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2022

Document Version:
Publisher's PDF, also known as Version of record

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PEDIATRICS | FACULTY OF MEDICINE | LUND UNIVERSITY

Printed by Media-Tryck, Lund 2022 NORDIC SWAN ECOLABEL 3041 0903

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FACULTY OF MEDICINE

Department of Clinical Sciences
Pediatrics

Lund University, Faculty of Medicine
Doctoral Dissertation Series 2022:129
ISBN 978-91-8021-291-5
ISSN 1652-8220

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LUND
UNIVERSITY

DOCTORAL DISSERTATION
by due permission of the Faculty of Medicine, Department of Clinical Sciences, Lund University, Sweden.
To be defended on 7th of October at 09.00 at Segerfalksalen

Faculty opponent
Professor Emeritus Sture Andersson
Children's Hospital, University of Helsinki
Helsinki, Finland

Organization	Document name
LUND UNIVERSITY Faculty of Medicine Department of Clinical Sciences Pediatrics	Doctoral dissertation
	Date of issue 2022-10-07
Author: Cecilia Hagman	Sponsoring organization

Title and subtitle

Very preterm infants and later lung function – impact of perinatal inflammation, club cell secretory protein and bronchopulmonary dysplasia

Abstract

Background: In high-income countries, survival is nowadays the most probable outcome after very preterm birth. However, despite advances in neonatal care, morbidity and long-term disability remain mostly unchanged. Bronchopulmonary dysplasia (BPD) is a chronic lung disease that develops in a substantial proportion of very preterm infants. Reduced lung function and pulmonary complications have been described to persist into childhood and even adolescence, as a consequence of BPD and prematurity.

Aim: The intention of this thesis was to investigate inflammatory and protective biomarkers during the early neonatal period and their association with short and long term respiratory morbidity in very preterm infants. Extensive lung function testing was performed at school age and compared to term born children.

Methods: Studies of three prospective cohorts of very preterm infants (born 2001-2007). Analysis of biomarkers such as club cell protein (CC16), and different cytokines from blood, tracheal aspirate (at birth, 6, 24 and 72 hours postnatally) and gastric fluid obtained after birth. Registration of respiratory data during hospitalization in the neonatal period. Assessment of lung function by spirometry, impulse oscillometry, body plethysmography, diffusion capacity and inert gas washout were performed at 12 years of age and a questionnaire was collected from caregivers at this time-point.

Results: Perinatal inflammation and decreased levels of CC16 were associated with severity of neonatal lung disease, a diagnosis of BPD and airway obstruction at 12 years of age. Very preterm born children showed airflow limitations, a higher total airway resistance, an increased ventilation inhomogeneity and lower diffusion capacity compared to children born at term. Boys born preterm had more airflow limitations than girls. In preterm infants, lung deficits were to a large extent reversed by bronchodilator inhalation.

Conclusion: Children born very preterm are at risk of lung function impairment at school-age, boys more than girls. Risk factors are to a large extent unknown, but these studies show that not only severe lung disease and diagnosis of BPD but also early inflammation and a low expression of CC16 at birth may be of importance for later lung function. Lung function deficits were to a large extent reversed by bronchodilator inhalation, emphasizing the importance of including lung function testing in follow-up. Comprehensive monitoring strategies as well as an individualized approach seems to be vital for the future of these vulnerable patients.

Key words: preterm, perinatal inflammation, neonatal, club cell secretory protein, bronchopulmonary dysplasia, lung function, spirometry, impulse oscillometry, diffusion capacity, N_2-washout, body plethysmography, fractional exhaled nitric oxide, reversibility

Classification system and/or index terms (if any)

Supplementary bibliographical information		**Language** English, Swedish
ISSN 1652-8220, Lund University, Faculty of Medicine Doctoral Dissertation series 2022:129		**ISBN** 978-91-8021-291-5
Recipient's notes	**Number of pages** 96	Price
	Security classification	

Signature  **Date** 2022-09-01

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Coverphoto by Daniele Levis Pelusi on Unsplash

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Pediatrics

ISBN 978-91-8021-291-5
ISSN 1652-8220

Printed in Sweden by Media-Tryck, Lund University, Lund 2022

To Truls and Måns

Your assumptions are your windows on the world.
Scrub them off every once in a while,
or the light won't come in.

Alan Alda

Table of Contents

Papers included in the thesis

This thesis is based on the following papers, hence referred to as Paper I-IV:

I. **Hagman C,** Björklund LJ, Hellgren G, Tufvesson E, Hansen-Pupp I: Club cell secretory protein (CC16) in gastric fluid at birth and subsequent lung disease in preterm infants. *Pediatric Pulmonology 2018;53:1399-1406.*

II. **Hagman C,** Björklund LJ, Bjermer L, Hansen-Pupp I, Tufvesson E: Perinatal inflammation relates to early respiratory morbidity and lung function at 12 years of age in children born very preterm. *Acta Paediatrica 2021;110:2084-2092.*

III. **Hagman C,** Björklund LJ, Bjermer L, Hansen-Pupp I, Tufvesson E: Lung function deficits and bronchodilator reversibility at 12 years of age in children born very preterm compared with controls born at term. *Submitted.*

IV. **Hagman C**, Björklund LJ, Hansen-Pupp I, Tufvesson E: Low club cell secretory protein in tracheal aspirate of very preterm newborns is associated with airway obstruction at school-age. *Submitted*

Abbrevations

ATS	American Thoracic Society
AUC	Area under the curve
AX	Area under the reactance curve between 5 Hz and the resonance frequency, F_{res}
BPD	Bronchopulmonary dysplasia
CC16	Club cell secretory protein
COPD	Chronic obstructive pulmonary disease
DL_{CO}	Diffusion capacity of the lung for carbon monoxide
ERS	European Respiratory Society
EXPRESS	Extremely Preterm Infants in Sweden Study
FIRS	Fetal inflammatory response syndrome
FEV_1	Forced expiratory volume in 1 sec
FVC	Forced vital capacity
FEF_{25-75}	Mean forced expiratory flow between 25% and 75% of FVC
FeNO	Fractional exhaled nitric oxide
F_{res}	Resonance frequency
GA	Gestational age
GLI	Global Lung Initiative
HFNC	High flow nasal cannula
IFN	Interferon
IL	Interleukin
IOS	Impulse oscillometry
IQR	Interquartile range
KCO	Diffusion coefficient for carbon monoxide

LCI	Lung clearance index
LLN	Lower limit of normal
LUFF	LungfunktionsUndersökning av För tidigt Födda barn
MBW	Multiple breath washout
MMP	Matrix metalloproteinase
NAVA	Neurally adjusted ventilatory assist
nCPAP	Nasal continuous positive airway pressure
NEC	Necrotizing enterocolitis
NOS	Nitric oxide synthases
ppb	Parts per billion
RDS	Respiratory distress syndrome
ROP	Retinopathy of prematurity
R_{tot}	Total flow resistance of the airways
R_{insp}	Inspiratory flow resistance of the airways
R_{exp}	Expiratory flow resistance of the airways
R_5, R_{20}	Respiratory resistance at 5 and 20 Hz
R_5-R_{20}	Frequency dependence of resistance
RV	Residual volume
SGA	Small for gestational age
S_{cond}	Ventilation heterogeneity of conducting airways
S_{acin}	Ventilation heterogeneity of intra-acinar airways
TA	Tracheal aspirate
TNFα	Tumor necrosis factor alpha
TLC	Total lung capacity
VA	Alveolar volume
VC	Vital capacity
X_5	Respiratory reactance at 5 Hz

Introduction

During my time as a paediatrician, I have always been interested in neonatology, physiology, embryology, pulmonology and how it all is related to each other. When on-call at late nights, I could stand beside an infant in the incubator and notice the breathing pattern, how changes in mechanical ventilation affected saturation, heart rate, motility and the general condition. While learning and getting the clinical touch, I always thought of how the puzzle pieces fit together.

When I had the opportunity to be included in a research project about very preterm infants and their development regarding lung function at school age, and to what extent early inflammatory biomarkers could have an impact for future respiratory health, I was excited.

This thesis is the result of the process and work in trying to find some more pieces to the puzzle that form our knowledge of neonatal respiratory diseases and the lung developmental changes over time in childhood.

Background

The Respiratory System

The respiratory system is complex and includes a wide range of different types of airways, lung parenchyma and pulmonary vessels. The main function is to distribute oxygen to the body and to remove carbon dioxide. The surface for gas exchange is regarded to be about the size of a tennis court, all this in the limited space of the thoracic cavity. The airway tree consists of approximately 23 generations of branches. Two different classification systems are used to separate and name the different compartments of the lung.

The first principle of division of airways into central and peripheral (large or small), where the first eight generations constitute the central part of the airway and are defined as having an inner diameter of > 2 mm with walls containing cartilage. The peripheral airways on the other hand are composed of non-cartilaginous bronchioles which lacks mucous glands, and have an inner diameter of < 2 mm. They are lined with surfactant which reduces surface tension and prevent closing on expiration and at low lung volumes.

The airway could also be divided into conducting and intra-acinar airways, the border being where the branching of the terminal bronchioles results in two or more respiratory bronchioles, around the 16^{th} generation. In the conducting airways there is no gas exchange, and the surface is lined with bronchial epithelial cells. Gas exchange occurs in the intra-acinar airways where the alveoli begin to appear, surrounded by a network of capillaries. In the alveoli there are three layers of the air-blood barrier where the actual gas exchange take place. The three layers of cells consist of 1) type I and type II alveolar cells which are part of the squamous epithelium of the alveolus, 2) the endothelial cell representing the capillary epithelium and 3) the shared fused basement membrane. Type II alveolar cells produce surfactant. The last generation of alveoli form blind ending alveolar sacs (1). Due to respiratory movements and mechanical challenges throughout life, the alveoli must be protected from over-distension as well as collapse by inherent stabilizing factors. To ensure mechanical stability of the parenchyma, the lung consists of two components, connective tissue fiber network, and the surfactant system (2).

Finally, to further optimize the function of the lung it is protected by the thoracic cavity, which gives help and support during inhalation and exhalation. The diaphragm is the main respiratory muscle, and the intercostal muscles of the chest wall also play an essential role by generating the force for breathing. Altogether they expand and contract the internal space of the thorax to ensure adequate respiratory effort.

Normal lung development

The development of the lung is a process that begins early in the embryonic life and continues after birth, into childhood and even to early adulthood. Both the structural and vascular development are closely related, and they progress simultaneously. These events of antenatal growth in the human fetus are divided into 5 stages.

The **embryonic phase** is the first of the five stages and occurs as early as 3-4 weeks of gestation. The lung develops as an outgrowth of the ventral wall of the primitive foregut, sulcus laryngotrachealis. Epithelial cells from the foregut endoderm invade the surrounding mesenchyme to form the trachea, lung primordia. Within days the development continues with subdivision into the right and left main bronchi. The left one is directed more laterally whereas the right one is directed more caudally. Subsequently, at around the 5th week there is a development into lobes, three endodermal buds on the right side and two on the left side, and segmental bronchi. These buds will further divide into segments of the individual pulmonary lobes at the end of this stage. The vasculogenesis with pulmonary arteries and veins develop from the 6th aortic arch and grows around the airway buds.

