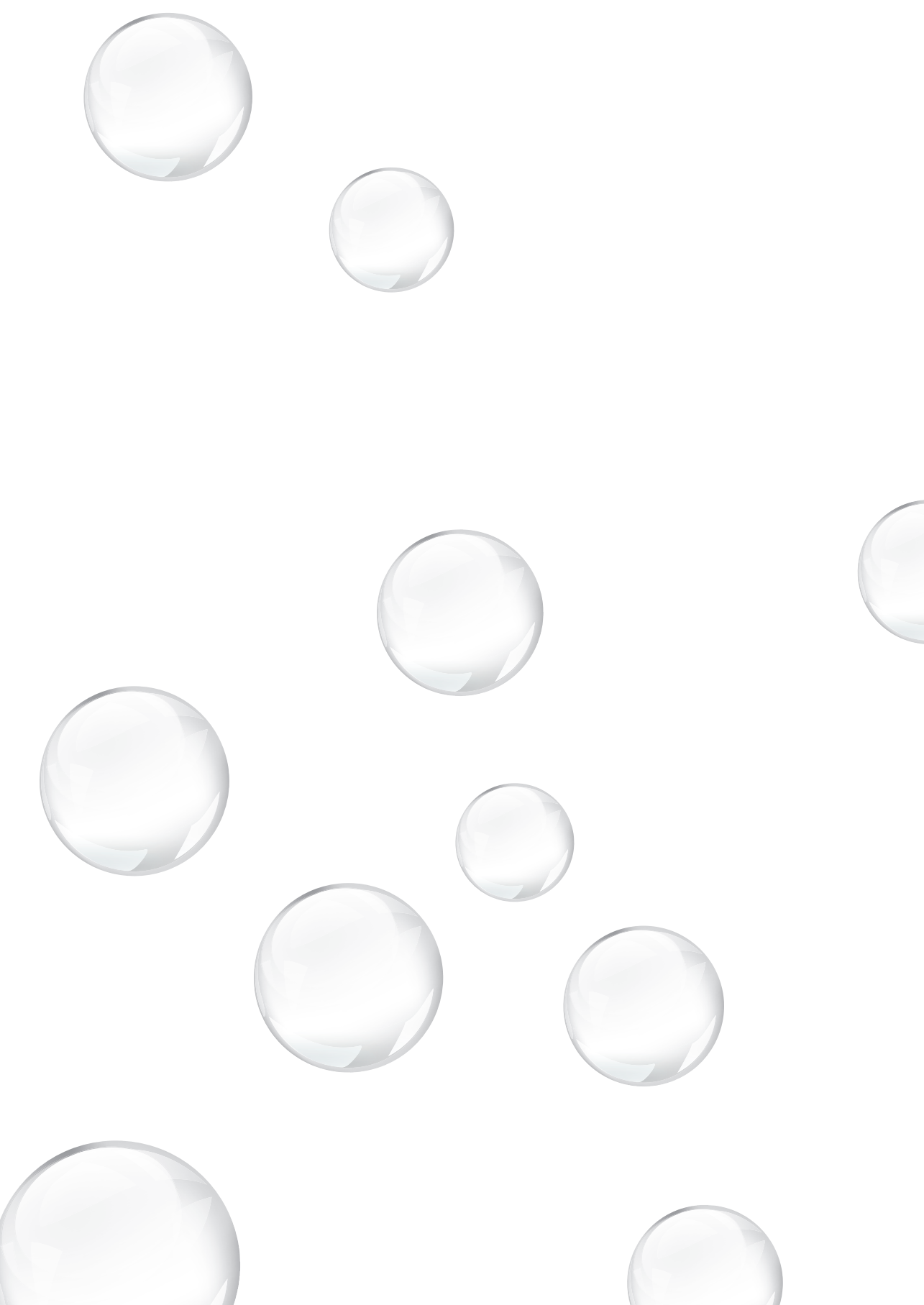
## SUPPLEMENTAL METHODS

### PRAGMA-BPD scoring method

The PRAGMA-BPD scoring is system is a quantitative structural lung disease computed tomography scoring system for BPD derived by the PRAGMA-CF scoring system (Am J Respir Crit Care Med. 2015 May 15;191(10):1158-65). The PRAGMA-BPD score assesses lung structure according to five major categories and according to the following scoring priority order: hypoattenuation, hyperattenuation, bronchial wall thickening, normal lung structure and architectural distortion. Hypoattenuation refers to lung parenchyma with reduced CT attenuation (dark appearance) either in the inspiratory or expiratory scans. This category includes mosaic perfusion, emphysema, trapped air, bullae and cysts. Hyperattenuation refers to lung parenchyma with increased CT attenuation (white appearance) either in the inspiratory or expiratory scans. This category includes consolidation, atelectasis, linear and subpleural triangular opacities. Bronchial wall thickening is defined when the ratio between bronchial wall thickness and outer diameter of the adjacent pulmonary artery is greater than 33% (J Pediatr 2004;144:154-161). The lung parenchyma not fulfilling the criteria of these three categories falls within the fourth category of normal lung structure. Finally architectural distortion, defined as abnormal displacement of bronchi, vessels, fissures and/or septa caused by diffuse or localized lung disease, is scored per lung segment. Each lung consists of 10 segments, where the medial basal segment is considered a separate segment on the left side. According to the number of lung segments involved, the architectural distortion is defined as mild (less than 7 lung segments), moderate (7-13 lung segments) and severe (more than 13 lung segments).

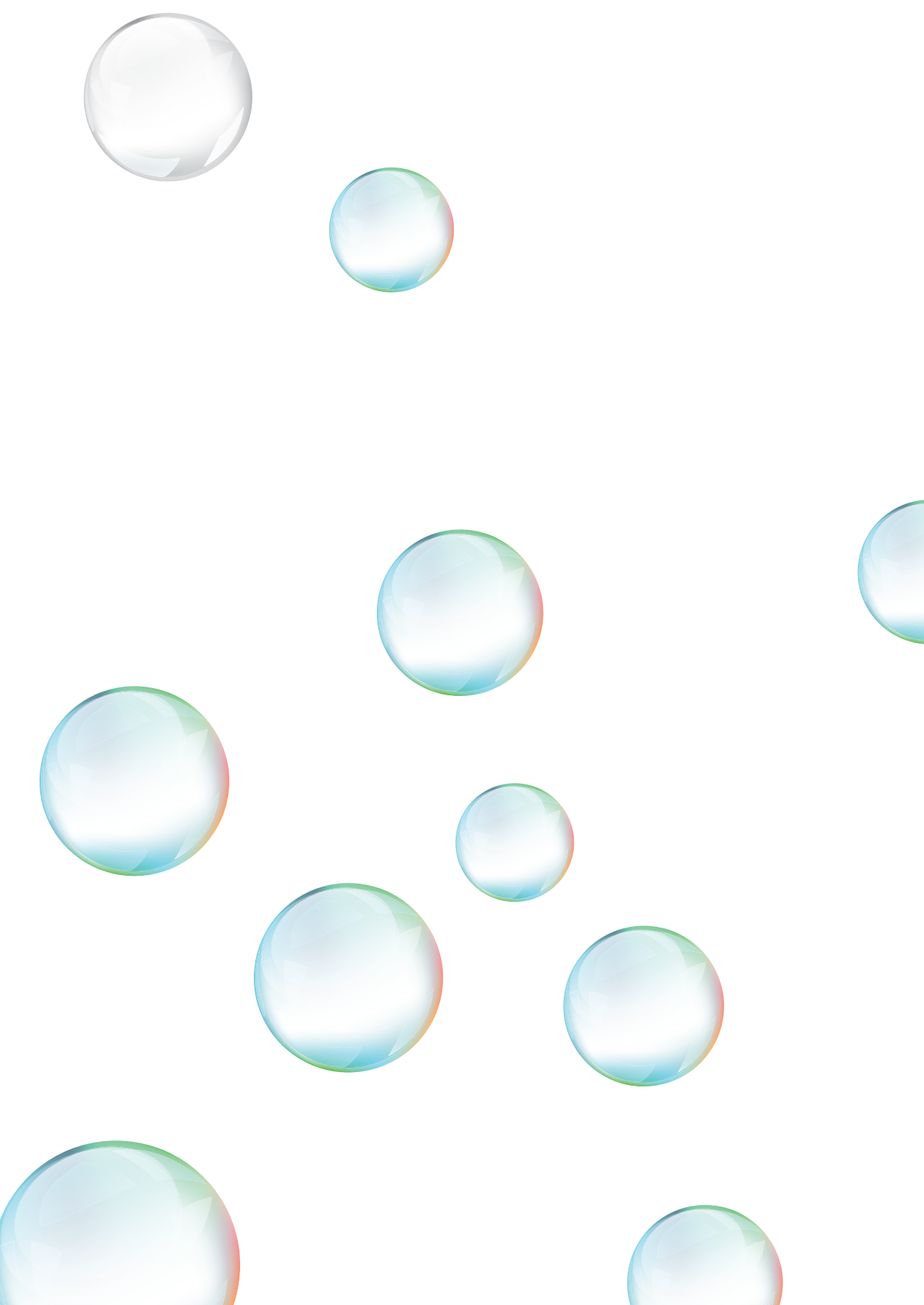
### CT BPD protocol

BPD protocol was performed using the 256-rows SOMATOM Definition Flash or 384-rows SOMATOM Force scanners (Siemens, Erlangen, Germany) with the following parameters: kV 90, 10-20 reference mAs, pitch 3, CAREDose4D. The following reconstructions were acquired: axial 1 and 3 mm lung window (kernels BI57 and Br64), axial 3 mm mediastinal window (Br40) and 2 mm sagittal and coronal multiplanar reformats. Computer tomography index (CTDI) ranged between 0.2 to 0.5 mGy.



# Part IV

## Reflection



# 9 General discussion

Today's children will grow up to be the adults of tomorrow, and the management of respiratory diseases in children will influence adult lung health [1]. In this thesis we focused on three groups of patients: children with asthma, cystic fibrosis (CF) and bronchopulmonary dysplasia (BPD). These diseases have in common that inflammation, destruction and (defective) repair mechanisms play a central role in their pathogenesis. Impairment of lung development has lifelong consequences on lung function. Consequently if we are able to improve respiratory health in a very early phase of life, this will have lifelong benefits.

There are several approaches possible to accomplish this goal. First, prediction and early diagnosis offers the opportunity of early treatment and maybe prevention of progression towards more severe disease. This has been the case in CF where newborn screening led to better lung function, nutritional status and improved survival in screened patients in early adulthood [2]. Second, more insight in the underlying pathophysiology of pediatric respiratory diseases is needed to develop new treatments or preventive strategies. Third, disease phenotyping may lead to personalized treatment. Last, better monitoring of disease progression with timely interventions may lead to better outcomes.