The next stage is the **pseudoglandular phase** which is between gestational weeks 7-17. During this stage the bronchial buds undergo a rapid dichotomous branching. By the end of this phase the formation of conducting airways and terminal bronchioles is complete, that is the first 16 generations. The pattern in which branching, and division is due to epithelial-mesenchymal interactions. During the 10th week of gestation, the bronchial walls are filled up with cartilage, smooth muscle cells and bronchial glands. Also, the early pseudo-stratified epithelium is replaced by columnar cells (ciliated epithelial cells) proximally and cuboidal cells (immature type II pneumocytes) distally and lung fluid begins to appear.

During the **canalicular phase** (gestational weeks 17-27), two important steps of the development of the lung can be detected: differentiation of type I and type II pneumocytes and the formation of the alveolar capillary barrier. Synthesis and storage of surfactant protein can be detected from around 24 weeks of gestation meaning that a possible platform for gas exchange is established, together with the alveolar capillary bed. Furthermore, the acinar structures including respiratory bronchioles, alveolar ducts and primitive alveoli are formed. These represent the proper respiratory part of the lung, the pulmonary parenchyma. At this stage,

surfactant deficiency is inevitable if preterm birth occurs, even though variety amongst individuals is plausible.

In the **saccular phase,** which is between weeks 28-36, the division of the airway is almost complete. Enlargement of peripheral airways, that is cluster of sacs develop on the terminal bronchiole forming the last generation of conducting airways and the terminal respiratory part of the bronchial tree. Saccules and thinning of the airway walls develop to ensure an increased surface area for gas exchange. Type II pneumocytes start to secrete surfactant into the fetal lung fluid and laminar bodies containing surfactant appear. In the interstitial space collagen and elastic fibers/cells can be detected and the fibroblasts start to produce extracellular material at the end of this stage.

The last phase during the fetal human lung development is the **alveolar phase** and this extends from gestational week 36 until 2-3 years of age. Formation of secondary septa in the saccules to thin-walled structures with capillary bed give rise to the definitive cup shaped alveoli. These alveoli are lined by a central sheet of connective tissue which support a thin layer of capillary network on either side. The refining of double capillary walled secondary septa and multiplication of alveoli continue into childhood and maybe also into early adolescence (Figure 1) (3).

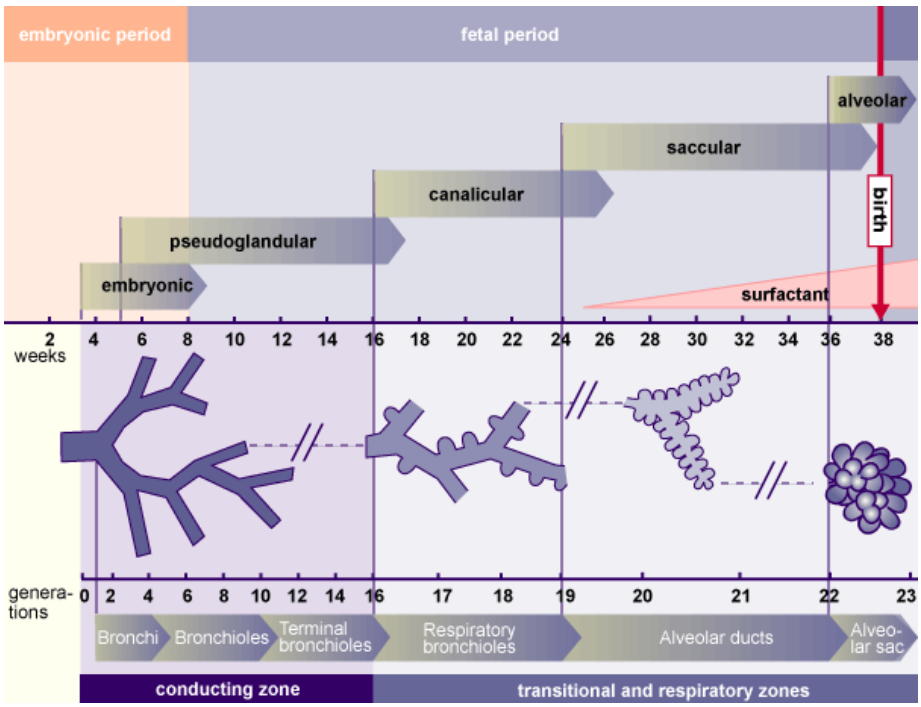

Figure 1. Embryology and development of the human lung. Uploaded from slideshare.net. Origin unknown.

Abnormal lung development - Bronchopulmonary dysplasia

Bronchopulmonary dysplasia (BPD) is a frequent complication after very preterm birth and remains the leading cause of respiratory morbidity among these infants. The disease was described in 1967 by Northway et al (4). Initially, the term hyaline-membrane disease was introduced and was described as a lung injury in preterm infants resulting from oxygen and mechanical ventilation. This was the first description of respiratory distress syndrome (RDS). The term "old BPD" refers to the pathological findings of airway injury, inflammation, epithelial metaplasia, smooth muscle hypertrophy, and parenchymal fibrosis seen in infants dying from the disease before the introduction of surfactant, and was the chronical disease following hyaline membrane disease, according to Northway. With newer strategies for neonatal respiratory care the concept of BPD has changed into “new BPD”, which is characterized by an alteration or arrest of lung development with fewer and larger alveoli and decreased pulmonary microvascular development (Figure 3). Today BPD continues to be a major problem despite the use of antenatal steroids, surfactant replacement therapy, gentle non-invasive ventilation techniques, permissive hypercarbia and judicious use of oxygen. Reduced lung function and pulmonary complications have been described to persist into childhood and even adolescence. According to a Swedish national survey, the EXPRESS study, 62% of infants born before 27 weeks of gestation, were diagnosed with BPD, and 14% had a severe form (5).

BPD is defined as supplemental oxygen requirements and/or mechanical ventilatory support at 36 weeks postmenstrual age, but the definition vary depending on which week the infant is born, according to Table 1 (6). The mechanism by which BPD occurs is multifactorial and most likely factors act additively and/or synergistically to promote injury (7). Interference with or inhibition of alveolar and vascular development is the key to impaired function. Pulmonary inflammation plays an important role in the pathogenesis of BPD, and an imbalance between pro-inflammatory and anti-inflammatory mediators in the developing neonatal lung has been suggested to contribute to the development of BPD (8, 9). The premature infant is also unable to temper the inflammatory response caused by extrauterine insults such as mechanical ventilation, sepsis and oxygen. These forces initiate a cascade of pro-inflammatory cytokines that lead to the development of significant inflammatory changes and chronic lung injury (10-15).

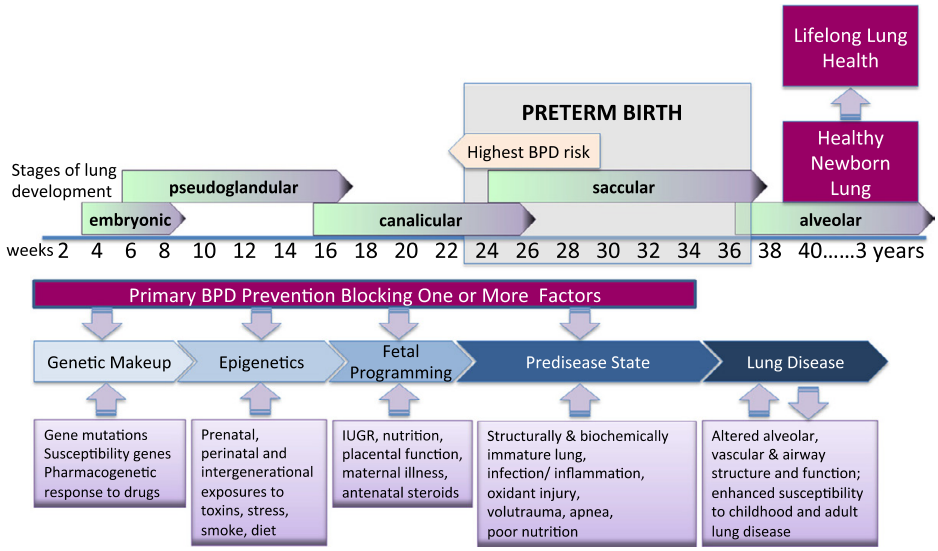

Figure 2. Etiology of bronchopulmonary dysplasia.

Several different BPD definitions have been developed to further maximize the possibility to predict severity of future respiratory morbidity and lung function impairments, as well as ensure effective preventive and treatment strategies for respiratory complications following preterm birth (16-18). A high degree of sensitivity for predicting short-term outcome is abundant in most of the used definitions, but a more objective and accurate description of BPD regarding both clinical symptoms and the heterogeneity among these infants is missing (19). The most commonly used definition for infants born before 32 weeks of gestation is based on the need for supplementary O_2 therapy and respiratory support at 36 weeks postmenstrual age (Table 1).

Table 1. Definition of bronchopulmonary dysplasia

Modified from Jobe and Bancalari (6)

Gestational age	<32 weeks	≥32 weeks
Treatment with oxygen >21% for at least 28 days **plus**		
Mild BPD	Breathing room air at 36 wk PMA or at discharge, whichever comes first	Breathing room air at 56 days postnatal age or at discharge, whichever comes first
Moderate BPD	Need for <30% oxygen at 36 wk PMA or at discharge, whichever comes first	Need for <30% oxygen at 56 days postnatal age or at discharge, whichever comes first
Severe BPD	Need for >30% oxygen and/or positive pressure at 36 wk PMA or at discharge, whichever comes first	Need for >30% oxygen and/or positive pressure at 56 days postnatal age or at discharge, whichever comes first

BPD = bronchopulmonary dysplasia; PMA = postmenstrual age; wk = weeks

There have been some discussions recently about the original BPD definition since new modes of respiratory support such as high flow nasal cannula has been introduced during recent years. Moreover, the criteria for supplemental oxygen may vary between sites. In another model Jensen et al (20), defines BPD only according to the type of respiratory support at 36 weeks of gestation, with no references to oxygen requirement. This definition has been shown to correlate with adverse outcome, both in terms of respiratory morbidity and death. Walsh et al (21) have a somewhat different approach with a physiological definition for establishing a diagnosis of BPD using an oxygen reduction test (ORT).

In the future, a definition of BPD with more accurate predictivity is warranted for an independent evaluation and management of different treatment options.

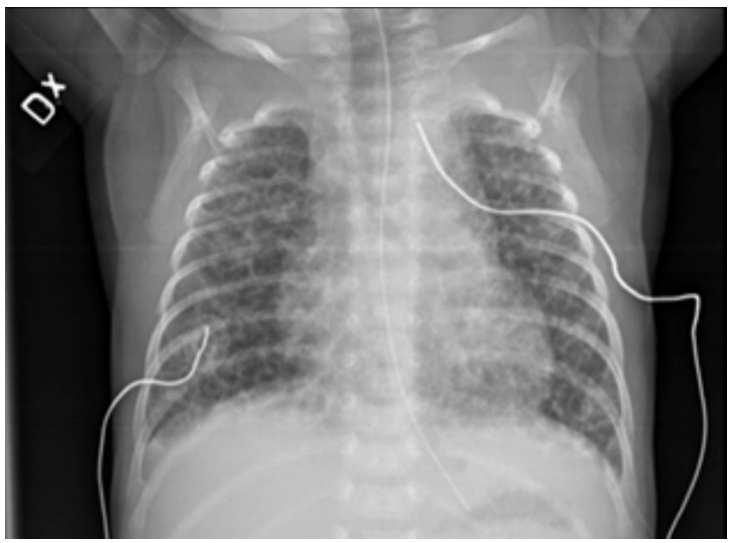

Figure 3. Chest X-ray showing severe BPD with diffuse parenchymal involvement, in a very preterm infant.

The preterm lung - factors for impaired lung development

Maternal risk factors

Smoking and other environmental factors

It is well known that smoking during pregnancy and maternal exposure to second-hand cigarette smoke (SHS) is associated with an increased risk of preterm birth, fetal growth restriction and low birth weight (22, 23). Smoking prevalence during pregnancy is commonly based on self-reported information and is believed to be underestimated in some surveys. In general, smoking prevalence among young women has been reduced during recent decades.

Tobacco smoke contains thousands of different substances, where most likely one or more plays a role in the pathogenesis of smoke related lung disease. Nicotine is one of the most interesting, since it easily crosses the placental barrier, and levels in the fetal blood and fetal lung seem to correspond with levels in the maternal blood. Shortly after birth infants to mothers who smoked showed altered tidal breathing pattern, decreased passive respiratory compliance as well as decreased forced expiratory flow. Longitudinal data with lung function tests from prospective birth cohorts reveal persistent deficits in pulmonary function, in childhood, adolescence and adulthood, with long-lasting permanent structural changes and alterations that may have an impact on lung morbidity trajectories (24-26).

During childhood and the first years of life, children to women who smoke during pregnancy, have an increased risk of hospitalization due to various respiratory symptoms (27, 28). Since infants and children have a higher breathing rate and in preterm infants also an immature lung development, they are more vulnerable for adverse outcome regarding later respiratory health (29).

An increased risk of respiratory illness and a diagnosis of asthma are factors that are important determinants for childhood and adolescence pulmonary function (26, 30-32). Several studies have shown more hospital admissions during childhood as well as an increased risk for recurrent wheezing (33-35), in infants to mothers who are smoking during pregnancy.

Furthermore, recent research has determined that children who are exposed to second-hand smoking or prenatal tobacco smoke have changes in levels of enzymes, hormones and expressions of genes, micro RNAs and proteins. In combination with genetic predisposition this may implicate later health effects (36). In utero exposure to air pollution, called particulate matter (PM) may influence and have adverse consequences for respiratory health. Outdoor PM exposure is associated with low birth weight and preterm birth (37, 38). Also, there is evidence that PM emissions

from household combustion of solid fuels as well as air pollution from agricultural burning and wildfires may affect birth outcome (39). Regarding respiratory symptoms there are some studies indicating a reduction in lung function (40) and an increased risk of lower respiratory tract infection at early age (41).

Fetal and neonatal risk factors

Preterm birth

Preterm birth is defined as birth before 37 weeks of gestation. There are three sub-categories within this categorization:

- Extremely preterm, before 28 weeks gestation
- Very preterm, before 32 weeks gestation
- Moderate to late preterm 32 to 36 weeks gestation

In Sweden approximately 115 000 children are born yearly. Of these, 5-6% are born before 37 weeks of gestation, about 1% are born very preterm and 0.3-0.4% are born extremely preterm (42). In high-income countries, such as Sweden, survival is nowadays the most probable outcome after very preterm birth. But despite advances in obstetric and neonatal care, such as administration of antenatal glucocorticosteroids, which is thought to stimulate lung maturation and endogenous surfactant production, and surfactant instillation in the lungs at birth, short- and long-term pulmonary morbidity remains mostly unchanged. Multiple pre- and postnatal events may have an impact on the developing lung. Mechanisms for these changes are disrupted alveolar growth, both the angiogenesis and alveolarization, inflammation, insufficient production of surfactant and an overall immature contexture of airways, interstitial and vascular structures (6, 43).

Fetal or intrauterine growth restriction (IUGR) and small for gestational age (SGA)

Among infants born preterm, weight deviation, classified either as intrauterine growth restriction or as small for gestational age, is common and is present in nearly a third of the preterm births (44). The definition for IUGR are infants with birth weight and/or birth length below the 10^{th} percentile for gestational age with an associated pathological umbilical artery blood flow detected by prenatal Doppler ultrasound. This is often due to placental dysfunction in utero (45, 46). SGA is defined as a birthweight of more than two standard deviations below the mean according to gestational age (47). Hence, IUGR and SGA are not synonymous. An infant born SGA could be constitutionally small and not suffer from IUGR, and vice versa an infant with IUGR does not necessarily need to be born SGA. Prematurity in combination with IUGR or SGA have additive effects on adverse outcome both in terms of short-and long-term lung development and is associated with decreased

lung function, reduced lung compliance and increased chest wall compliance during childhood and adolescences (48-50).

Preeclampsia

Preeclampsia is a systemic, multiorgan disorder unique to pregnancy and associated with significant maternal and neonatal morbidity and mortality (51).

In normal pregnancy the placenta invades the uterine wall and creates a low resistance vascular system for the fetus, which optimizes the intrauterine environment. In preeclampsia this process of remodeling is defective and results in placental ischemia, oxidative stress, formation of micro-embolies and may also disrupt fetal hematopoiesis (52). Due to insufficiency in the interspace between uterus and placenta the blood flow to the fetus is compromised. Antepartum diagnosis includes blood pressure greater than 160 mmHg (systolic) or 110 mmHg (diastolic), associated with proteinuria, multiorgan involvement, thrombocytopenia, pulmonary oedema or oliguria (53). Despite a multitude of strategies to treat preeclampsia, preterm delivery is a common consequence of this disease.

Preeclampsia may be responsible for the enhancement of BPD as well as necrotizing enterocolitis (NEC) seen in this population.

Chorioamnionitis and fetal inflammatory response syndrome

The maternal inflammatory response to an intrauterine infection is chorioamnionitis. The clinical findings being maternal fever, elevated white blood count, tender uterus by palpation, and amniotic fluid showing elevated pro-inflammatory cytokines, signs of positive microbial culture or PCR and/or inflammatory cells. Histologically, the findings are granulocyte infiltration and necrosis in the choriodecidual space, chorioamniotic membranes, amniotic fluid or in the umbilical cord. Histological chorioamnionitis has been shown to have a higher predictivity for perinatal comorbidities than chorioamnionitis defined by clinically diagnosis (54).

Fetal inflammatory response syndrome (FIRS), is the fetal counterpart and is characterized by fetal vasculitis, meaning presence of leukocytes in the blood vessel walls of the chorion and in the umbilical cord (funisitis). Elevated concentrations of IL-6 in fetal plasma are associated with an onset of preterm labour and multiorgan fetal involvement. Intra-amniotic infection has been considered as the major cause of both acute chorioamnionitis and funisitis. However, recent evidence indicates that sterile intraamniotic inflammation could as well be associated with these disorders (55). Sterile fetal inflammation, neonatal sepsis, BPD, periventricular leukomalacia (PVL) and cerebral palsy are all short- and long-term complications associated with maternal chorioamnionitis.

Furthermore, data supports a link between prenatal inflammation and later airway disease in infancy and childhood. In a large cohort of more than 500 000 children,

clinical chorioamnionitis before preterm birth was associated with an increased risk of childhood asthma (56). Histological chorioamnionitis was associated with a more pronounced expiratory flow obstruction at lung function testing in a cohort of infants with varying degrees of prematurity, (57). In a long-term follow-up study of very preterm infants, FIRS was associated with both early childhood wheezing and asthma beyond 5 years of age (58).

Genetic factors

The degree to which genetics contribute and the number of genetic factors involved in the etiology of a specific disorder, determines the individual susceptibility for that disease. The surfactant system includes a variety of known mutations including primary deficiency of one or more of the surfactant proteins (SP-B, SP-C or ABCA3) which all progress to severe respiratory distress syndrome (RDS) (59). Also, abnormalities of lamellar bodies (the structure where surfactant is stored in the alveolar type II cells) without any surfactant protein deficiency may cause severe lung disease (60). Bhandari et al (61) showed that among monozygotic twins with preterm birth, genetic factors, contributed to an increased susceptibility for BPD.

Sex differences

Preterm boys are more susceptible to several different respiratory disorders during prematurity than girls, such as distress syndrome (RDS), the development of BPD and more ventilatory and circulatory support early in life (62-64). This male disadvantage seems to persist into early adolescence where males with preterm birth showed an increased airway obstruction (65). Male sex has also been associated with a deterioration in lung function during adolescence (66). In prematurely born adults at 19 years of age asthma and respiratory symptoms were reported more frequently in females but were more common in both prematurely born females and males as compared to controls (67). In a follow-up at 11-14 years of age of 319 participants in the United Kingdom Oscillation Study, all born before 29 weeks gestation, expiratory flows during spirometry were significantly worse in males than in females; however, reversibility was not reported and there were no term-born controls (65).

Metabolic factors

The field of metabolomics is a different approach in understanding the complex aetiology of multifactorial diseases such as BPD. Metabolomics is the quantitative analysis of many different low molecular metabolites found in a specific cell, organ or organism, including substrates of a defined metabolic pathway. Changes in the metabolite's composition (as in a disease) reflect the interaction between a specific pathophysiological state, the genetic predisposition and environmental stimuli, creating an unique fingerprint of an organism (68). In a study of amniotic fluid, infants who developed BPD had reduced levels of S-adenosyl methionine. This

metabolite act in the reaction and formation of glutathione, which is a powerful antioxidant for free radicals and oxygen reactive substances (69). Other studies have shown similar patterns using urine in the prediction of BPD (70, 71).

Respiratory strategies

Strategies for mechanical ventilation in the preterm infants have shifted during the past decades. Since children and especially preterm infants have different respiratory physiology compared to adults, there are some obstacles to consider. Pharynx, larynx, trachea and the bronchial tree are more compliant in the preterm neonate and these differences may lead to airway collapse during forceful inspiration. Epiglottis is large and positioned high in the pharynx and close to the soft palate with subsequent lower airflow resistance in the nasal passage. This could be one explanation for infants' breath preferentially through their nose. Diameters of the airway are narrow and higher airway resistance may occur. The preterm lung lacks accessory interalveolar communications and have fewer alveoli which might increase the risk of atelectasis and decrease the ability for elastic recoil during management of the airways. A surfactant-deficient lung is characterized by poor compliance, reduced volume, atelectasis, ventilation-perfusion defects and hypoxia. Due to immaturity of the respiratory control system, breathing pattern of preterm infants are often irregular and periodic. This could lead to severe apneoas. Also, ventilatory response to hypercapnia and hypoxia may be delayed in onset. Finally, the thorax cavity is compliant and deformable, which is visible as intercostal, sternal and supraclavicular recessions during respiratory distress (72).

Oxygen

Fetal life evolves in a hypoxic environment and an adequate access of oxygen during the embryonic, fetal and postnatal period is essential for normal growth and development. During fetal to neonatal transition, the initiating of breathing immediately after birth induces cardiorespiratory as well as metabolic changes. Pulmonary vascular resistance decreases, and the right ventricular cardiac output is redirected to the lungs where the blood gets oxygenated. Arterial oxygenation saturation rises over a period of 10 to 15 minutes in the healthy newborn (73). Supplemental oxygen is used to facilitate this transition or later in the neonatal period. Oxygen saturation target is 90 to 94%. These recommendations have remained the same during the last years. Targeting lower saturations (85 to 89%), reduces the risk for severe retinopathy of prematurity (ROP) but increases the risk of neonatal mortality and necrotizing enterocolitis (NEC) (74). Hyperoxia may contribute to the development of BPD, due to oxidative stress and alterations of cell structure and function (75).