In this thesis we focused on biomarker identification as biomarkers may be essential in prediction and early diagnosis of respiratory diseases, but can also contribute to insight in underlying pathophysiology, disease phenotyping and monitoring of disease progression and treatment effect. To identify biomarkers we used different approaches. In **chapter 3 and 4** we investigated biomarkers in exhaled breath of children with asthma and CF with the use of a newly developed technique enabling both fast detection and molecule identification. In **chapter 5 and 6** we investigated biomarkers in tracheal aspirates (TAs) of preterm infants by means of liquid chromatography tandem mass spectrometry (LC-MS/MS), proteome assay and multiplex immunoassay (Luminex). We choose to detect biomarkers in exhaled breath and TAs, as both can be obtained routinely in a clinical setting and are minimally invasive which is particularly important in children. Finally, in **chapter 7 and 8** chest CT scans and polysomnography have been studied as outcomes in infants with severe BPD, which may however also be 'alternative biomarkers' that may predict outcome of BPD. The main findings of each chapters are discussed below, as well as the challenges that we encountered while working in an interdisciplinary field of research. In addition, the relevance of our findings and their potential scientific and clinical implications are placed in a broader context. Finally, future research perspectives with respect to biomarker research in pediatric respiratory diseases will be discussed.

## DIFFERENT APPROACHES TO TEST BIOMARKERS IN PEDIATRIC RESPIRATORY DISEASES

Biomarkers are broadly defined as "any substance, structure, or process that can be measured in the body or its products and influence or predict the incidence of outcome or disease". The ideal biomarker is easy to obtain with minimal discomfort or risk to the patient, is cheap, has high sensitivity and specificity, good reproducibility and repeatability, and can be used for one or preferably more clinical applications such as diagnosis, disease phenotyping, prognosis, or monitoring. Biomarkers in respiratory diseases can be measured systemically (e.g. in blood) or locally in the lung and airways (e.g. in endobronchial biopsies, bronchoalveolar lavage fluid (BALF), sputum, exhaled breath and exhaled breath condensate). To investigate a specific aspect of a disease, single biomarkers can be useful, like the fraction of exhaled nitric oxide (FeNO) which reflects eosinophilic airway inflammation in asthma [3]. However, single markers do not reflect the complexity of most respiratory diseases in which genetic, environmental and epigenetic factors interact with each other and may lead to inflammation, infection, damage and repair. Therefore, the last decade a systems biology approach including genomics, proteomics and metabolomics, has become popular to enable the analysis of numerous potential biomarkers at the same time and allow for the discovery of new pathophysiological pathways without a priori hypothesis.

In this thesis we investigated biomarkers in exhaled breath and TAs by using techniques which enabled us to detect multiple biomarkers at the same time. For identification of multiple biomarkers at once two approaches may be followed. First, one might use an unselected approach to study biomarker profiles which offers the possibility to discover new pathophysiological processes of lung health and disease. The disadvantage of such a 'fishing expedition' is that it is time consuming, costly and may lead to side paths that are not relevant. The second approach is preselection of biomarkers based on current knowledge. This approach, which we used in our studies, enables to investigate biomarkers in specific pathways, previously shown to contribute to the disease in question. In **chapter 4** we investigated volatile exhaled compounds (VOCs) present in exhaled breath of children with asthma and CF. We studied a specific broad wavelength spectrum containing the absorption profiles of hydrocarbons, since previous studies have identified hydrocarbons as the most discriminating VOCs between patients with and without asthma or CF. In **chapter 5** we used a proteome profiling and Luminex assay to measure multiple cytokines, chemokines and growth factors in TAs of preterm infants. Both assays contained a preselected set of biomarkers known to be important in angiogenesis, since angiogenesis is a driving force of alveolarisation and disruption of microvascular development in premature lungs has recently been postulated as a critical factor in the alveolar development in BPD. In **chapter 6** we measured a specific set of sphingolipids in

TAs of preterm infants using liquid chromatography tandem mass spectrometry (LC-MS/MS). Previous studies in mice indicated the importance of ceramides in inflammation and repair processes in the developing lung.
We speculate that the use of 'omics' techniques, measuring multiple biomarkers at once with or without a priori hypothesis, may improve stratification of patient groups to a level of individual decision making, contributing to personalized medicine.

## EXHALED BREATH ANALYSIS IN DIAGNOSIS AND MONITORING OF ASTHMA AND CYSTIC FIBROSIS

Exhaled breath analysis is a noninvasive method to assess potential biomarkers for diagnosis and management of respiratory diseases in childhood, and exhaled breath can be very easily obtained in children of all ages.

### Broadband quantum cascade laser-based spectroscopy

**Chapter 3** describes how we developed a broadband quantum cascade laser-based spectroscopic system for exhaled breath analysis, in cooperation with the Optics research group at the Delft University of Technology, the Netherlands. This technique allows for detection of VOCs in exhaled breath samples, with an absorption spectrum in a specific, broad wavelength region. Advantages of this technique over the more commonly used gas chromatography-mass spectrometry (GC-MS) technique are especially the limited need for preprocessing and the relatively fast detection without the need for highly trained personnel. The main advantage over the use of sensor-based detection methods, like the electronic nose (eNose), is the possibility of VOC identification with the use of molecular databases. VOC identification may be helpful for further understanding of underlying pathophysiological processes of respiratory disease. Current disadvantages of this new method are the still bulky equipment and the relatively low sensitivity.
We performed a first pilot study, in which we analyzed exhaled breath samples of healthy children, children with stable asthma and children with stable CF (**chapter 4**). We found overall poor short- and long-term repeatability. This could be explained by the fact that we investigated a large spectroscopic range comprising 8612 individual wavenumber points, and because daily life factors (e.g. eating, drinking, exercise, variable composition of ambient air) influence exhaled breath content and were not standardized. However, despite the variability of the spectra we were able to detect differences between exhaled breath profiles from healthy children and from children with chronic airway inflammation due to asthma or CF.
We propose that, before further clinical studies can be undertaken, the sensitivity and repeatability of this technique need to be improved. In general, laser-based methods for

trace gas detection, in our case in exhaled breath samples, are based on de Beer-Lambert law and the absorbance, which is determined by the wavenumber corresponding to the highest molar absorption coefficient, the interaction distance and the concentration. During the development of our set-up we mainly focused on increasing the interaction distance within the multipass cell in order to improve the sensitivity. To further increase the sensitivity it is also possible to apply preconcentration of samples, as was done with GC-MS. However, whether or not a higher sensitivity will lead to better performance remains to be shown. Improving the repeatability with regards to exhaled breath analysis is far more challenging, since this is due to numerous factors, including the width of the wavelength spectrum, the amount of noise, and the variability of the composition of exhaled breath due to numerous environmental factors. Furthermore, there is a need for further validation of this technique by internal validation, mainly comparing this technique with other exhaled breath techniques such as GC-MS and the eNose, and by external validation, by demonstrating the reproducibility of this technique in other target populations. Finally, the equipment needs to be adapted to make it applicable in a clinical setting. Key element in this process of miniaturization is the multipass cell used to increase the interaction distance. The use of hollow waveguides (HWG) is an attractive alternative allowing to confine the laser light in thin channels where small volumes of the exhaled breath sample can be introduced [4]. HWGs are available with internal diameters of 1 mm and volumes lower than 100 microliters. They allow interactions distances of up to 4 meters [5]. This relatively small interaction distance is not a limitation because in the HWGs the laser fills the complete volume of the cell, which increases the probability of interaction between the laser light and the molecules [4,6]. As an example, Charlton *et al.*[4] reported an effective increase in the sensitivity of their absorption system by a factor 1.2 when compared to their results obtained with a 3 meter long multipass cell. Taken together, when preconcentration can be combined with broadband quantum cascade laser-based spectroscopy with the use of HWGs, in order to reduce the equipment in size for practical use in a clinical setting, this might proof to be an attractive technique for exhaled breath profiling with the possibility to characterize the individual VOCs.

## Clinical application of exhaled breath analysis

The analysis of VOC profiles in exhaled breath, 'breathomics', is a potential tool in respiratory medicine. Yet, despite decades of research, 'breathomics' is still not incorporated in clinical practice, and the biological origin of most VOCs is largely unknown.

In **chapter 2** we summarized the methodological issues and clinical applications of exhaled breath and EBC analysis in children with respiratory diseases. Lack of standardization of the collection- and measurement procedures and differences in the selection of subjects probably explains the high variability and low reproducibility of exhaled

biomarkers between studies. Currently, analysis of biomarkers in exhaled breath and exhaled breath condensate (EBC) is still a research tool and not validated for clinical use. Several steps are required before exhaled breath analysis can be implemented into clinical practice [7,8]. First, there is a need for longitudinal studies investigating exhaled breath biomarkers in real life, to validate their diagnostic potential. Second, the lack of standard collection and analysis methods clearly hampers the validation that is necessary before clinical application. Standardization of both collection and analysis techniques will allow for pooling of data and will facilitate external validation. An attempt to standardize these factors between studies is currently underway by a European Respiratory Society Task Force and a collaborative open-source development project (www.breathe-free.org). Last, identifying the biological sources of individual VOCs, (e.g. systemic, upper or lower respiratory tract, environmental, instrumental) and knowledge of the factors that influence their levels in exhaled air may further improve their diagnostic accuracy.

**Future directions**

In clinical practice, diagnosis and monitoring relies on complex integration of different parameters, including clinical history, physical examination, functional tests, imaging, blood tests and histopathology. Integrating clinical knowledge with data from basic research is a complex process, but is now being developed as 'systems medicine' [9]. Current health care is moving from being reactive to preventive and the aim of system medicine is to intervene at an early stage to control symptoms and prevent or reduce future risks. Since many diseases have their origin in childhood, in other words during growth and development, the pediatric field can be a pioneer in the development of a systems medicine approach. This requires interdisciplinary collaboration between various disciplines who not always speak the same language, and integration of data from molecular and cell biology, physiology, clinical medicine, and epidemiology. In the near future, also personal health profiles acquired through e-health, wearable devices and health apps will potentially produce a wealth of personal data and contribute to individualized treatment and monitoring. Furthermore, a systems medicine approach stresses the need for computational and bioinformatics infrastructures to deal with proper management and interpretation of large scale multidimensional data sets and to incorporate the temporal dimension of both biological and clinical data. It will be a major challenge to select those patients who are likely to benefit from such a multidisciplinary systems medicine approach, and to educate clinicians to work with them and keep a keen eye on possible flaws and drawbacks.

As an example, in September 2015, a population-based study started in the Netherlands, using the spiroNose, a standardized method which integrates eNose technology with spirometry [10], with the primary aim to gather exhaled breath profiles in a central database together with anonymized patient characteristics. This database will be

coupled to a self-learning algorithm with an accessory app (www.breathcloud.org). This project uses a 'systems medicine' approach combining clinical data with lung function and breathomics. However, whether or not this kind of technically demanding approaches will pay off in terms of feasibility, discriminatory power, reproducibility and costs, remains to be shown. In order to bring forward metabolomics of exhaled breath and EBC, international research networks should urgently work together to standardize collection methods, analysis techniques and interpretation.

## BIOMARKERS FOR BRONCHOPULMONARY DYSPLASIA DEVELOPMENT: TWO TARGETED APPROACHES

The second part of this thesis focused on biomarkers in preterm born children with bronchopulmonary dysplasia (BPD). Currently, the two best established clinical risk factors for BPD are gestational age (GA) at birth and birth weight [11]. However, for an individual preterm born child it remains difficult to predict whether he or she will develop BPD. Therefore, there is a clear need to identify biomarkers or predictors that discriminate between preterm children who will or will not develop BPD. Such biomarkers may also give new insights in the pathophysiology of the disease and guide the design of new preventive and/or therapeutic strategies. The last decades various inflammatory biomarkers in TAs or blood have been investigated for their predictive value for BPD, but so far results have been negative or could not be reproduced [12]. Biomarkers could be detected in amniotic fluid, cord blood, blood and/or in samples from the respiratory tract, specifically bronchoalveolar lavage fluid (BALF) or TAs. In **chapter 5 and 6** we have investigated multiple biomarkers in TAs of preterm infants. We evaluated the levels of cytokines and growth factors (**chapter 5**), and sphingolipids (**chapter 6**) in TAs over time. This longitudinal aspect is a strength of our studies since lung development, injury and repair are dynamic processes. We focused on the first postnatal week, because we aimed to identify early markers of BPD with the main goal to enable the development of early intervention and prevention strategies.

## ROLE OF MACROPHAGE-DERIVED ANGIOGENIC GROWTH FACTORS IN BRONCHOPULMONARY DYSPLASIA

Histology of lungs from patients who died from BPD show impaired alveolarisation but also remarkable abnormalities in the pulmonary vascular network with a reduction in small arteries, abnormal distribution of capillaries in the distal lung, and intrapulmonary arteriovenous connections [13-15]. Disruption of microvascular development in very imma-

ture lungs has been postulated as a critical factor in the abnormal alveolar development in BPD [13,16,17]. Traditionally, the classical angiogenic pathways associated with vascular morphogenesis, such as vascular endothelial growth factor (VEGF) and Angiopoietin-1 (Ang-1), have been implicated in BPD pathogenesis [18]. Although VEGF was shown to be a key mediator of lung angiogenesis and alveolarisation in mice [19,20], human studies on the correlation between these factors and clinical outcome show more variable results [21,22]. Placenta-mediated pregnancy complications like pregnancy induced hypertension (PIH) and preeclampsia with fetal growth restriction have been found to be associated with BPD in very preterm infants independently of GA and birth weight [23]. Maternal blood levels of antiangiogenic factors like soluble fms-like tyrosine kinase 1 (sFlt-1) arising from the placenta are increased in PIH and preeclampsia [24]. These factors might also play a role in impaired angiogenesis in the fetus. Furthermore, in mice, overexpression of proangiogenic factors like placental growth factor (PlGF) resulted in increasing type II alveolar cell apoptosis causing enlarged airspaces and pulmonary emphysema similar to BPD pathology [25]. In humans, higher PlGF cord blood levels have been found in preterm infants who developed BPD [26]. Unfortunately PlGF was not analyzed in our studies. The current paradigm suggests that imbalanced circulating proangiogenic and antiangiogenic factors could impair vasculogenesis in fetal lungs, which may lead to general disorders in lung development. However, the signals linking alveolar and vascular growth in the developing lung are still not well understood.

In **chapter 5** we performed a targeted biomarker search within angiogenesis related factors using a proteome profiling assay. We measured multiple both classical and alternative growth factors known to be involved in physiological and aberrant angiogenesis. The proteome profiling assay is an easy method to quickly screen samples on the presence of multiple factors. However, this assay only allows semi-quantitative measurement and needs a lot of material (1.5 ml per membrane). Therefore in our study we had to pool multiple aliquots. Using this screening assay we identified the presence of multiple both classical and alternative angiogenic growth factors of which some were not described before in BPD. To further investigate differences between children with and without BPD we measured exact concentrations of a selected group of angiogenesis related growth factors, chemokines and cytokines in TAs of preterm born infants using a multiplex assay (Luminex) and ELISA. We found that TAs of preterm infants who do not develop BPD contain higher concentrations of M2-related chemokines, M1/pro-inflammatory cytokines and of a selection of proangiogenic factors including EMMPRIN, GDF15 and HGF, compared to TAs of infants who later developed severe BPD. The high expression of EMMPRIN [27], GDF15 [28], and HGF are of special interest since these factors promote angiogenesis via modulating hypoxia-inducible factor (HIF) family. In utero, lung development occurs under hypoxic conditions and HIF has shown to play an important role in branching of the epithelium and in the development of the pulmonary vasculature.

Under hypoxic conditions in utero, HIF activates proangiogenic factors including VEGF and Ang 1 and 2 [29]. After birth the hypoxic environment is acutely exchanged for a normoxic and in case of preterm birth often hyperoxic environment causing acute downregulation of HIF. The presence of high numbers of HIF inducing factors such as GDF15 and EMMPRIN may therefore play an important role in preservation of postnatal angiogenesis independent of oxygen tension.

We next studied cell number and phenotype present in TAs. Differential cell count on cytospins showed a significant different distribution of epithelial cells, granulocytes and monocytes/macrophages over time. The monocytes/macrophages were abundantly present in TAs of preterm infants especially at later time points, day 5 and 7 after intubation. We found that the macrophages in TAs express both CD68 and intermediate levels of CD14, and therefore recently differentiated from monocytes. Subsets of monocytes and macrophages, which express so-called M2 markers, are well-known for their functions in angiogenesis. These subsets were consistently higher in TAs of children without BPD than of those with severe BPD. Of interest M2 macrophages have been found in neonatal lungs at the tips of alveolar septa where they seem to guide branching morphogenesis suggesting a role in ensuring normal lung growth and development [30]. We therefore formulated a new hypothesis: M2-proangiogenic macrophages and their products protect preterm born infants from BPD development. To test this hypothesis we set up a more mechanistic study and investigated monocytes/macrophages in the lungs of neonatal mice during the alveolarisation phase of lung development using flowcytometry, RT-PCR and functional studies. We found that neonatal pulmonary monocytes during alveolarisation display a similar gene expression profile as embryonic macrophages and/or tumor-associated macrophages (TAMs) with strong gene expression of M2-related factors, pro-inflammatory cytokines and proangiogenic genes. This indicates that M2 immunity is a default immune state during postnatal lung development and that the M2 profile in TAs of children without BPD reflects the normal microenvironment in the lung during postnatal alveolarisation, although a more efficient and active response to injury is not excluded. An improved understanding of the role of these monocytes/macrophages under homeostatic and inflammatory conditions is of clinical relevance. It supports research into modulation of monocytes and macrophages and their derived products in the context of lung development to provide new future prevention or treatment modalities for BPD.

### Role of sphingolipids in bronchopulmonary dysplasia

The sphingolipid metabolism is suggested to be involved in various lung diseases [31]. Sphingolipids are important structure-bearing constituents of the cell membrane and function as regulatory molecules in cell proliferation and cell death, endothelial barrier function, angiogenesis, and immune response [32,33]. Previously, we observed a transient

increase in ceramide concentrations in BALF of a mouse model of BPD during the first 2-4 weeks of hyperoxia. Supplementation of D-sphingosine, a synthetic precursor of sphingosine-1-phosphate (S1P), during normoxic recovery of hyperoxia-induced lung damage accelerated normalization of ceramide concentrations and improved the hyperoxia-induced alveolar arrest in this mouse model [34]. These findings suggest that ceramides are involved in the development of BPD and might be early biomarkers for BPD, and a target for therapeutic intervention. We translated our findings from this preclinical study into a human exploratory study and showed that ceramide profiles were indeed detectable in TAs of preterm infants, changed over time, and were significantly different between infants who did and did not develop BPD (**chapter 6**). However, a multivariable analysis showed that ceramide profiles over time had no additional predictive value over known clinical predictors of BPD like GA at birth, birth weight, and total days of mechanical ventilation. An increased initial ceramide-triggered apoptotic signal seemed to be present in all preterm infants exposed to mechanical ventilation and hyperoxia. Furthermore, a late increase in ceramides was present in those preterm infants who did not develop BPD, suggesting that this increase might function as a proliferation signal. These results suggest a potential role of ceramides in BPD development. It is therefore worthwhile to further investigate the role of ventilation- and hyperoxia-induced ceramide production in epithelial apoptosis as a mechanism playing a role in pulmonary apoptosis and inhibition of alveolar development in preterm infants with BPD. Further research should also focus on discovering which enzymes in the sphingolipid pathway are responsible for the differences in ceramide levels. Specific enzyme inhibitors may be studied in mouse models for their potential to prevent hyperoxia induced lung injury, like acid sphingomyelinase inhibitors have shown to normalize ceramides and inflammation in cystic fibrosis [35].



**New potential pathways in BPD development**

In **chapter 5 and 6** of this thesis we described potential new pathways playing a role in BPD development. The pathogenesis of BPD is heterogeneous and complex and involves interaction among many genes, proteins and their environment. Interestingly, cytokines, growth factors (**chapter 5**) and sphingolipids (**chapter 6**) have been shown to interfere in the regulation of S1P. Numerous cytokines and growth factors are able to influence the sphingolipid signaling pathway, mainly through regulation of either the expression or activation of sphingosine kinase [36]. For example, endothelin-1 is able to activate the sphingomyelin pathway, thereby increasing the amount of ceramides and subsequently induce apoptosis [37]. On the contrary, both hepatocyte growth factor (HGF) and epidermal growth factor (EGF) attenuated ceramide-induced apoptosis [38,39].Conversely, S1P can also trans activate growth factor and cytokine signaling cascades [40-42]. For example, an increase in ceramides disrupted the S1P/ceramide homeostasis and resulted in a re-

duction of HIF-1α and VEGF expression, inducing lung cell apoptosis, whereas increased S1P restored the expression of HIF-1α and VEGF and prevented lung cell apoptosis in a rat model [42]. In addition, inhibition of sphingosine kinase 1(SphK1) blocked TNF-α induction of IL-8 in lung epithelial cells, also supporting a role for the interaction between sphingolipids and cytokines in the initiation of lung inflammation [43]. Furthermore, it has been shown that S1P regulates macrophage function [44]. Recently, a potential link between S1P signaling system and defective monocyte-derived or alveolar macrophage phagocytic function and resultant chronic inflammation was suggested in patients with COPD [45]. In **chapter 5** we also found a major role for alternatively activated monocytes and monocyte-derived macrophages and their produced cytokines, chemokines and growth factors in BPD development. These interactions between S1P, macrophages and their derived cytokines, chemokines and growth factors may also potentiate an effect on angiogenesis and alveolarisation in the developing lung.

Our results described in **chapter 5 and 6** consistently suggest a role for early apoptosis and late proliferation in the development of BPD. However, the alterations in expression of these factors that we observed in patients who developed BPD do not necessarily imply causality. Furthermore, the presence of these factors in TAs might not be a direct reflection of mechanisms occurring in the alveoli or lung tissue. Currently, studies examining lung tissue of BPD patients are scarce and only involve postmortem studies [46]. Endobronchial biopsies have been shown to be feasible and safe in children with asthma [47]. However, endobronchial biopsies in children with BPD will probably not contribute to further understanding of the underlying pathology since the structural abnormalities are present in the peripheral part of the lungs. For this purpose more postmortem studies in preterm infants who died because of respiratory insufficiency are needed. To gain better insight in the regulation of inflammation, angiogenesis, apoptosis and proliferation, and their effects and interactions during normal and impaired lung growth as seen in preterm birth we still depend on *in vitro* models and animal studies.