Surfactant

Surfactant lowers surface tension in the peripheral units of the lung and is vital for pulmonary adaptation after birth. Already in 1929, the German physiologist Kurt

von Neergaard concluded that “a lower surface tension would be useful for respiratory mechanisms” and that “surface tension as a force counteracting the first breath of the newly born should be investigated further” (76). Production of alveolar surfactant begins around 23 to 24 weeks of gestation and reaches physiological sufficient values around 35 weeks of gestation. This means that the preterm infant has a relatively transient deficiency of surfactant (77).

Exogenously administrated surfactant improves gas exchange and significantly improves survival for preterm infants with respiratory distress syndrome (78, 79) and is nowadays an established treatment. The most commonly used exogenous surfactant on a world-wide scale, and the only one available in Sweden, is poractant alfa (Curosurf), a natural porcine surfactant developed by Tore Curstedt and Bengt Robertson in Stockholm and first used in a human infant in 1983. Endotracheal instillation as a bolus dose is the routine administration, for surfactant replacement therapy. One technique used is INSURE (Intubation, SURfactant, Extubation), in which the endotracheal tube in most cases can be removed after instillation (80). Nowadays, minimal invasive approaches for surfactant administration have been introduced, in which the infant is breathing spontaneously and where surfactant is instilled into the trachea via a thin catheter. Different techniques are applied, LISA (less invasive surfactant administration) or MIST (minimal invasive surfactant therapy) (81-83). Recently, further one technique for surfactant instillation has been introduced where surfactant is instilled via a laryngeal mask into the trachea, but this procedure still needs to be evaluated (84).

Ventilatory support

The ventilators initially used for infants were rough and had a large dead space, where most of the ventilator time in use surpassed the lung volume in the neonate. Since they initially were adapted for use in the adult population, they were unable to synchronize with the irregular and more shallow breathing pattern in the infant. This required sedation of the child. Also, tracheostomy was the preferred strategy before the introduction of endotracheal tubes (85). These ventilation strategies could result in barotrauma, where the lung is overdistended or volutrauma, where the lung is inflated with a greater volume than the total lung capacity (TLC). On the other hand, ventilation with too small volumes, below functional residual capacity, causes atelectotrauma due to collapse and reexpansion of peripheral airways (86).

The first ventilator designed for pediatric and neonatal management was introduced for clinical use in 1980. Approximately 50% of preterm infants with gestational age < 28 weeks require intubation and mechanical ventilation, where the smallest and most immature infants are in greatest need (87). Today, different modes of synchronized ventilation and volume targeted ventilation are used to minimize lung injury both in the short perspective but also for future long-term respiratory function. Using non-invasive ventilation techniques (NIV), for primary respiratory support has reduced the incidence of BPD as well as grade III-IV intraventricular

haemorrhage, pneumothorax and ventilator duration (88, 89). Nasal continuous positive airway pressure (nCPAP) may replace intubation and has become widely used as the first line of treatment for preterm respiratory support (90). Other more recently developed respiratory strategies include high flow nasal cannula (HFNC), as primary support and as weaning from mechanical ventilation, and neurally adjusted ventilatory assist (NAVA), in which the ventilator uses the electrical activity of the diaphragm (Edi), to generate appropriate breaths and assists ventilated infants (91).

Pharmacological treatment

Inhalation of bronchodilators and corticosteroids are used as prevention and treatment for BPD, but only short-term effects, like improvements in airway resistance and compliance, have been demonstrated (18, 92, 93). Drugs of choice are ipratropium bromide, salbutamol/albuterol and/or budesonide (94).

Systemic corticosteroids have been used for many years to facilitate extubation and shorten the course of severe lung disease in preterm infants. The use of early dexamethasone to prevent BPD was discredited because of a high risk of cerebral palsy. A recent meta-analysis, including 62 studies and 5559 neonates, identified several regimes of systemic or inhaled corticosteroids that can prevent BPD and suggested that a moderately early initiated (8-14 days) medium cumulative (2-4 mg/kg) dose of dexamethasone is most appropriate (95). In Swedish neonatal care, betamethasone is used instead of dexamethasone, but the proportion of children treated varies widely among centers. Recently, low-dose early hydrocortisone has been introduced in some Swedish centres as prophylaxis for BPD in infants born before 28 weeks according to the so-called PREMILOC protocol (96). When the infants included in this thesis were born, betamethasone was most often started in infants still on mechanical ventilation with a high oxygen requirement at 10-14 days of age (97, 98).

Antileukotrienes are a relatively new approach for preventing chronic lung disease but still lacks evidence and randomized trials are needed, both for short-term and long-term outcomes (99).

Caffeine is used for its ability to increase the respiratory drive, diaphragm contractility and therefore reducing apnoea of prematurity, the need for mechanical ventilation, enhance extubation success and decrease the risk of BPD (100).

Diuretics are thought to improve respiratory effort. Excessive hydration could be associated with pulmonary oedema and thereby increased need for respiratory support and subsequently an increased risk for BPD development. Furosemide, the most used diuretics, is a loop diuretic that improves lung compliance, and decreases pulmonary vascular resistance and interstitial oedema. The optimal time-point for administration of furosemide most likely being in infants > 3 weeks of age, where no inconsistent effects are detectable (94, 101).

Inhaled nitric oxide (iNO), is a pulmonary vasodilator and has beneficial effects on lung parenchyma, bronchi and pulmonary vasculature. Criteria for using iNO are high oxygenation index (rescue), prophylactic in intubated preterm infants and as later enrolment for prevention of BPD (102). However, long-term effects and safety have not yet been sufficiently evaluated.

Nutrition

A relatively large proportion of particularly extremely preterm infants are at risk of growth failure. It is a challenge to maintain an adequate nutrition where the needs of both a high energy and protein intake is met and where the volume intake is acceptable. These infants need a nutritional strategy which includes early aggressive parenteral nutrition and initiation of concentrated feedings of energy and nutrients. Optimal nutrition plays an essential role in preventing the development of BPD. Fortified own mother's milk, fortified donor milk or preterm enriched formulas are necessary to achieve adequate growth, as well as functional nutrient supplements (vitamins, minerals and trace elements) are important. Dedicated nutritional support both during hospitalization and after discharge are vital since children with BPD may suffer from gastroesophageal reflux and poor coordinated feeding (103).

Morbidity during childhood and adolescence

Asthma and allergy

Asthma is a common chronic inflammatory disorder of the airway that can implicate individuals of all ages, usually beginning in childhood and persisting into adolescence and adulthood. It is characterized by airway obstruction and bronchial hyperresponsiveness. An accurate definition has been defined by the Global Initiative for Asthma (GINA) (104, 105):

> "Asthma is a chronic inflammatory disorder of the airways in which many cells and cellular elements play a role: in particular mast cells, eosinophils, T lymfocytes, macrophages, neutrophils and epithelial cells. In susceptible individuals, this inflammation causes recurrent episodes of wheezing, breathlessness, chest tightness and coughing, particularly at night or in the early morning. These episodes are usually associated with widespread but variable airflow obstruction that is often reversible either spontaneously or with treatment. The inflammation also causes an associated increase in the existing bronchial hyperresponsiveness to a variety of stimuli. Reversibility of airflow limitations may be incomplete in some patients with asthma."

According to this definition, asthma is not a single disease but consists of different phenotypes with large variation in course and outcome. Children born preterm, both with and without a diagnosis of BPD, may display different degrees of impaired gas exchange, bronchial hyperresponsiveness and asthma-like symptoms, such as wheezing (106, 107). Hospitalization for respiratory symptoms in early childhood

is increased and a diagnosis of asthma is common, even though asthma and chronic lung disease following preterm birth most likely are two separate entities (108, 109). Some important characteristics following BPD and asthma seem to overlap, but the casual pathology, risk factors, management of treatment and natural history are somewhat different. In the EPICURE-study, long-term follow-up of preterm infants born < 26 weeks gestational age, 25% had an asthma diagnosis at the age of 11 (110). Regardless, future evaluation of BPD and asthma-like symptoms may include family history, lung function testing, signs of airway inflammation and characterizing respiratory symptoms by using the asthma control test (questionnaire) for requiring information to different strategies to successfully treat and manage this increasing group of individuals.

Infections

Preterm infants are more sensitive to lower respiratory infections since they have an underdeveloped immune system and/or anatomical changes that might compromise their normal defences. Furthermore, factors affecting the environment in utero, such as maternal atopy, antibiotic exposure and infections might worsen the outcome from common infections and hospitalization is more common in these groups of children. Special attention and strategies should be managed since an increased risk for chronic respiratory symptoms and decreased lung function could persist into adolescence and adulthood (58).

Physical activity

Overall, physical activity in childhood and adolescence is vital to avoid future health risks, including obesity, metabolic and cardiovascular disease. For children born preterm there seem to be even more advantages to maintain a healthy lifestyle. Tikanmäki, M et al (111) have reported lower muscle fitness among children born both very preterm and moderate preterm compared to controls. Furthermore, young adults born preterm perceived themselves less fit than controls. In this study, there were no differences in cardiorespiratory outcome.

There is evidence that prematurity per se reduces exercise capacity in young adults, as an independent risk factor regardless of other known markers for physical activity, such as socioeconomic status and current BMI, where the exercise capacity seemed to increase linear with advancing gestational age (112). Moreover, a low birth weight, also appears to predict low capacity as well. Several other studies present similar results (113-115). For pediatricians and other health care professionals these groups of children require particular focus on promoting physical activity to enhances both respiratory outcome and muscular fitness as a preventive measure for future general health.

Protective and inflammatory biomarkers in the lung

Club cell secretory protein (CC16)

Club cell secretory protein (CC16) is the most abundant protein in normal airway secretions and is mainly produced by non-ciliated club cells in the bronchial and bronchiolar epithelium. It has anti-inflammatory properties and protects the lung from oxidative stress.

In amniotic fluid within the fetus, CC16 is detectable from 15 weeks of gestation and concentrations increase 25-fold up to term age. Production of CC16 in the fetal lung reaches a plateau already at 30 weeks of gestation, which is different from the temporal pattern for production of surfactant where levels increase during pregnancy even after 30 weeks. Very preterm infants may be born with a relative CC16 deficiency and may have an inadequate ability to protect themselves against the development of a pro-inflammatory state in the lung. The presence of CC16 has been suggested to be a marker of bronchial epithelial growth and development of pulmonary airways (116). CC16 is believed to facilitate extrauterine pulmonary adaptation (117). The levels of CC16 in plasma increase significantly from a median level of 15 ng/mL at birth to 96 ng/mL at the age of 4 months. Thereafter, the concentration of CC16 decreases to 20 ng/mL at 18 months of age and continues to decrease to 7 ng/mL at the age of 3 years (118). Normal value in adults is 7 ng/mL (119).

The human CC16 gene is located on chromosome 11q12.3, where several regulatory genes of allergy and inflammation exist. Studies reveal that factors such as gender, age, obesity, renal function, diurnal variation, and exercise regulate CC16 levels in circulation.

Within the normal lung, club cell proliferation maintains the facultative progenitor cell pool (self-renewal) and restores terminally differentiated cells of the conducting airway epithelium (ciliated cells). The unique features of lung epithelial maintenance and repair suggest that chronic lung disease could be treated through interventions that stabilize the club cell pool or by cell replacement strategies that restore this abundant cell type, in that club cells act as stem cells (120). CC16 has anti-inflammatory properties and functions as a natural immunosuppressor, inhibiting fibroblasts, monocyte and neutrophil chemotaxis, phagocytosis as well as regulating the activity of secretory and intracellular phospholipase A2 (PLA2) (121, 122). PLA2 is an enzyme that may promote inflammation and cause inactivation of surfactant.