*In vitro* models like pericyte-endothelial cell co-cultures (**chapter 5**) can be used to study the mechanisms by which the identified factors affect angiogenesis and/or epithelial repair. In addition, wound healing studies can be used, where human lung epithelial cells are grown in a monolayer and scratched to create a 'wound' to monitor through time-lapse microscopy the speed of wound closure in the presence of the introduced factors. In addition, epithelial cell proliferation assays can be performed to indicate whether observed effects are due to proliferation or migration. Besides these known *in vitro* models the laboratory of Pediatric Surgery of the Erasmus MC (dr. R.J. Rottier and dr. I.M. de Kleer) are currently involved in a consortium with the University of Twente (dr. A.A. Poot and prof. dr. D.F. Stamatialis), Maastricht University (dr. R.K. Truckenmuller) and Leiden University Medical Centre (prof. dr. P.S. Hiemstra) collaborating on the development of micro engineered 3D alveolar tissue using organ-on-a-chip technology, to

create a 'lung-on-a-chip'. In contrast to currently used 2D lung models, lung cells in this model are cultured on curved membranes, allowing exposure to air and liquid flow as well as mechanical forces. The model allows alveolar epithelial cells to be grown on one side of the porous membrane and microvascular endothelial cells on the other side. They are currently successful in growing alveolar cell lines into the lung-on-a-chip device. In the near future they aim to add human umbilical vein endothelial cells (HUVECs) and to use human induced pluripotent stem cells to generate both alveolar and endothelial cells for implementation. This lung-on-a-chip model can also be used to explore the effects of the identified growth factors on lung tissue repair. Finally the therapeutic potential of the most promising growth factors and their sources can only be studied in animal models. However current animal models for BPD, like the hyperoxia mediated mouse model, need further refinement to better mimic the human condition. Current animal models have relatively normal lungs, while the lungs of preterm born infants are exposed to intrauterine inflammation, growth abnormalities, antenatal corticosteroids and postnatal effects of for example mechanical ventilation, oxygen supplementation, persistent ductus arteriosus and infections.

**Current clinical outcome of preterm born infants with severe BPD**

The improved survival of extremely preterm born infants (< 28 weeks GA) results in an increasing incidence of BPD. Survivors of preterm birth and BPD have an increased risk for long term respiratory morbidity. These children experience more respiratory symptoms, such as wheeze, cough, dyspnea, and reduced exercise capacity, than children without BPD [48-51]. Furthermore, severe BPD has been associated with persistent pulmonary hypertension in preterm infants [52,53] and severe BPD may be a risk factor for developing pulmonary hypertension at a later age [54]. Also, neurological and cognitive impairment persisting into adulthood are common in preterm infants with BPD [55]. Chest computed tomography (CT) studies show structural abnormalities in up to 85% of BPD patients (**chapter 7**). A recent meta-analysis showed that preterm infants with BPD have lower forced expiratory volume in 1 s ($FEV_1$) compared to children without BPD [56]. However, despite this significant morbidity and physical limitations adolescents with BPD experience a quality of life similar to that of healthy controls, which is in contrast to adolescents with asthma [57]. Most of the earlier studies included children who were born before 1990 and do not reflect todays BPD population. Furthermore, the relation between structural abnormalities of the lungs and airways and lung function in patients with BPD early and later in life is not clear. Since lung function tracks throughout life, it is important to recognize both structural and functional impairment early in life in order to identify those at risk for future respiratory morbidity, and to develop targeted preventive strategies. Therefore, there is a need for long-term follow up of preterm born infants and since children with BPD might have a higher risk for early development of

COPD-like disease in adulthood, we have the obligation to follow these children into adulthood and guarantee transition to a pulmonologist.

### The use of chest CT as a possible outcome measure for bronchopulmonary dysplasia

Chest CT is the most sensitive imaging technique to detect structural abnormalities in patients with BPD [58] and has been used as the primary endpoint in clinical studies on cystic fibrosis. In BPD there are no standardized universal CT scoring systems, and therefore we developed such a standardized CT protocol and quantitative scoring method, the PRAGMA-BPD scoring method (**chapter 7**). Our PRAGMA-BPD scoring method requires validation, including assessing intra-observer and inter-observer reliability, cross validation to other outcomes like quality of life and lung function, and standardization within and between different centers, before it can be used for future clinical trials. The ultimate goal of such a quantitative scoring system for BPD would be to improve clinical and pathophysiological phenotyping of individual patients, to predict long term respiratory morbidity, and to help developing new preventive measures to optimize lung development in early life. In our follow up study (**chapter 8**) we found structural lung abnormalities in almost all preterm infants with severe BPD at 6 months corrected age. However, we also found that overall around 90% of the total lung volume was still scored as normal, which can explain the relatively good lung functions found in some studies and the reported high quality of life. Still, BPD is a heterogeneous disorder and there are also a substantial number of preterm born children with BPD who do have severe structural damage and experience severe respiratory complications.

### Structural and functional abnormalities in infants with severe BPD

In **chapter 8** we present the first results of our prospective hospital-based cohort which includes preterm infants born ≤ 32 weeks GA diagnosed with severe BPD from September 2013 onwards. These children will be followed from birth until adulthood by a multidisciplinary team of pediatric specialists and undergo several investigations including chest CT scans to assess structural abnormalities and lung function tests to evaluate functional abnormalities (Table 1). In **chapter 8** we present data from 49 infants in whom we assessed lung structure on chest CT scans using the PRAGMA-BPD scoring system and performed overnight polysomnography (PSG) at 6 months corrected age.
We found that 95.5% of the infants had abnormal lung structure at 6 months corrected age. Although almost all infants with severe BPD in our study showed architectural distortion, the amount of normal lung volume was still quite high, namely 89.7 (85.6-93.2)%. However, some children had less than 45% normal lung volume. In the future chest CT may be used as an outcome measure for BPD. It is important to recognize abnormal lung structure early in life in order to identify those children with the most

severe disease, who are at risk for future severe respiratory morbidity and should be monitored more strictly.

We found that 74% of the infants had an abnormal oxygen desaturation index (ODI) at 6 months corrected age. Our PSG results are consistent with previous studies [59] and indicate that infants with severe BPD might be more vulnerable for rapid desaturation during apneas, perhaps because of decreased ventilatory reserve. We speculate that increased ODI may be unfavorable for lung development, growth and neurocognitive development and suggest overnight PSG registration before cessation of supplemental oxygen.

Associations of lung structure measures with ventilatory function were only weak and disappeared after correction for GA. Also in CF studies weak correlations between lung structure and lung function were shown, probably due to the localized and inhomogeneous aspect of the disease. In BPD we find a similar inhomogeneous picture. Furthermore, lung imaging techniques are not able to detect functional changes and lung morphometrics are probably not sensitive enough to demonstrate relative small structural changes. Multiple breath washout techniques, body plethysmography and compliance measurements might be more sensitive to pick up subtle changes in lung function.

Our data showed that especially GA at birth influences both lung structure and ventilatory function at later age. Preterm infants are born when lung development is still in the canalicular or saccular stage, characterized by vascular and alveolar simplification. This suggests that poor lung development at the time of preterm birth and during exposure to mechanical ventilation and supplemental oxygen is crucial for the development of BPD.

Finally, in addition to higher GA at birth also increased early postnatal growth positively influenced both the total and normal lung volume, and ventilatory function reflected in less severe oxygen desaturations in preterm infants with severe BPD. These results suggest that postnatal alveolar growth is positively influenced by overall weight gain. The underlying mechanisms for the associations between infant weight growth and lung structure and function are unclear. The lower lung function might be explained by smaller airways in preterm born infants. Another possible explanation for the observed associations between infant weight gain and lung structure and function is low grade systemic inflammation, associated with obesity. Both the increased amount of pro-inflammatory cytokines producing macrophages present in adipose tissue and the adipokines and leptin secreted by adipose tissue itself could lead to this systemic low grade inflammation [60]. Recently, it was shown that a coordinated expression of pro-inflammatory cytokines like interleukin (IL)-6 and tumor necrosis factor (TNF)-α plays a protective role and might positively influence lung maturation [61-63]. In **chapter 5** we also found higher concentrations of pro-inflammatory cytokines in preterm infants pro-

tected from BPD, which is in line with this hypothesis. Besides the influence of postnatal growth it would be interesting to evaluate whether intrauterine growth patterns are also associated with lung structure and function impairment. It has recently been found that fetal growth restriction, more than birth weight, predisposes for impaired lung development [23]. Furthermore, the question remains whether postnatal growth would have been better when prolonged supplemental oxygen was given to these preterm born infants with a positive influence on both total and normal lung volume and ventilatory function at 6 months corrected age.

Our prospective cohort provides the opportunity to follow preterm born infants with severe BPD until adult age in a multidisciplinary team, assessing both lung structure and respiratory function and their clinical consequences over time. Detailed information on prenatal and postnatal environmental factors is collected prospectively. These data allow us to gain more insight in structural and functional lung development of preterm infants with severe BPD and the influence of postnatal growth until adulthood. When we will continue to track these individuals trough their adult life's, in the future we might be able to answer that question whether today's children with severe BPD will grow up to be the early COPD-like patients of tomorrow.

## Future directions

BPD is a serious complication of preterm birth with long-term respiratory morbidity.

BPD is a multifactorial disease, but the exact pathophysiology is still unclear. Therefore, for an individual preterm born child it remains difficult to predict whether he or she will develop BPD. A lot of different pathways seem to be involved in postnatal alveolarisation, including growth factors involved in angiogenesis, inflammatory cells and cytokines, and the sphingolipid pathway. Biomarker research may lead to more insight in the underlying pathophysiology of BPD and may guide the design of new preventive and/or therapeutic strategies.

The most important factor in the prevention of BPD is the prevention of preterm birth and prenatal etiological factors for preterm birth like pregnancy induced hypertension (PIH), preeclampsia and chorio-amnionitis. Since GA at birth, birth weight and intrauterine growth restriction are important clinical risk factors for BPD development, it would be interesting to investigate prenatal treatments which interfere with poor placentation. Currently, there is no treatment for poor placentation once it has developed. A few promising interventions are now emerging such as nitric oxide donors (organic nitrates, S-nitrosogluthathione), sildenafil citrate, maternal VEGF gene therapy injected locally into the uterine arteries, hydrogen sulphide donors, statins and plasma exchange or plasmapheresis [64]. In the absence of real treatment, current obstetric practice focuses on prevention of poor placentation and preterm birth. For example, maternal smoking has been associated with preterm birth and subsequently BPD development [65] and

smoke-free legislation has been shown to be associated with a 10% reduction in preterm births [66]. Studies in mice have suggested that also maternal diet, mainly a high fat diet, during pregnancy can cause placental inflammation and result in alveolar simplification, thereby increasing the risk of BPD development [67].

Besides prevention of preterm birth, a lot of research focused on postnatal measures to prevent BPD development. Since invasive mechanical ventilation is one of the major contributors to the development of BPD in preterm infants less invasive ventilation was thought to reduce the incidence of BPD [68]. A recent trial of CPAP instead of invasive mechanical ventilation in the first day of life resulted in a significant reduction of BPD or death of very low birth weight infants [69]. Other strategies of gentle ventilation include intubation solely to administer surfactant, early extubation and nasal intermittent mandatory ventilation. Given the fact that less invasive ventilator strategies are often successful, TAs will not be feasible anymore to obtain and alternative methods to sample the newborn airways, like exhaled breath and EBC, should be explored.

The introduction of antenatal corticosteroids have shown positive effects on lung development: reducing inflammation and stimulating surfactant synthesis and lung epithelial differentiation in the developing lung [70]. Postnatal dexamethasone has been used for some time to prevent BPD development, however, in 2001 a meta-analysis revealed a relation between the use of early dexamethasone and poor neurodevelopmental outcome, especially cerebral palsy [71]. This resulted in a general concern about the use of steroids in preterm infants even for late targeted use. Recently, animal data have suggested that hydrocortisone might have a less detrimental effect on the brain than dexamethasone [72]. Retrospective studies seem to indicate that hydrocortisone is indeed effective in reducing BPD, without causing serious adverse effects in humans [73]. Currently, a randomized double-blind placebo-controlled trial investigating if hydrocortisone is safe and effective in reducing the incidence of the combined outcome death or BPD at 36 weeks PMA in chronically ventilated preterm infants is conducted in 15 NICUs in the Netherlands and Belgium (SToP BPD trial, www.neonatologiestudies.nl/stopbpd/). Also local administration of budesonide by either intratracheal instillation or inhalation has been studied in the prevention of BPD [74]. Animal models confirmed that local delivery of budesonide may have favorable effects on the developing lung without the neurodevelopmental side effects [75].

Studies on how to treat children with established BPD are scarce and current treatment options are limited and symptomatic. Ideally, a new treatment for BPD should promote postnatal lung growth and target the repair of lung injury. There are a number of interesting emerging therapies focusing on treatment for BPD:

First, stem cell therapy: stem cellls, primitive multipotent cells with the ability to self-renew and give rise to several differentiated cellular phenotypes, are currently a 'hot topic' in BPD research. Over the last years, animal studies using mesenchymal stem

cells (MSCs) as treatment for BPD showed positive results, which have led to the first trial in humans [76]. In this study no serious adverse events or acute toxicity of stem cell treatment were observed. Currently, a major problem is the lack of standardization of preparing MSCs populations which makes it difficult to compare studies. Data on long term outcome of stem cell therapy are lacking.

Second, microbiome: another new field of research is exploring the role of the respiratory microbiome, the community of micro-organisms colonizing the upper and lower airways, in respiratory diseases. Molecular methods such as 16S rRNA gene sequencing provide improved sensitivity to detect micro-organisms compared with traditional culture-based techniques [77]. Current evidence shows that the colonization of the airways begins very early in life, probably already in utero [78]. Decreased diversity of the respiratory microbiome in preterm infants has been associated with BPD [79]. The microbiome might influence the development of BPD by stimulating pro- or anti-inflammatory responses. Prophylactic azithromycin therapy was found to be associated with a significant reduction in BPD in preterm infants [80]. However, further studies are needed to provide more information on pharmacokinetics and potential harmful effects, before routine use of azithromycin in the neonatal population. Most likely, the respiratory microbiome is influenced by the same environmental factors that have been shown to influence the gut microbiome like delivery mode, breastfeeding and the use of antibiotics [81]. Further studies are needed to improve our understanding about the bacteria-host interactions playing a role in BPD development.

In BPD research, the movement of potential preventive and therapeutic measures from bench to bedside is complicated due to the difficulty of extrapolating animal data to human preterm infants, the complex multifactorial etiology, and the fact that most human studies are of small sample size [82]. In this respect, a biobank may be extremely helpful. Collection of maternal blood, placental material, amniotic fluid, umbilical cord blood, and blood, urine and TAs obtained from the infants after birth, together with prospective clinical data regarding pregnancy, delivery and postnatal environmental factors could contribute to future research of BPD prevention. There is also a need for adequate long-term clinical follow-up studies of preterm infants. Due to treatment changes and increasing survival over time, outcome will constantly change, necessitating the continuous collection of new data in order to evaluate the long-term outcome of present treatment strategies. In addition, careful documentation of the clinical evolution might facilitate the identification of different phenotypes and the association with (epi)genetic markers [83-86].

To summarize, biomarkers may help in the diagnosis, monitoring and management of respiratory diseases in childhood. The continuous search for new biomarkers as part of a systems medicine approach may help to identify new pathways and possibly develop new treatments. There is a need for integration of basic and clinical data requiring interaction between various disciplines. Last, long term follow-up from childhood until adulthood is needed to document the course of respiratory diseases, and unravel the influence of preventive measures, treatment, and exposures.

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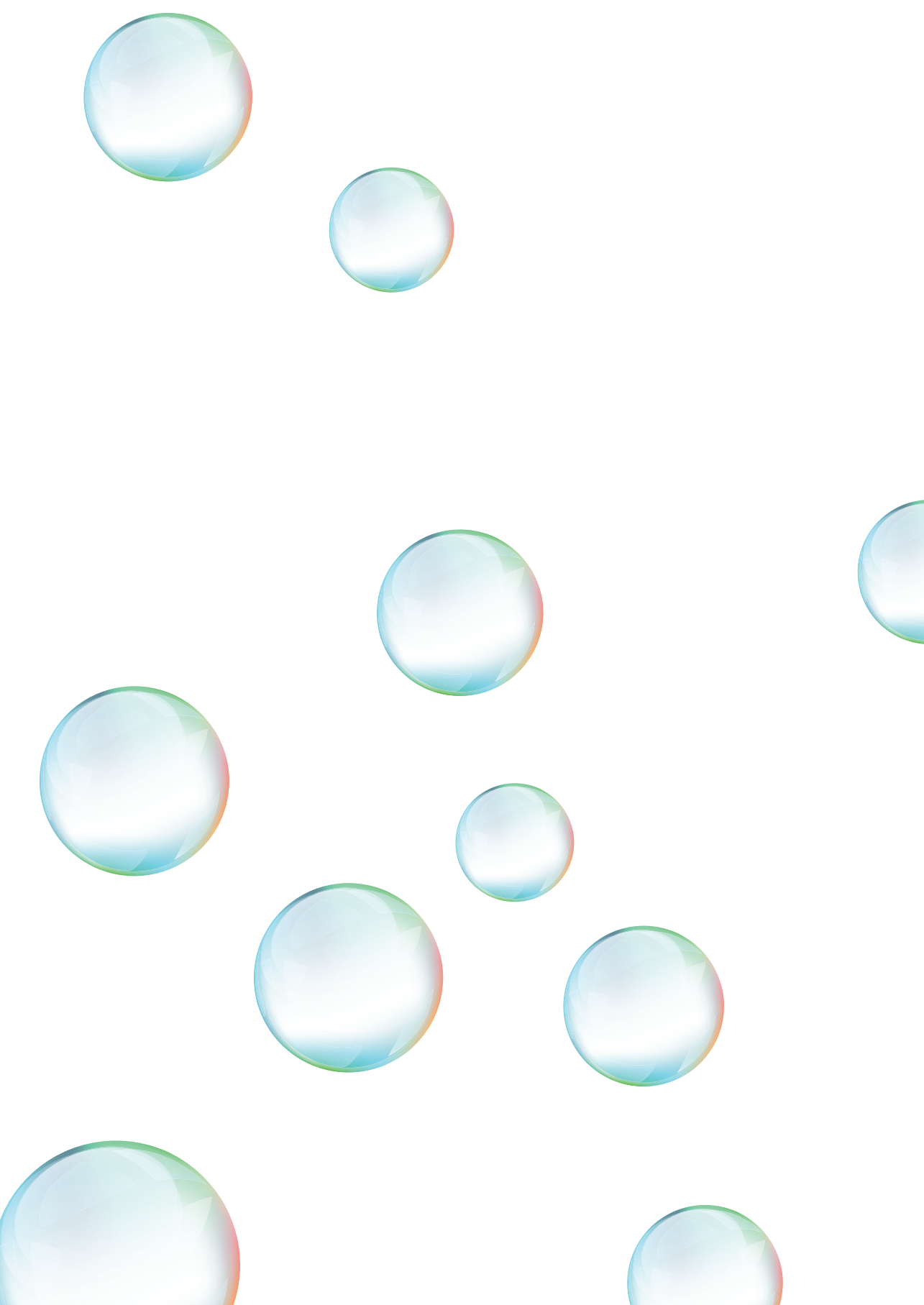


# 10 Summary
# Samenvatting

## SUMMARY

Today's children will grow up to be the adults of tomorrow and respiratory diseases in children and their management will have impact on adult lung health. Biomarkers can play a role in the prediction and early diagnosis of respiratory diseases, but can also contribute to gaining more insight in underlying pathophysiology, disease phenotyping and monitoring of disease progression and treatment effect. In this thesis, we focused on these different aspects with the ultimate goal to improve the outcome of pediatric respiratory diseases.

### Asthma and cystic fibrosis: exhaled breath analysis with laser-based spectroscopy

Previous studies on exhaled breath analysis in diagnosis and management of children with respiratory disorders are reviewed in **chapter 2**. In this review methodological issues and clinical applications of exhaled breath and exhaled breath condensate (EBC) analysis in children with respiratory diseases are discussed. Methodological issues can be divided in precollection (e.g. physiological and environmental factors), collection (e.g. breathing maneuvers and devices), and postcollection (e.g. storage and analysis techniques) conditions. Lack of standardization of the collection- and measurement procedures and differences in the selection of subjects probably explain the high variability and low reproducibility of exhaled biomarkers between studies. Currently, besides the fraction of exhaled nitric oxide (FeNO), analysis of biomarkers in exhaled breath and EBC is still a research tool and not validated for clinical use.

In **chapter 3** we present a new technique to detect volatile organic compounds (VOCs) in exhaled breath: broadband quantum cascade laser-based spectroscopy. This technique was developed in cooperation with the Optics research group at the Delft University of Technology, the Netherlands. Broadband quantum cascade laser-based spectroscopy allows for detection of VOCs in exhaled breath samples, with an absorption spectrum in a specific, broad wavelength spectrum. Advantages of this technique over the most commonly used gas chromatography-mass spectrometry (GC-MS) technique are especially the limited need for preprocessing and the relatively fast detection without the need for highly trained personnel. The main advantage over the use of sensor-based detection methods, like the electronic nose (eNose), is the possibility of VOC identification with the use of molecular databases.