Current findings indicate that CC16 not only may reflect the pathogenesis of pulmonary diseases, but also could serve as a potential biomarker in several lung diseases and may be a promising treatment for chronic obstructive pulmonary

disease (COPD) (123). CC16 transfers passively across the air-blood barrier, along a concentration gradient (124, 125). Circulating CC16 rises in various pulmonary diseases characterized by increased epithelial permeability, such as acute respiratory distress syndrome (126), idiopathic interstitial pneumonia (127), and sarcoidosis (128) as well as after exposure to environmental air pollutants (129) or fire smoke (130).

Lower levels of CC16 in blood and airways have been associated with prevalence and severity of COPD (131). In addition, low serum CC16 was associated with a faster subsequent decline of forced expiratory volume in one second (FEV_1) among patients with COPD in the ECLIPSE (132) and Lung Health Study (133).

CC16 and the preterm lung

Early detection of increased serum level of CC16 as a marker of lung injury may be important, for interventions aiming to minimize lung injury, that is, by using more lung-protective ventilatory strategies (134) or certain pharmacology agents (135). Some studies show that low cord blood CC16 concentrations in preterm infants independently predict the development of BPD (136). Low CC16 levels may reflect early lung injury, which contributes to the severity of RDS and progress towards BPD.

Preterm neonates at risk for developing BPD show an enhanced inflammatory reaction in the lungs and an associated increase in pulmonary microvascular permeability (137). Local downregulation of CC16 has been associated with inflammatory lung disease and increased concentrations of CC16 can indicate injury to alveolar-capillary integrity (138). CC16 increase acutely in the blood of ventilated preterm neonates during the initial phase of mechanical respiratory support proportionally to respiratory disease severity and this increase could persist in those infants who later develop BPD (139).

In a study of preterm infants with RDS, Levine et al (140), found that recombinant human CC16 seemed to reduce pulmonary inflammation and was suggested to be a possible future therapeutic agent. The inhibition of cellular infiltrates was maximal on day 3. There was also a reduction in vascular permeability and protein leak in the infants who were treated with recombinant human CC16.

For the future CC16 is a biomarker which may be included in the battery of parameters collected in the preterm infant at birth.

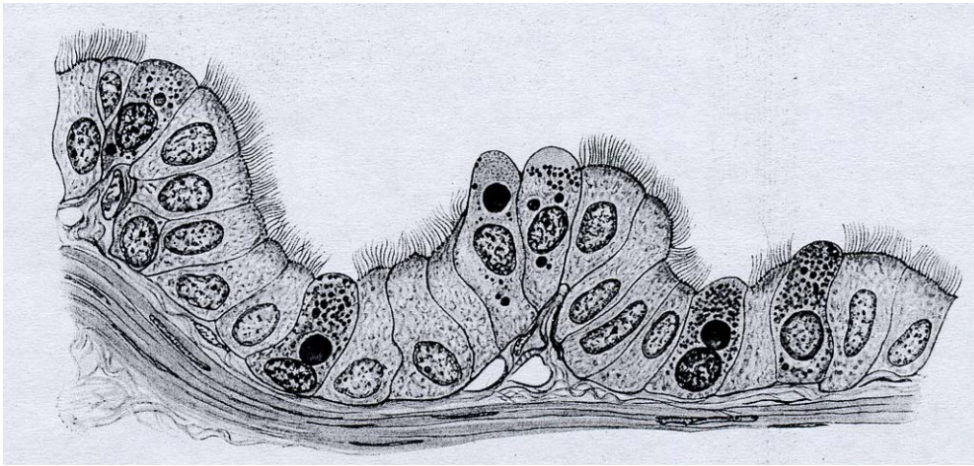

Figure 4. Histological view of club cells in the bronchial epithelium
The picture comes from Max Clara's original description of club cells, published in 1937. Original copyright owner unknown.

Cytokines

The immune system is divided into two separate response pathways. The innate immune system of the lung acts as an immediate non-specific response to foreign antigens and pathogens. It consists of phagocytic cells such as macrophages, monocytes and neutrophils, as well as mediators. The adaptive immune system mediates a response that is antigen specific but slower than the innate immune system. It works through activation of lymphocytes. Interactions between and within the adaptive and the innate immune system is regulated by specific proteins called cytokines. These have a molecular mass of less than 30 kDa. Cytokines act by binding to specific receptors on immune cells with high affinity, hereby regulating the intensity and duration of the inflammatory response in general as well as in the preterm lung. Interleukins (IL) are grouped into families based on their structure or the receptors. They do not act alone, almost always they operate in an environment with other mediators such as tumour necrosis factor-alfa (TNF-α), chemokines and other growth factors, such as insulin-like growth factor-1 (IGF-1).

In our studies, we measured the *pro-inflammatory* cytokines (IL-1β, IL-2, IL-6, IL-8, IL-12, TNF-α and IFN-γ) and the *anti-inflammatory* cytokines (IL-4 and IL-10). Furthermore, monocyte chemoattractant protein-1 (MCP-1) and matrix metalloproteinase-9 (MMP-9) were analyzed. For overview of origin and function of these biomarkers see Table 2.

Table 2. Characteristics of measured inflammatory biomarkers (141).

IL1-β	Important inflammatory mediator mainly produced by activated macrophages. Activates lymphocytes. Acts together with TNF-α.
IL-2	Produced by T lymphocytes. Promotes growth and activity of T-cells.
IL-6	Secreted by macrophages. Initiate acute phase protein synthesis and production for neutrophils. Support B cell division. Also act as a hematopoetic progenitor.
IL-8	A potent chemotactic factor produced by various cell types which promotes recruitment of neutrophils and stimulates angiogenesis.
IL-12	Induce production of interferon-gamma (IFN-γ) and TNF-α. Promotes differentiation and proliferation of T-cells.
TNF-α	Early and abundant mediator in inflammation. Produced by many different cell types. Participate in vasodilation, edema formation and leukocyte adhesion to epithelium through expression of adhesion molecules.
IFN-γ	Activator of macrophages. Expression is induced by IL-12 and IL-18.
IL-4	A multifunctional immunoregulatory cytokine produced primarily by mast cells, Th2 cells, eosinophils and basophils. Enhances IgG and IgE production. Co-stimulates B-cell proliferation and the differentiation of B-cells into plasma cells.
IL-10	A potent anti-inflammatory cytokine produced by a multitude of immune cells. Downregulates expression of pro-inflammatory cytokines and enhances B-cell proliferation and production of antibodies. Inhibits antigen presentation and Th1 cells.
MCP-1	A chemokine also referred to as chemochine ligand 2 (CCL2). Produced by several celltypes. Attracts or enhances the expression of other inflammatory factors or cells, such as monocytes and macrophages.
MMP-9	An enzyme which regulates breakdown and remodeling of extracellular matrix and basement membranes and plays a role within action of neutrophiles.

When the preterm lung is exposed to mechanical ventilation and oxygen, an inflammatory response could be initiated where neutrophils and macrophage invade the airways. This may trigger a delay or an arrest of both alveolarization and angiogenesis (142). Maternal chorioamnionitis and the subsequent fetal inflammation response syndrome (FIRS), initiate increased levels of IL-1β, IL-6 and IL-8 in the preterm infant and has been associated with development of BPD (143). The response to inflammation by the anti-inflammatory cytokine IL-10 may be impaired in immature lungs (144).

Tracheal aspirate (TA) is one indicator of inflammation and microvascular permeability in the preterm lung and an increase in inflammatory markers in TA may start a development of BPD already in the first week of life (145). In a study by Iwatani et al, TA volume was significantly higher in ventilated infants as a measure of more inflammation and permeability and also a predictor of BPD (146).

Several studies continue to map the complexity and coherence for cytokines and the neonatal lung, regarding etiology, development and course of disease (9, 145, 147-150).

Fractional exhaled NO (FeNO)

Nitrogen oxide (NO) is synthesized in the human airways by various cells in the lung, predominantly vascular endothelial cells, inflammatory cells, epithelial cells and airway nerve cells, through oxidation of the amino acid L-arginine to L-citrulline. This reaction is catalysed by an enzyme called nitric oxide synthases (NOS).

Three distinctive forms of NOS can be detected, two of which are constitutive isoforms, neuronal NOS (nNOS) and endothelial NOS (eNOS). These isoforms produce lower amounts of NO continuously and are both involved in physiological processes within the lung as vasodilators, bronchodilators and neurotransmitters. Working as a pluripotent mediator its role is complex and still not fully understood. The third isoform is inducible NOS (iNOS), and this form can produce high concentrations of NO in airway inflammation as a non-specific defence mechanism against pathogens. iNOS is said to act as a pro-inflammatory mediator and may cause oxidative tissue damage. High concentrations of NO measured in exhaled breath can be found in patients with asthma especially those with an atopic phenotype, and combined with eosinophilic inflammation (151). Furthermore, FeNO may be used to predict and evaluate pharmacological treatment with steroids and to monitor airway inflammation (152).

Different techniques can be used to measure exhaled NO, including chemiluminescence, electrochemical detection and laser spectroscopy (153). For clinical purposes, the most used technique is the electrochemical, which is also available as a portable device for close follow-up at home. According to ERS standard, measurements of FeNO < 25 ppb (parts per billion) are assumed to indicate absence of eosinophilic airway inflammation, while FeNO > 50 ppb suggest an ongoing inflammation. Values between 25 and 50 ppb should be judged together with clinical symptoms (154). For children FeNO <20 ppb indicate absence of airway eosinophilic inflammation, while measurements above 35 ppb suggested that such inflammation was present (152).

Various both exogenous and endogenous factors have been shown to influence on FeNO levels in humans (155). Constitutive factors including age, height and gender seems to affect the measured values. In children, increased airway epithelial surface area during growth results in a 100% increase in levels between 7 to 17 years of age, from 7 to 14 ppb (156). Also, in male gender, the larger airway epithelial surface in relation to body size increases the measured FeNO by 15-25% (157). Exogenous factors include tobacco smoke, air pollution and airway inflammation. A high dietary intake of nitrate may increase the measured FeNO by 100% (158).

Also, other underlying conditions with eosinophilic inflammation that is IgE-sensitation like allergic rhinitis could lead to increased FeNO levels. Even COPD may have this effect. Because of the above-mentioned limitations FeNO should not be used exclusively as a diagnostic tool but rather as a complement to other measurements of airway impairment.

Analysis of exhaled NO at different flow rates could further improve more detailed information in NO production in both bronchial and alveolar compartments (159, 160).

Lung physiology and measurements

Lung function testing is an important part of the diagnosis, management and understanding of airway and respiratory disorders, as well as an objective method to ensure rectified results for follow-up. This includes information concerning

- Lung growth and development of the immature lung
- Quantitative measurements of severity of disease
- Interpretation of physiological processes and an opportunity to follow changes over time
- Effects of various interventions and treatments
- To some extent consider epidemiological evaluation and detect risk factors for disease

Dynamic lung volumes

Spirometry is the standard for assessing and follow lung function over time. It is readily available and is an invaluable screening test for general respiratory health, both in clinical settings and in research. Indications for performing spirometry vary from evaluating symptoms, signs or abnormal laboratory tests of the lung, measure the effect of a disease on pulmonary function, assess prognosis, pre-operative risks, and to ensure therapeutic interventions (161).

Spirometry requires cooperation between the subject performing the test and the examiner. The result obtained depends on technical skills as well as personal factors. Children are regarded mature enough when reaching the age of 5 to 6 year, with individual differences.