The pilot study described in **chapter 4** explored the clinical applicability of this new technique in healthy children and children with asthma and cystic fibrosis (CF). We found overall poor short- and long term repeatability. However, despite the variability of the spectra we were still able to detect differences between exhaled breath profiles from healthy children and from children with chronic airways inflammation due to asthma or

CF. We propose that, before further clinical studies can be undertaken, the sensitivity and repeatability of this technique need to be improved.

### Bronchopulmonary dysplasia: early markers in tracheal aspirates and clinical follow up

The improved survival of extremely preterm born infants (< 28 weeks gestational age, GA) may result in an increasing incidence of bronchopulmonary dysplasia (BPD). In Europe, the current incidence of BPD ranges from 4% in infants born at a GA of 31 weeks to 56% in infants born before 26 weeks GA. Children with BPD are at increased risk of long-term respiratory morbidity as a result of impaired lung development. The exact pathogenesis of BPD remains unclear and treatment modalities are limited and only treat symptoms. Altogether, it is important to gain more insight in the pathophysiology of this chronic lung disease. Identification of biomarkers for BPD development may result in future early prediction and diagnosis and the development of new prevention and intervention strategies. In addition, since BPD is a heterogeneous disease it is also important to recognize both structural and functional impairment early in life and identify those children most at risk for future respiratory morbidity.

In **chapter 5 and 6**, we investigated potential biomarkers in tracheal aspirates (TAs) of preterm born infants with and without BPD. Recently, disruption of microvascular development in very immature lungs has been postulated as a critical factor in the abnormal alveolar development in BPD. In **chapter 5** we used a proteome profiling and Luminex assay to measure multiple growth factors, cytokines and chemokines in TAs of preterm infants. We found higher levels of several M2-type macrophage-related angiogenic growth factors and pro-inflammatory cytokines in serial TAs of preterm infants who did not develop BPD. Furthermore, we found significantly higher gene expression of the M2-related genes MRC1 and CD206 in infants without BPD. Our findings support a role for M2-like macrophages in angiogenesis and tissue homeostasis during alveolarisation. We speculate that M2 macrophages and their products may be potential therapeutic targets for prevention and treatment of BPD. In **chapter 6** we focused on a specific set of sphingolipids in TAs of preterm infants using liquid chromatography tandem mass spectrometry (LC-MS/MS). Previous studies in mice indicated the importance of ceramides in inflammation and repair processes in the developing lung. We found that an early increase and subsequent decrease in ceramides in TAs of preterm infants exposed to mechanical ventilation and supplemental oxygen seems to predispose for BPD development. Therefore, ceramide profiles in TAs may be a new early marker for BPD and the ceramide pathway in general might also be a potential new target for future prevention or treatment of BPD.

In addition, we evaluated the results of an improved clinical monitoring program for preterm children with severe BPD. Chest computed tomography (CT) scans and poly-

somnography have been studied as 'alternative biomarkers' that may predict outcome of BPD. In **chapter 7** we reviewed the literature regarding chest CT studies in patients with BPD. We found that structural abnormalities are present in up to 85% of BPD patients and the extent of these abnormalities seem to correlate with BPD severity and lung function. There are no standardized universal CT scoring systems available for assessment of structural pulmonary abnormalities in BPD patients. Therefore, we developed such a standardized CT protocol and quantitative scoring method, the PRAGMA-BPD scoring method, with the primary aim to enable the use of chest CT scans as a clinical outcome measure and predictor for long-term structural abnormalities and respiratory morbidity in BPD patients.

In **chapter 8** we described the results of chest CT scans and polysomnography measurements in children with severe BPD at 6 months corrected age and showed that the extent of structural abnormality was associated with ventilatory function impairment during sleep. Both higher GA at birth and higher birth weight were associated with better lung structural outcomes and ventilatory function. Also, increased weight gain between 2 and 6 months of corrected age was associated with better oxygenation and weight at 6 months was independently associated with more healthy lung volume and less severe oxygen desaturations during sleep.

In **chapter 9** the main findings of our studies and their scientific and clinical implications are discussed and recommendations for future research are given. We conclude that biomarkers may be helpful not only in the diagnosis, monitoring and management of respiratory disease in childhood, but also in identifying new pathways which may eventually result in new preventive or therapeutic measures. Furthermore, long-term follow up from childhood until adulthood is necessary in order to document the course of respiratory diseases, and to evaluate the influence of preventive measures, treatment, and exposures. In order to reach these goals there is a need for integration of basic and clinical data requiring collaboration and interaction of various disciplines.

## SAMENVATTING

Aandoeningen van longen en luchtwegen op kinderleeftijd en de behandeling daarvan kunnen van grote invloed zijn op de gezondheid van volwassenen. Biomarkers, of wel meetbare indicatoren van een bepaalde biologische toestand of ziekte, kunnen helpen bij het stellen van een vroege diagnose en bij het monitoren van longziekten. Daarnaast kunnen biomarkers bijdragen aan de kennis over de pathofysiologie van longziekten en daarmee ook aan nieuwe behandelingen. In dit proefschrift worden deze verschillende aspecten van het gebruik van biomarkers toegepast met als doel het verbeteren van de toekomst van kinderen met longziekten.

### Astma en taaislijmziekte: analyse van uitademingslucht met behulp van laser spectroscopie

**Hoofdstuk 2** geeft een overzicht van de literatuur op het gebied van het gebruik van uitademingslucht voor het diagnosticeren en behandelen van kinderen met longziekten. In dit hoofdstuk worden de methodologische uitdagingen en klinische toepasbaarheid van uitademingslucht en -condensaat (afgekoelde druppeltjes uitademingslucht) bediscussieerd. Methodologische uitdagingen kunnen worden onderverdeeld in uitdagingen vóór de verzameling van uitgeademde lucht en -condensaat (zoals fysiologische en omgevingsfactoren), tijdens de verzameling (zoals ademhalingsmaneuvers en verzamelapparatuur) en na de verzameling (zoals opslagmethoden en techniek van analyseren) gerelateerde condities. De hoge variabiliteit en lage reproduceerbaarheid van de resultaten van huidige studies kunnen worden verklaard door het gebrek aan standardisatie van verzamel- en analysemethoden en de selectie van proefpersonen. Momenteel is behalve het meten van de fractie van stikstofmonoxide (FeNO), de analyse van biomarkers in uitademingslucht en -condensaat niet gevalideerd voor gebruik in de dagelijkse praktijk.

In **hoofdstuk 3** beschrijven we een nieuwe techniek om vluchtige organische componenten (VOCs) in uitgeademde lucht te meten: breedband quantum cascade laser spectroscopie. Deze techniek maakt gebruik van het feit dat elk molecuul licht van een bepaalde specifieke golflengte absorbeert. De opstelling werd ontwikkeld in samenwerking met de afdeling Optica van de Technische Universiteit Delft, Nederland. Met behulp van deze breedband laser spectroscopie techniek is het mogelijk om een breed scala aan VOCs te detecteren en te identificeren in uitgeademende lucht. Voordelen van deze nieuwe techniek over de huidig meest toegepaste techniek, gas chromotografische scheiding van de verschillende componenten en detectie met behulp van massa spectrometrie (GC-MS), zijn dat voorbehandeling van de monsters niet nodig is, en dat de techniek relatief snel en makkelijk is. Het voordeel ten opzichte van het gebruik van de

electronische neus (eNOse), is de mogelijkheid tot het identificeren van de verschillende VOCs door gebruik te maken van moleculaire databanken.
Het onderzoek beschreven in **hoofdstuk 4** laat de eerste resultaten zien van de klinische toepasbaarheid van deze nieuwe techniek in het detecteren van VOCs in uitgeademde lucht van gezonde kinderen en kinderen met astma en taaislijmziekte. Er bleek sprake van een matige reproduceerbaarheid op zowel de korte als de lange termijn. Ondanks de variabiliteit waren we wel in staat verschillen te detecteren tussen de uitgeademde lucht van gezonde kinderen en kinderen met chronische luchtwegontsteking als gevolg van astma of taaislijmziekte. Echter, de sensitiviteit en de reproduceerbaarheid van deze techniek moet worden verbeterd, alvorens er klinische vervolgstudies kunnen worden gedaan.

### Bronchopulmonale dysplasie: vroege biomarkers in luchtwegaspiraten en klinische follow up

De incidentie van bronchopulmonale dysplasie (BPD) is de laatste jaren niet gedaald, ondanks betere behandelmethodes van te vroeggeboren kinderen. Dit komt waarschijnlijk doordat meer extreem vroeg geboren kinderen (< 26 weken zwangerschapsduur) overleven. De huidige Europese incidentie van BPD varieert tussen 4% van de kinderen geboren bij een zwangerschapsduur van 31 weken, tot 56% van de kinderen geboren bij een zwangerschapsduur van 26 weken. Kinderen met BPD hebben een hoger risico op lange termijn longgerelateerde problemen ten gevolge van een afwijkende longontwikkeling. De exacte pathogenese van BPD is tot nu toe onduidelijk en behandeling is beperkt tot het bestrijden van symptomen. Het is belangrijk om meer inzicht te krijgen in het ontstaan van deze chronische longziekte. Het gebruik van biomarkers kan bijdragen aan een vroege detectie van te vroeggeboren kinderen die een hoger risico hebben op het ontwikkelen van BPD, en mogelijk ook in de ontwikkeling van nieuwe preventieve en behandelstrategieën. Daarnaast is het belangrijk om zowel structurele als functionele longafwijkingen vroeg in het leven vast te stellen en daarmee de kinderen met het hoogste risico op longproblemen in het latere leven te identificeren.
In **hoofdstuk 5 en 6** hebben we potentiële biomarkers onderzocht in luchtwegaspiraten van te vroeggeboren kinderen met en zonder BPD. Recent werd geopperd dat een verstoorde ontwikkeling van het vaatbed in onderontwikkelde longen een kritische factor is in het ontstaan van een abnormale ontwikkeling van longweefsel zoals gezien wordt bij BPD. In **hoofdstuk 5** hebben we gebruik gemaakt van proteome profiling, Luminex en ELISA technieken om multipele groeifactoren, chemokines en cytokines die van invloed kunnen zijn op vaatontwikkeling te meten in luchtwegaspiraten van vroeggeboren kinderen. Te vroeggeboren kinderen die geen BPD ontwikkelen bleken hogere waardes van M2-type macrofagen gerelateerde groeifactoren en cytokines in luchtwegaspiraten te hebben dan te vroeggeboren kinderen die wel BPD ontwikkelen.

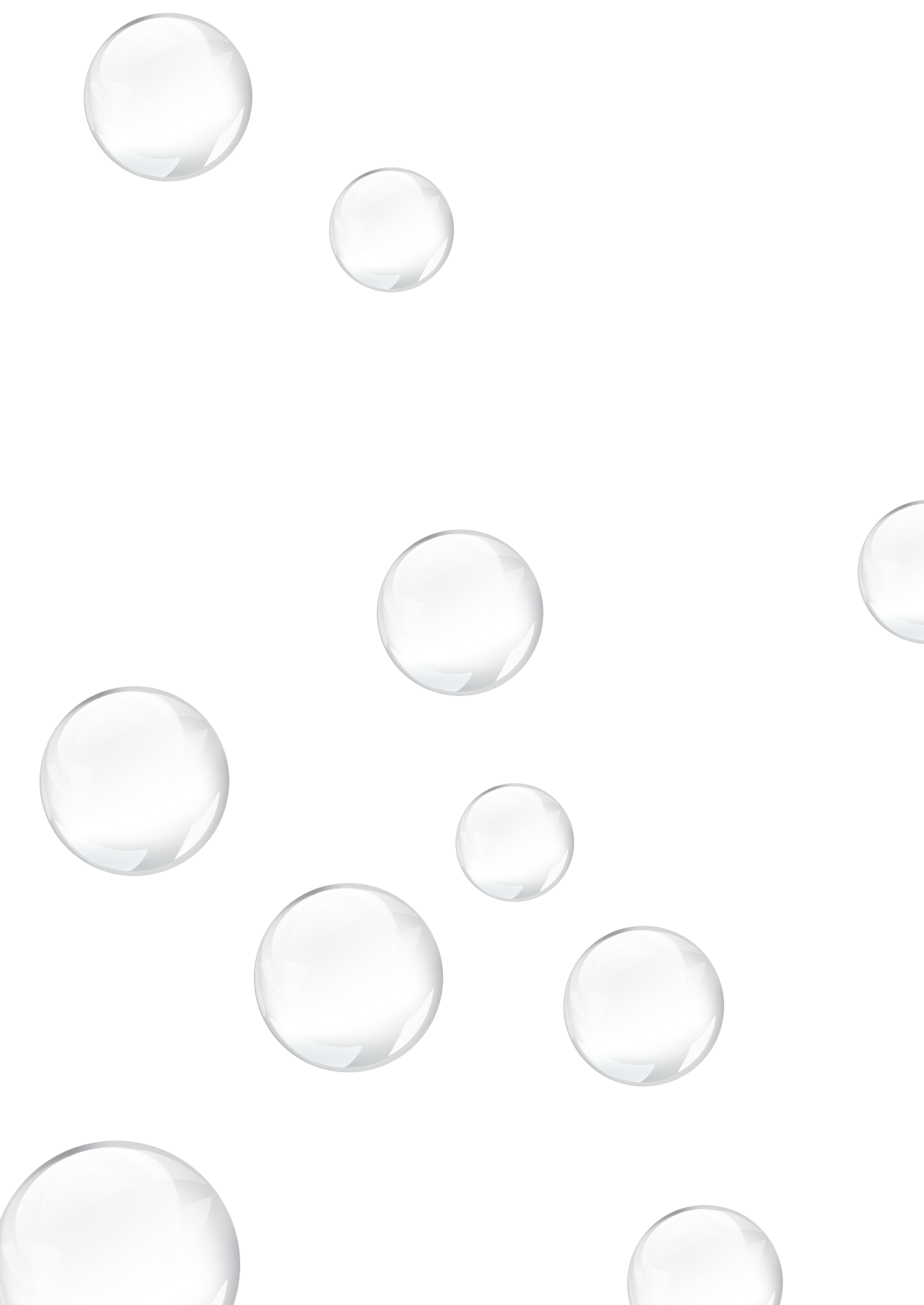
Daarnaast kwamen ook bepaalde M2-gerelateerde genen (MRC1 en CD206) hoger tot expressie in luchtwegaspiraten van te vroeggeboren kinder zonder BPD. Deze bevindingen suggereren een potentiële rol van M2-type macrofagen in de vaatontwikkeling en daarmee de ontwikkeling van longweefsel. We speculeren dat M2-type macrofagen en hun gerelateerde groeifactoren en cytokines potentiële nieuwe aangrijpingspunten zijn voor preventie en behandeling van BPD. In **hoofdstuk 6** hebben we ons gericht op het meten van sfingolipiden, bestandsdelen van celmembranen, in luchtwegaspiraten van te vroeggeboren kinderen met behulp van vloeistof chromotografische scheiding van de verschillende componenten en detectie met behulp van tandem massa spectrometrie techniek (LC-MS/MS). Eerder werd bij muizen aangetoond dat met name ceramides, een bepaald soort sfingolipide, een belangrijke rol spelen in zowel onstekings- als herstelprocessen gedurende de longontwikkeling. Wij hebben aangetoond dat een vroege toename en daaropvolgende afname in ceramides in luchtwegaspiraten van te vroeg geboren kinderen voorspellend zijn voor het ontwikkelen van BPD. Het ceramide profiel in luchtwegaspiraten lijkt een nieuwe vroege marker voor BPD ontwikkeling en interventies gericht op ceramides kunnen in de toekomst mogelijk ook leiden tot nieuwe preventieve of behandelstrategieën voor BPD.

In de **hoofdstukken 7 en 8** bespreken we de eerste resultaten van een nieuw klinisch follow up programma voor te vroeggeboren kinderen met ernstige BPD. In dit klinische follow up programma wordt op de leeftijd van 6 maanden, gecorrigeerd voor de vroeggeboorte, een CT-scan van de longen gemaakt en een slaap-ademhalingsonderzoek (polysomnografie) verricht, als 'alternatieve' biomarkers die mogelijk inzicht kunnen geven in de lange termijn uitkomst van BPD. **Hoofdstuk 7** geeft een overzicht van de literatuur op het gebied van CT-scans van de longen bij patiënten met BPD: structurele longafwijkingen zijn aantoonbaar in 85% van de BPD patiënten en de uitgebreidheid van de afwijkingen correleert met de ernst van de BPD en longfunctieafwijkingen. Er is momenteel geen gestandaardiseerde universele CT-scoringsmethode beschikbaar voor de beoordeling van structurele longafwijkingen bij patiënten met BPD. Daarom hebben wij de PRAGMA-BPD scoringsmethode ontwikkeld, met als primaire doel om CT-scans van BPD patiënten in de toekomst te kunnen gebruiken als uitkomstparameter in klinische studies. Daarbij kunnen CT-scans van de longen mogelijk ook lange termijn structurele longafwijkingen en daarmee geassocieerde respiratoire morbiditeit voorspellen.

In **hoofdstuk 8** beschrijven we de resultaten van long CT-scans en polysomnografie in kinderen met ernstige BPD op de gecorrigeerde leeftijd van 6 maanden oud. De uitgebreidheid van de structurele afwijkingen op de CT-scans is geassocieerd met de mate van beperking in ademhalingsfunctie zoals gemeten met behulp van polysomnografie. Een grotere gewichtstoename tussen de gecorrigeerde leeftijd van 2 en 6 maanden bleek geassocieerd met betere zuurstofvoorziening. Een hoger gewicht op de gecor-

rigeerde leeftijd van 6 maanden was geassocieerd met meer gezond longweefsel en minder dalingen in zuurstofverzadiging tijdens slaap.

In **hoofdstuk 9** worden de resultaten van onze studies bediscussieerd en aanbevelingen voor toekomstig onderzoek gedaan. We concluderen dat biomarkers een rol kunnen spelen in de diagnose, monitoring en behandeling van kinderlongziekten. Daarnaast kunnen biomarkers ook belangrijk zijn in het ontrafelen van onderliggende pathofysiologische processen en daarmee een bijdrage leveren aan het ontwikkelen van nieuwe preventieve en behandelmethodes. De lange termijn follow- up van kinderen met longziekten tot in het latere volwassen leven is van groot belang om inzicht te krijgen in het beloop van longziekten over de tijd en de invloed van preventieve en behandelstrategieën daarop. Om deze algemene doelen te kunnen bereiken is het belangrijk fundamentele en klinische onderzoeksgegevens met elkaar te integreren, dit vereist nauwe samenwerking tussen onderzoekers in het laboratorium, klinische onderzoekers, artsen en (ouders van) patiënten.



# A

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PhD portfolio
Dankwoord

## LIST OF ABBREVIATIONS

| | |
|---|---|
| Ang | Angiopoietin |
| ADMA | Asymmetric dimethylarginine |
| AHI | Apnea hypopnea index |
| AMP | Adenosine monophosphate |
| ATP | Adenosine triphosphate |
| BAL | Bronchoalveolar lavage |
| BALF | Bronchoalveolar lavage fluid |
| BPD | Bronchopulmonary dysplasia |
| CAI | Central apnea index |
| CAs | Central apneas |
| CCL | C-C motif chemokine |
| CDH | Congenital diaphragmatic hernia |
| Cer | Ceramide |
| CerDiHy | Dihydroceramides |
| CF | Cystic fibrosis |
| CFTR | Cystic fibrosis transmembrane conductance regulator |
| COPD | Chronic obstructive pulmonary disease |
| CPAP | Continuous positive airway pressure |
| CT | Computed tomography |
| CXCL | C-X-C motif chemokine |
| CXCR2 | CXC chemokine receptor 2 |
| cysLT | Cysteinyl leukotrienes |
| EB | Exhaled breath |
| EBC | Exhaled breath condensate |
| EGF | Epidermal growth factor |
| EMAP-II | Endothelial monocyte activating polypeptide-II |
| EMMPRIN | Extracellular matrix metalloproteinase inducer |
| eNO | Exhaled nitric oxide |
| eNose | Electronic nose |
| FeNO | Fractional exhaled nitric oxide |
| FEF75 | Forced expiratory flow after 75% of vital capacity |
| FEV1 | Forced expiratory volume in 1 second |
| FGF | Fibroblast growth factor |
| FRC | Functional residual capacity |
| FVC | Forced vital capacity |
| GA | Gestational age |
| GC-FID | Gas chromatography-flame ionization detection |

| | |
|---|---|
| GC-MS | Gas chromatography-mass spectrometry |
| GC-ToFMS | Gas chromatography-time of flight mass spectrometry |
| GDF15 | Growth differentiation factor 15 |
| GFR | Green fluorescent protein |
| GM-CSF | Granulocyte-macrophage colony-stimulating factor |
| $H_2O_2$ | Hydrogen peroxide |
| HELLP | Hemolysis elevated liver enzymes and low platelets |
| HETE | Hydroxyeicosatetraenoic acids |
| HGF | Hepatocyt growth factor |
| HIF | Hypoxia inducible factor |
| HUVEC | Human umbilical vein endothelial cells |
| HWG | Hollow waveguides |
| ICS | Inhaled corticosteroids |
| IGF-1 | Insulin-like growth factor 1 |
| IL | Interleukine |
| IQR | Interquartile range |
| LCI | Lung clearance index |
| LC-MS/MS | Liquid chromatography tandem mass spectrometry |
| LIF | Leukemia inhibitory factor |
| LT | Leukotriene |
| LTRA | Leukotriene receptor antagonist |
| LX | Lipoxin |
| MAI | Mixed apnea index |
| MAs | Mixed apneas |
| MBW | Multiple breath washout |
| MCP | Monocyte chemoattractant proteins |
| MCT | Mercury cadmium telluride |
| MDApp | Minimum detectable absorption per point |
| MDAps | Minimum detectable absorption per scan |
| MIP | Macrophage inflammatory proteins |
| MMP | Metallomatrix protease |
| MRI | Magnetic resonance imaging |
| MSCs | Mesenchymal stem cells |
| 3-NT | 3-nitrotyrosine |
| NO | Nitric oxide |
| NOS | Nitric oxide synthase |
| NOx | Metabolites of nitrogen oxides |
| NREM | Non-rapid eye movement sleep |
| oAHI | Obstructive apnea hypopnea index |