One parameter is forced vital capacity (FVC), which is the volume change between a full inspiration and a maximal forced expiratory volume. FVC represents the size of the lungs but does not include the volume left after airway closure, the residual volume (RV). Other parameters, most frequently used in children are forced expiratory volume in 1 second (FEV_1) and peak expiratory flow (PEF). These are both measurements of flow and they are dependent of resistance of the airways. But since resistance of the airways differ between central and peripheral segments it is believed that FEV_1 mostly reflects central changes in the bronchial tree. To evaluate

smaller and more peripheral airways the forced expiratory flow between 25% and 75% of the expired volume (FEF_{25-75}) in combination with FEV_1/FVC is believed to give better reflection of the physiology. FEF_{25-75} is also known as the maximum mid-expiratory flow. The ratio FEV_1/FVC may give an indication of the connection between lung volumes and the size of the airways. Other ratios for clinical purposes are FEF_{25-75}/FVC and the dysanapsis ratio, calculated according to Duke et al (162), which are used to describe the discrepancy in size between airways and lungs. Vital capacity (VC) is the volume in the lung after a full unforced slow inspiration preceded by a complete expiration. For assessing central obstruction as sign of increased airway resistance, spirometry is the method of choice, but for small airway pathophysiology and changes over time it needs to be performed in combination with other more sensitive lung function tests.

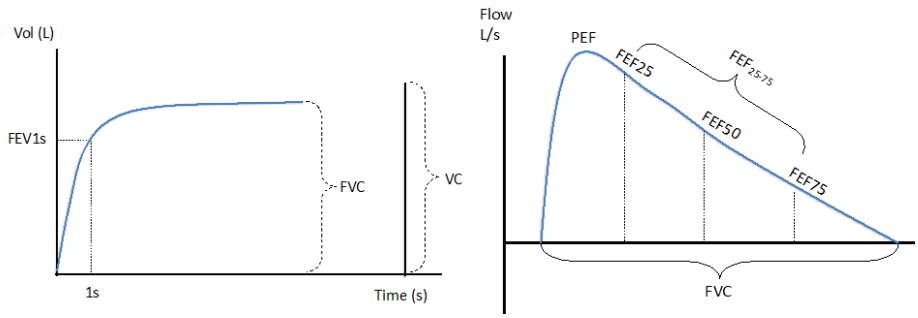

Figure 5. Spirometry parameters.
A graphic representation of different spirometry parameters.

Prematurity and spirometrical values

Several studies on effect of preterm birth on later FEV_1 have been performed. Kotecha et al (163) did a systematic review and meta-analysis and found an increased risk of deficits in FEV_1 as percent of predicted in all preterm-born survivors, also the deficits were greater in infants with BPD. Another review (164), evaluated long-term effects of BPD and outcome in school-aged children. They concluded that airway patency was decreased in BPD survivors compared with term controls, while total lung capacity and functional residual capacity were normal or only slightly reduced. Later studies have been somewhat more conflicting regarding relationship between BPD and pulmonary outcome. Prematurity per se most likely has a greater impact than first assumed (165, 166).

Static lung volumes

Body plethysmography is a method to obtain information of the volume of air that remains in the lungs after a maximal exhalation. This includes residual volume

(RV). The volume of gas inhaled or exhaled during the normal respiratory cycle is called the tidal volume (V_T). Total lung capacity (TLC) is the volume of gas after maximal inspiration, that is the sum of all volume compartments in the lungs. Another parameter is functional residual capacity (FRC), which represents the volume of gas present in the lung at end-expiration during tidal breathing.

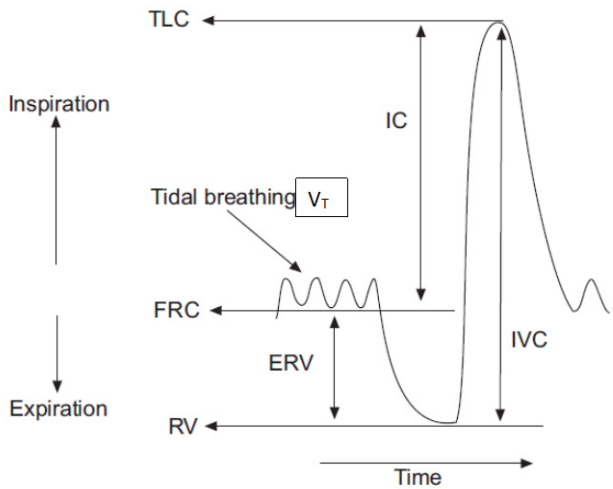

Figure 6. Lung volumes
A graphic representation of the different lung volumes. Modified from Miller et al **(161)**

Body plethysmography is mainly used for measurements of static lung volumes and airway resistance. It is regarded to have good reproducibility and is sensitive to early changes in lung mechanics. All measurements of plethysmography are based on Boyle's law, meaning that when a constant mass of gas is compressed or decompressed, the gas volume will decrease or increase so that the product of volume and pressure at any given time is constant, within the condition of an isothermal environment (167). For clinical purposes this means that the subject is placed in a closed chamber and the measurements are performed where all the above conditions are met, called a body box. The obtained values rely on detecting changes in box pressure in combination with either differences of mouth pressure or with flow rate under defined breathing conditions (168).

Hyperinflation is an abnormally large persisting gas volume in the lung remaining after full expiration and could be a sign of air trapping. This is seen as an increase in RV in relation to TLC, and the subsequent ratio RV/TLC increases as an early sign of changes in the asthmatic subject.

In children, lung volumes are related to body size, with standing height being the most important factor. In general, lung growth seems to fall behind the increase in height during childhood and adolescence so that in puberty there is a shift in the relationship of lung volume and height. Measurements are all done in accordance with European Respiratory Society/American Thoracic Society standards (ERS/ATS).

Prematurity and lung volumes

Static lung volumes do not seem to be affected in children born very preterm (164, 169). This is in line with our studies as well. However, the ratio RV/TLC, a measurement of air trapping, was higher in children born preterm compared to children born at term, which could indicate changes in the peripheral airways. Cazzato et al (170) also found an elevated RV/TLC in children with a diagnosis of BPD.

Diffusion capacity

DL_{CO} (diffusion capacity of the lung for carbon monoxide) is a measurement to evaluate and assess the lungs' ability to transfer inspired gas by the bronchial tree to the blood. To estimate lung volumes by different gas dilution techniques were developed in the beginning of 2000^{th} century (159, 171). Various gases have been studied since the development of the method, but today the most utilized gases are carbon monoxide (CO), methane (CH_4) or helium (He). CO has high affinity for haemoglobin and follows somewhat the same pathway as oxygen (O_2) to finally bind to haemoglobin. Therefore, the absorption of CO from inhaled air is dependent on all the aspects that follow O_2, that is ventilation, state of the alveolar-capillary membrane, cardiac output and levels of haemoglobin. DL_{CO} is the product of the rate constant for uptake of CO per unit barometric pressure from alveolar gas (KCO) and alveolar volume (VA). KCO is usually written as DL_{CO}/VA, which indicates the efficiency of CO transfer by alveoli.

There are three different techniques to measure DL_{CO}, single breath method, intrabreath method and rebreathing technique. By far the most assessable and used for clinical purposes and research is single breath technique. In single breath determination the DL_{CO} is measured during a 10 s breath hold at maximal inspiration, *i.e.*, at TLC. In absolute terms, this is said to represent total lung capacity (TLC). The alveolar lung volume during breath holding is measured simultaneously by using methane as a biological inert tracer gas. At the same time KCO is determined (172).

Changes in DL_{CO} parameters may indicate pathological processes in the lung. Decreased DL_{CO} and KCO is seen in chronic obstructive pulmonary disease (COPD) with emphysema due to alveolar destruction. Also, smoking may cause a decrease

in DL_{CO}. The mechanism for these changes is alveolar and/or microvascular damage and destruction which lead to loss of alveolar or capillary surface area. Reduced DL_{CO} and KCO is also seen in interstitial lung disease and pulmonary fibrosis due to thickening of the alveolar-capillary membrane. In asthmatic subjects an incomplete alveolar expansion without compromising the alveolar structure could increase KCO, most likely depending on better perfusion of different parts of the lung. This is a limitation of the gas dilution technique since the measuring only takes place in the parts of lung where there is a communication directly with the surrounding air.

Prematurity and diffusion capacity

Lower diffusion capacity among children born very preterm has been shown in numerous studies (170, 173, 174), both in children with a former diagnosis of BPD and those without BPD.

Resistance and reactance

Pathological changes in the small airways may be present in a variety of different lung diseases such as BPD, COPD and asthma. These small, often inaccessible airways are frequently involved at an early time-point in the development of lung disease and may show pathology before any clinical symptoms and/or changes in other lung function tests or imaging have occurred. Sometimes they are referred to as “the quiet zone” of the lung (175).

Furthermore, throughout airway generations there is a reduction of the length and diameter of the airway. Exponential increase in airway numbers lead to an increase in cross-sectional area with every subsequent generation. This contexture of the respiratory system has two effects on airway physiology. 1) At any given flow, the velocity of gas within the lung decreases with more peripheral generations in the bronchial tree. This means that in the proximal airway the velocity is high, but in the more distal airway the flow is laminar and to a greater extent more dependent on gas density (176). At the interface between the conducting and acinar airways there is a change of flow, from connective to diffusion along a concentration gradient. 2) In a healthy subject the resistance to airflow in the small airways constitutes between 10 to 25% of total airway resistance (177).

To maintain an equitable ventilation to different parts of the lung, small airway resistance is independent of lung volume, whilst the reverse is true for large airway resistance. These arrangements make it possible to maintain low airflow and minimal work of breathing. In disease, small airway resistance increase. The are several reasons for small airway obstruction and hence increased resistance, is due to several mechanisms, including mucus, reduction of luminal diameter from inflammatory changes, smooth muscle hypertrophy or airway thickening. Also, loss

of structural support of the airway may cause collapse and thereby increased resistance. In physics, these structural changes are a result of Pouseille's law that states that the resistance to flow is inversely proportional to the fourth power of radius. In impulse oscillometry (IOS), pressure impulses are sent through the respiratory system at a spectrum of frequencies, and measurements provide information about the mechanical properties of large as well as small airways. Since this method only requires tidal breathing, no forced maneuvers and is non-invasive, it is suitable for children. IOS measures the respiratory impedance, which includes resistance and reactance, measured over a range of frequencies. Higher frequencies, that is > 20 Hz, travel shorter distances, generally in the larger airways, while lower frequencies < 15 Hz reaches deeper into the lung parenchyma and smaller airways. Resistance at 5 Hz (R_5) reflects total resistance and resistance at 20 Hz (R_{20}) represents central resistance. Peripheral resistance is subsequently measured as R_5-R_{20}.

During childhood, age and height have an impact on resistance and reactance. Development and growth of the lungs imply an increasing diameter of the bronchus/bronchioles and the number and size of alveoli. This gives decreasing values of resistance at all frequencies and X_5 shows lower negative values.

If small airways are obstructed resistance become frequency dependent. Reactance (X) is the sum of two components, elastance and inertance. Both are dependent on oscillation frequency. X_5 reflects the elastic recoil of the peripheral airways. Elastance is regarded as the back pressure created *after* some of the flow has occurred, while inertance is the opposing force in the airways that builds up *before* the flow takes place (178). Pathological changes of the elasticity in the lung, such as fibrosis or hyperinflation, result in more negative values of X_5. The resonant frequency (F_{res}) is where the pressure and elastic recoil cancel out and reactance is zero. Changes in these two parameters cause alteration in the area under the reactance curve (AX) as measured from X_5 to F_{res} (179-181). Reference values used in this thesis are based on Dencker et al (182).

Prematurity and impulse oscillometry

In a study by Malmberg et al (183), forced oscillation technique (FOT) showed a higher respiratory resistance and lower reactance in school-aged children with BPD. Similar results were found by Vrijlandt et al (184). In boys born moderate to late preterm an increased frequency dependency of resistance (R_5-R_{20}) and AX were measured at 16 years of age (185) and longitudinal data from IOS have showed an increased peripheral airway resistance over time in a group of preterm children with BPD compared to those without BPD (186).

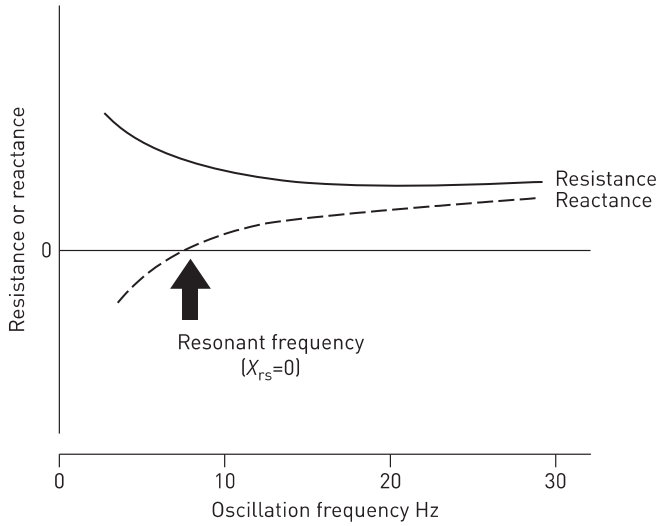

Figure 7. Impulse oscillometry parameters
Diagram of frequency dependence of resistance and reactance. Reprinted with permission of the ©ERS 2022. European Respiratory Journal. Copyright © ERS 2022 All rights reserved. King, GG et al 2020/Technical Standards for respiratory oscillometry/Eur Respir J/2020;55:1900753 **(181)**

Ventilation inhomogeneity

Inert gas washout (IGW) is based on the principle that gas is transported and mixed in the lung. It is a technique that can detect pulmonary ventilation inhomogeneity. The respiratory tree is divided into conducting (generation 0-16) and acinar (generation 17-23) airways. Small or peripheral airways are defined as those with a luminal inner diameter of < 2 mm, which equals to generation 8-23. In the conductive airways there is a linear gas flow, and the velocity is relatively high. Further caudally, the airways divide, the total cross-sectional diameter increases and linear gas flow velocity decreases. In healthy subjects, distribution of normal ventilation occurs by convection and diffusion. At the level of transition from conducting to respiratory airways, diffusion becomes important as a driving factor for gas transport. Where the convection and diffusion are equal in force a "diffusion-convection front" is produced (187).

Currently, three main mechanisms of ventilation inhomogeneity are known: **convection-dependent** in the conducting airways, **diffusion-limited** related in the distal airways (acini) and interaction between convection and diffusion in the **diffusion-convection** zone (acinar entrance). In the alveoli (acinar compartment), a capillary meshwork embeds and forms a large surface for gas exchange (188, 189). Changes in small airway dimensions, that is different pathological processes affect

the distribution of ventilation and shown as an inhomogeneity. IGW analyses the composure of exhaled gas and determine the concentration of the inert gas, that is the gas that is not taken up into the bloodstream. In the initial studies, nitrogen (N_2) was used as the gas of choice, but in infants, inhalation of pure oxygen (O_2) could change the normal tidal breathing pattern and other inert gas came in use, such as sulphur hexafluoride (SF_6) and helium (He). Most important when using different gases are that they do not participate in the gas exchange or dissolve in the blood or other tissues. Also, it must be safe for the participants to inhale.

There are two different techniques for IGW. Initially, single breath washout (SBW) was used when performing a classical vital capacity (VC) maneuver with exhalation to residual volume (RV), followed by inhalation of 100% oxygen (or a mixture of gases containing SF_6 or He) to total lung capacity (TLC), and then exhalation from TLC to RV. An expirogram with four different phases is produced. The first phase represents the dead space, where no gas mixing takes place. The second phase comes from gas mixing with the rapidly increased concentration of N_2. Air from peripheral airways enter the mouthpiece, where the gas is analysed. Also called the bronchial (transitional) phase. The alveolar phase, which is the third phase the concentration of exhaled N_2 reaches a plateau and represents air from more peripheral lung segments. If there was an entirely even ventilation this plateau would be flat, but even in a healthy lung there are regional differences due to gravity and this gives rise to some uneven ventilation heterogeneity. But the oscillation in this phase is to some extent also dependent on the heart pulse. The fourth phase is believed to represent airway closure in the basal lung regions occurring as the residual volume (RV) is approached. This phase is also referred to as the closing volume which is defined as the volume between the start of phase four and RV (Figure 8). Initially the closing volume was considered a sensitive index of peripheral airway obstruction, but now the phase III slope is the preferred index. The steeper the slope, the more ventilation heterogeneity.

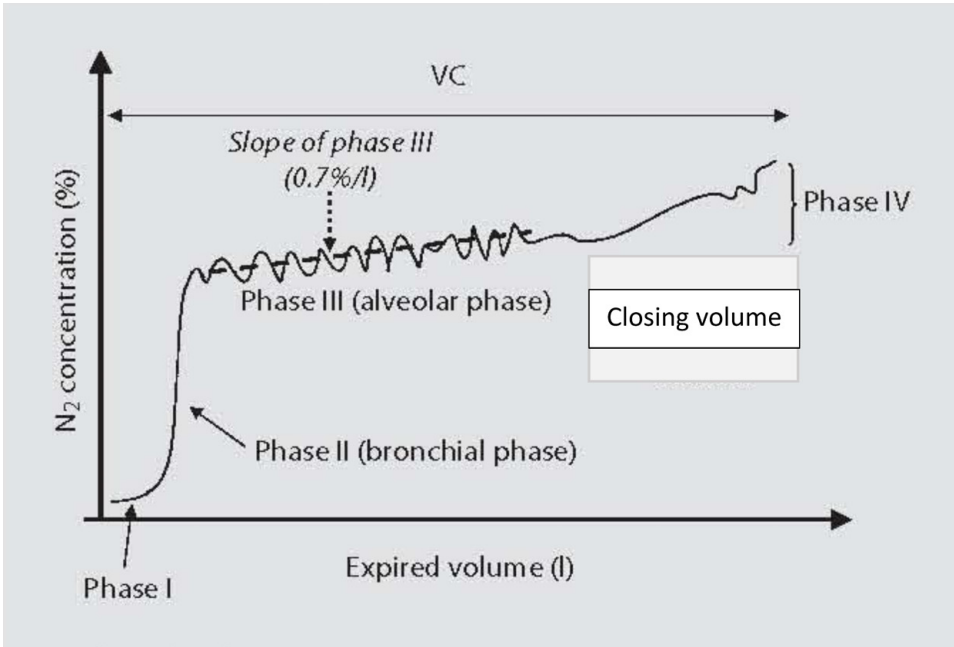

Figure 8. Curve of VC in single breath washout (SBW) in healthy subject.
Modified from Robinson et al **(188)**.

Multiple breath washout (MBW) is more time consuming but has the advantage of being more informative. It is useful for children since it requires minimal co-operation and co-ordination. Like IOS, MBW is performed during tidal breathing which eliminates potential effects on smooth muscle tone by manoeuvrers of deep inspiration and forced expiration. The test is performed until the end-tidal inert gas concentration reaches $1/40^{th}$ of the starting concentration. This corresponds to approximately 2.5% for N_2. When the total volume of expired air during a measurement equals the functional residual capacity (FRC), one turnover is said to have occurred. The total number of turnovers required to complete the test is called the lung clearance index (LCI). For clinical measurements, the number of lung volume turnovers required to lower the end-tidal N_2-concentration to 5% and 2.5% of the initial concentration is used ($LCI_{5.0}$ and $LCI_{2.5}$ respectively). The difference being that $LCI_{2.5}$ reflect the more peripheral units of the lung than $LCI_{5.0}$. Lung clearance index measures an overall ventilation inhomogeneity and provides no detailed information on where this heterogeneity arises.

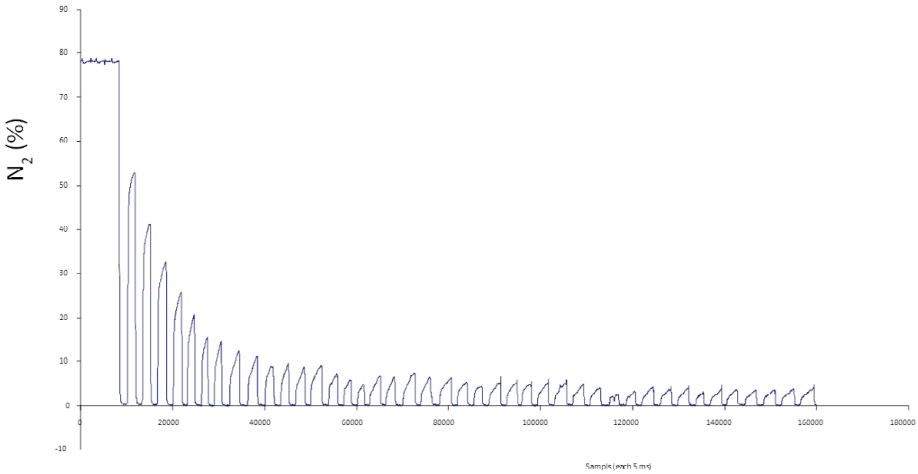

Figure 9. Lung Clearance Index in MBW.
The LCI is calculated as the number of lung volume turnovers (TO) required to clear the lungs of the inert marker gas (N_2) to 1/40th (=<2%) of the starting concentration.

Analysis of the N_2 concentration during different phases of each breath, from the alveolar phase III slope, provides more detailed information of where inhomogeneity arises. These measurements are called S_{cond} and S_{acin} and allows to dissect differences in conducting and intra-acinar airways, respectively. Sn_{III} is a term used to describe where the phase III slopes concentration is normalized. The first values of slope III are thought to reflect ventilation inhomogeneity within the diffusion-convecting front and represents the acinar airways (S_{acin}). The subsequent evolution of SIII values represent ventilation heterogeneity in the conductive airways since different time constants for filling and emptying parallel lung units in lung turnover 1.5 to 6.0, depends on dissimilar degree of obstruction and is called S_{cond}.