| | |
|---|---|
| OAI | Obstructive apnea index |
| OAs | Obstructive apneas |
| ODI | Oxygen desaturation index |
| OHs | Obstructive hypopneas |
| PCD | Primary ciliary dyskinesia |
| PDA | Persistent ductus arteriosus |
| PDGF | Platelet derived growth factor |
| PEF | Peak expiratory flow |
| PGD | Prostaglandin |
| PIH | Pregnancy induced hypertension |
| PlGF | Placenta growth factor |
| PMA | Postmenstrual age |
| PNNL | Pacific northwest national laboratory |
| ppmv | Parts per million by volume |
| ppbv | Parts per billion by volume |
| pptv | Parts per trillion by volume |
| PRAGMA-BPD | Perth-Rotterdam Annotated Grid Morphometric Analysis - Bronchopulmonary dysplasia |
| PSG | Polysomnography |
| PTR-MS | Proton transfer reaction-mass spectrometry |
| QCL | Quantum cascade lasers |
| RAST | Radioallergosorbent test |
| REM | Rapid eye movement sleep |
| RFP | Red fluorescent protein |
| RNS | Reactive nitrogen species |
| rms | Root mean square |
| ROS | Reactive oxygen species |
| RT-PCR | Reverse transcription polymerase chain reaction |
| SaO2 | Mean oxygen saturation |
| SaO2 nadir | Deepest oxygen desaturation |
| SD | Standard deviation |
| S1P | Sphingosine-1-phosphate |
| sFLT-1 | Soluble fms-like tyrosine kinase-1 |
| sICAM-1 | Soluble intracellular adhesion molecule-1 |
| SIFT-MS | Selected ion flow tube-mass spectrometry |
| SphK1 | Sphingosine kinase 1 |
| SPT | Sleep period time |
| TAMs | Tumor-associated macrophages |
| TAs | Tracheal aspirates |

| | |
|---|---|
| TEC | Thermo-electric cooled |
| TGF-β | Transforming growth factor beta |
| Th | T helper |
| TIB | Time in bed |
| TIMP-1 | Tissue inhibitors metalloproteinase-1 |
| TLC | Total lung capacity |
| TNF-α | Tumor necrosis factor alpha |
| TST | Total sleep time |
| TURBO DECCS | Transportable unit for research on biomarkers obtained from disposable exhaled condensate collection systems |
| VEGF | Vascular endothelial growth factor |
| VOC(s) | Volatile organic compound(s) |

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## ABOUT THE AUTHOR

Esther van Mastrigt was born on August 28, 1984 in Capelle aan den IJssel, the Netherlands. In 2002 she passed her pre university education (Gymnasium) at the Emmaus College in Rotterdam and started her medical training at the Erasmus University in Rotterdam. In her second year of medical school, Esther was invited to participate in the Master of Science program in Clinical Epidemiology at the Netherlands Institute for Health Sciences. During this program she worked for one year at the Generation R study under the supervision of dr. Carmelo Gabriele and prof. dr. Johan C. de Jongste. As part of this research training she also attended a summer school in Epidemiology at John Hopkins Bloomberg School of Public Health, Baltimore, MD, USA. Esther received both her Master's degree in Medicine and in Clinical Epidemiology in 2007. In February 2009 she obtained her medical degree (cum laude) and started working as a pediatric resident (ANIOS) at the medium care department of Erasmus MC-Sophia Children's Hospital. In 2010 she started her PhD study under the supervision of dr. Mariëlle W. Pijnenburg and prof. dr. Johan C. de Jongste at the department of Pediatric Respiratory Medicine of the Erasmus MC-Sophia Children's Hospital. In 2011 she was awarded a grant from ZonMW (AGIKO-stipendia) and combined her research with pediatrics training at the Erasmus MC-Sophia Children's Hospital (prof. dr. Matthijs de Hoog). In 2012 she received a grant of the Sophia Foundation for Scientific Research for her research project 'Sphingolipid Profiles In Neonates (SPIN-study)' at the department of Neonatology, Erasmus MC-Sophia Children's Hospital under the additional supervision of prof. dr. Irwin K.M. Reiss. During her PhD training she followed a Research Master Program Infection and Immunity at the Molecular Medicine Postgraduate School and received her Master's degree in 2014. In January 2016, Esther continued her residency training in Pediatrics at the department of Pediatrics, Maasstad Hospital, Rotterdam (dr. Michael Groeneweg). In leisure time, Esther loves to sport (triathlon) and to travel. She lives together with Rob van Sundert.

## LIST OF PUBLICATIONS

**van Mastrigt E,** Kakar E, Ciet P, den Dekker HT, Joosten KF, Kalkman P, Swarte R, Kroon AA, Tiddens HAWM, de Jongste JC, Reiss IKM, Duijts L, Pijnenburg MW. Structural and functional respiratory impairment in infants with severe bronchopulmonary dysplasia. *Submitted*.

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Neerincx AH, Linders YAM, Vermeulen L, Belderbos RA, Mandon J, **van Mastrigt E**, Pijnenburg MW, van Ingen J, Mouton JW, Kluijtmans LAJ, Wevers RA, Harren FJM, Cristescu SM, Merkus PJFM. Hydrogen cyanide emission in the lung by *Staphylococcus aureus*. Eur Respir J 2016 Aug;48(2):577-9.

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## PHD PORTFOLIO

Name PhD student: E. van Mastrigt
Erasmus MC department: Pediatrics, divisions of Respiratory Medicine and Neonatology
Research School: MolMed
PhD period: 2010-2016
Promotor: Prof. dr. J.C. de Jongste and Prof. dr. I.K.M. Reiss
Co-promotor: Dr. M.W. Pijnenburg

| | Year | Workload (ECTS) |
|---|---|---|
| **1. PhD training** | | |
| **General courses** | | |
| - BROK (Basiscursus Regelgeving Klinisch Onderzoek) | 2010 | 1.0 |
| - Biomedical English Writing and Communication | 2014 | 4.0 |
| - CPO Symposium Monitoring and Patient Safety in Investigator Initiated Clinical Trials | 2011 | 0.4 |
| **Specific courses** | | |
| - Master of Science Infection and Immunity, MolMed | 2010-2014 | 40.0 |
| **Seminars and Workshops** | | |
| - Pediatric Research Days, Rotterdam | 2010-2014 | 1.6 |
| - Jonge Onderzoekersdag, NVK, Veldhoven | 2011 | 0.3 |
| - ERS School course 'Monitoring of Airway Diseases', Veruno, Italy | 2011 | 1.2 |
| - VSL Breath analysis, Delft | 2011 | 0.8 |
| - Breath Summit, Parma, Italy | 2011 | 1.2 |
| - Netherlands Respiratory Society (NRS) Young investigator Symposium, Utrecht | 2013 | 0.3 |
| **(Inter)national conferences and presentations** | | |
| - Physics@FOM conference, Veldhoven, Netherlands (*poster presentation*) | 2012 | 0.5 |
| - American Thoracic Society (ATS) conference, San Francisco, USA (*2 poster presentations*) | 2012 | 1.0 |
| - Longdagen, Utrecht, the Netherlands (*poster presentation*) | 2012 | 0.5 |
| - Sectie kinderlongziekten site visit, Brompton Hospital, London, UK (*oral presentation*) | 2013 | 1.0 |
| - American Thoracic Society (ATS) conference, San Diego, USA (*poster presentation*) | 2014 | 0.5 |
| - European Respiratory Society (ERS) conference, Amsterdam, the Netherlands (*2 poster presentations*) | 2015 | 1.0 |
| - Sectie kinderlongziekten site visit, University Hospital, Basel, Switzerland (*oral presentation*) | 2015 | 1.0 |
| - European Respiratory Society (ERS) conference, London, UK (*oral presentation)* | 2016 | 1.0 |

| | | |
|---|---|---|
| **Other** | | |
| - Research meetings department pediatric respiratory medicine | 2010-2015 | 4.0 |
| - Peer review of articles for international scientific journals | 2010-2015 | 1.0 |
| - Outpatient clinic, department pediatric pulmonology | 2010-2015 | 2.0 |
| **2. Teaching** | | |
| **Lecturing** | | |
| - Practical Inhalation medication (4rd year medical students) | 2011, 2013, 2014 | 0.3 |
| **Supervising Master's theses** | | |
| - Tidal breathing FeNO measurements: a new algorithm. RC de Groot (*published article*) | 2011 | 1.0 |
| - The clinical utility of reticular basement membrane thickness measurements in asthmatic children. L Vanlaeken (*published article*) | 2012 | 1.0 |
| - Polysomnography findings in infants with severe bronchopulmonary dysplasia at 6 months corrected age. J Pichardo (*submitted article*) | 2014 | 1.0 |
| **3. Scholarships, grants and prizes** | | |
| - Erasmus MC Trustfonds | 2010 | |
| - ZonMW AGIKO grant (nr.40-00703-98-11646) € 63.530 | 2011 | |
| - SSWO grant (nr. S13-24) € 200.000 | 2012 | |
| - NRS travel grant | 2015 | |

Definition abbreviations: ECTS: European Credit Transfer and Accumulation System; 1 ECTS represents 28 hours.

*De afgelopen jaren stond mijn leven in het teken van de combinatie van 3 onderdelen: onderzoek, klinische opleiding en privé. In feite redelijk vergelijkbaar met het trainen voor een triatlon. Drie verschillende disciplines, die je alle drie in perfectie wilt uitvoeren. Iets wat ik niet altijd als even makkelijk heb ervaren.*

*Allereerst het zwemmen, een technisch onderdeel waarbij veel komt kijken, net als bij het doen van onderzoek. Het uitdenken van een studie-opzet, het aanvragen van de financiering, de daadwerkelijke uitvoering met het includeren van patiënten en het verzamelen en verwerken van studiematerialen, het analyseren en interpreteren van alle gegevens, en uiteindelijk het schrijven van een artikel.*

*Dan het fietsen, leren door veel te doen, met vallen en opstaan, te vergelijken met een klinische opleiding tot kinderarts. Door in een groep van verpleegkundigen, arts-assistenten en kinderartsen te fietsen, leer je van elkaar, kan je elkaar van tijd tot tijd uit de wind houden, en kan je samen accelereren.*

*In de triatlon sport wordt hardlopen, het laatste onderdeel, gezien als het belangrijkste onderdeel voor succes. Net zoals ik de afgelopen jaren heb geleerd dat balans in je persoonlijke leven het belangrijkste is voor het behalen van welke eindstreep dan ook.*

*In een triatlonwedstrijd komen alle drie de disciplines samen, volgen ze elkaar op, op weg naar de eindstreep. Vandaag is het dan zover, ik mag gaan laten zien dat ik met succes een triatlonwedstrijd kan afronden. Met als beloning; een medaille, een omhelzing, felicitaties en een hapje en een drankje!*

## DANKWOORD

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