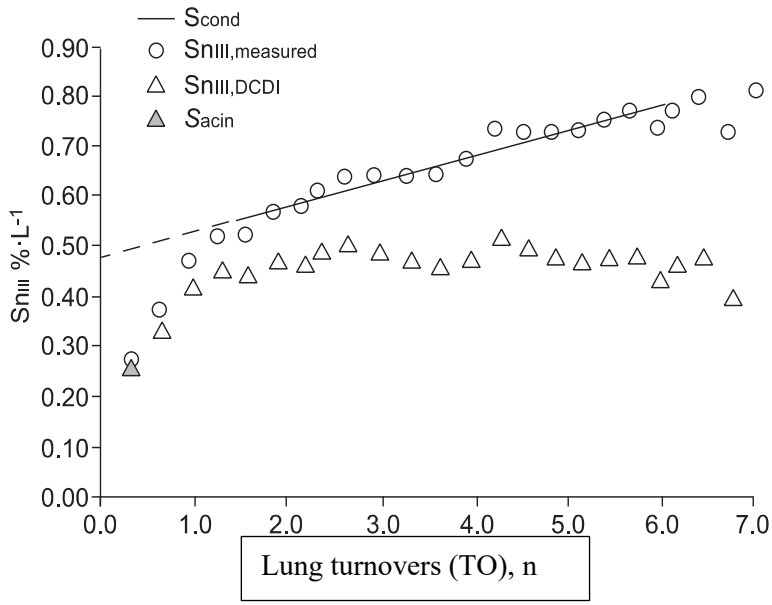

Figure 10. Multiple breath washout test illustrating phase III (alveolar) slope parameters.
Modified from Robinson, PD et al **(188)**

For children, S_{cond} and S_{acin} are corrected by multiplying to tidal volume (in litres) in each subject, since MBW normally calculate with a fixed tidal volume (1.0-1.3 litres) and respiratory rate10-12 breaths per minute. Higher values of LCI, S_{cond} and S_{acin} indicate more ventilation inhomogeneity. The model of S_{cond} and S_{acin} is theoretical and highly user dependent, and conclusions are complex mostly because of specific details on the locations of the pathological event. There are however some studies that have shown that dysfunction in the peripheral conducting airways could increase S_{cond} in patients with mild asthma (190, 191).

In conclusion, the MBW technique is sufficiently sensitive for early detection of impairment in the lungs that arises in the peripheral airways and the method can add information that cannot be obtained by other lung function tests or imaging of the chest (159, 189).

Prematurity and parameters from N_2-washout

More ventilation inhomogeneity has been shown in children born very preterm (173, 174), but there are relatively few studies until now.

Aims

The overall aim of this thesis was to investigate the impact of inflammatory and protective pulmonary biomarkers obtained in very preterm infants at birth on early respiratory morbidity and later lung function at 12 years of age, and in these infants evaluate lung function in relation to bronchopulmonary dysplasia, sex and term born infants.

Paper I

To evaluate if levels of CC16 in gastric fluid at birth in very preterm infants are related to severity of lung disease, measured as need for mechanical ventilation and BPD. Also, to relate markers of inflammation in tracheal aspirate to levels of CC16 in gastric fluid at birth.

Paper II

To determine if perinatal inflammation, quantified as plasma cytokine levels at birth and during the first 72 h of life, in very preterm infants, is associated with early lung disease, development of BPD, and lung function impairment at 12 years of age.

Paper III

To extensively assess lung function pattern at 12 years of age in three cohorts of very preterm infants in relation to term born controls and to evaluate the impact of BPD and sex on lung function measurements, as well as different airway symptoms.

Paper IV

To explore if an inflammatory imbalance, quantified as low CC16 and high pro-inflammatory cytokines in tracheal aspirate in very preterm infants early after birth would influence on persistent airway symptoms and lung function abnormalities at 12 years of age, and if those could be detected by pulmonary function testing.

Participants and methods

Study population

The study population consisted of three previously studied cohorts of preterm infants, and in one of the cohorts also term controls. The first two cohorts included very preterm infants. The third cohort consisted of extremely preterm infants, but also included term born controls. All preterm infants had previously been admitted to the Neonatal Intensive Care Unit in Lund (Figure 11) (192-194). Among the children not included for lung function testing at 12 years of age some could not perform lung function testing because of physical and/or mental disability, some had moved outside the reach of the study and some of the caregivers of the children declined participation. One child, a triplet, was included in cohort 1, in connection with the lung function testing, together with the siblings.

In the *first cohort*, 71 long-term survivors born very preterm in Lund 2001 to 2003 were included. Inclusion criteria were gestational age < 32 weeks, antenatal consent and no congenital anomalies. In this cohort 55 children participated in lung function measurements at 12 years of age. Among the 14 children not examined, some could not perform lung function testing because of physical and mental disability, some had moved outside the reach of the study and some of the children declined participation.

In the *second cohort*, 64 very preterm infants, born in Lund 2005-2007 were included. Inclusion criteria were similar to the first cohort except that gestational age was < 31 weeks. In this cohort 41 children underwent lung function testing at 12 years of age.

The *third cohort* was a subset of infants from a population-based study including all births before 27 weeks gestational age in Sweden during a three-year period from 2004-2007 (EXPRESS), together with matched term born controls selected from the Swedish Medical Birth Registry. At 12 years of age, 61 children born preterm and 56 term born controls performed lung function measures. There was an overlap between the two latter cohorts, where 21 children from the second cohort also took part in the population-based study.

Some families declined participation in the lung function study, resulting in a total of 136 preterm and 56 term born children being examined.

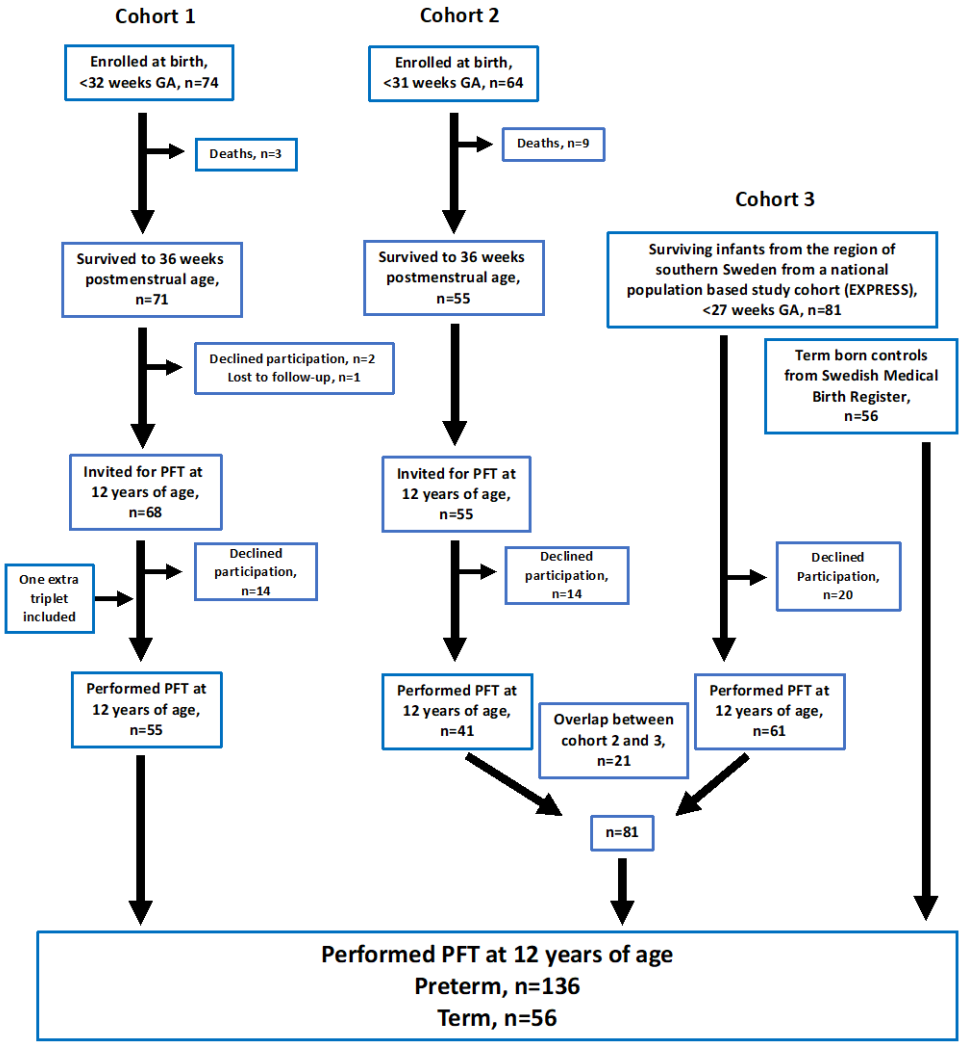

Figure 11. Flow chart of the included children.
PFT = pulmonary function test.

Study design

Paper I

Children from the second cohort participated (n=41). Antenatal and neonatal data were prospectively retrieved from medical records. Information regarding fraction of inspired oxygen (FiO_2), mean airway pressure in ventilated infants, blood gases at 6, 24 and 72 h of age, as well as total number of days on mechanical ventilation or continuous positive airway pressure (CPAP) were prospectively recorded.

Treatment with supplemental oxygen at 28 days of age and at 36 weeks postmenstrual age (PMA) was registered. BPD was defined as the infant having supplemental oxygen at 36 weeks postmenstrual age. Blood samples were obtained from the umbilical cord and from an arterial line at 24 h of age. Sampling of gastric fluid was collected within the first hour after birth during routine insertion of a nasogastric tube and before any enteral feeding. Tracheal aspirate was collected exclusively in infants on mechanical ventilation during a routine tracheal suctioning procedure within the first 24 h of age, but at the earliest 6 h after surfactant administration. The manoeuvre was performed using a tracheal suction set attached to a sampling tube. Concentrations of TNF-α, IL-1β, IL-6, IL-10, MCP-1, MMP-9 and CC16 were analysed in plasma and tracheal aspirate. CC16 was also analysed in gastric fluid.

Paper II

Children from the first cohort participated (n=55). Perinatal and neonatal data were prospectively recorded from the infant's birth until hospital discharge. Respiratory data including fraction of inspired oxygen (FiO_2), mean airway pressure in ventilated infants and blood gases were registered at 6, 24, 48 and 72 h of age and coordinated with blood sampling for cytokines (TNF-α, IFN-γ, IL-1β, IL-2, IL-6, IL-8, IL-12, IL-4 and IL-10) at three of these time points (6, 24 and 72 h). Cytokine burden was calculated as area under the curve (AUC) from birth to 72 h of life. Surfactant treatment, number of days with mechanical ventilation or CPAP as well as treatment with postnatal systemic corticosteroids were recorded. The accumulated dose of systemic corticosteroids from birth to discharge was calculated as hydrocortisone equivalents (mg/kg), assuming that 1 mg of betamethasone equals 35 mg of hydrocortisone. Lung function testing with spirometry, diffusion capacity of carbon monoxide of the lung and fractional exhaled NO was performed at 12 years of age. Relationship between this early life sampling and pulmonary function test at school age was presented.

Paper III

All described three cohorts participated, *i.e* 136 preterm born children and 56 term born children. Antenatal and neonatal data were collected prospectively. A history of respiratory symptoms, medication and allergy were obtained from a

questionnaire, which was completed by the accompanying parent/caregiver at the visit for lung function testing at 12 years of age. Extensive pulmonary function tests were performed including, spirometry, body plethysmography, impulse oscillometry, carbon monoxide diffusion capacity, and multiple breath washout. Comparisons according to lung function measurements were done between:
- children born very preterm vs children born at term,
- children born very preterm with vs without a previous diagnosis of BPD
- preterm and term born children according to male vs female sex.

All measurements were repeated 15 min after inhalation of 200 μg salbutamol, and reversibility was measured.

Paper IV

Children from the second cohort participated (n=41). Of these children 19 had samples from gastric fluid, 30 had samples from umbilical cord blood, 40 had plasma samples at 24 h of age and 20 had samples from tracheal aspirate. Registration of respiratory data was collected during hospitalization. Lung function testing included flow-volume spirometry, body plethysmography, impulse oscillometry, carbon diffusion capacity and multiple breath nitrogen washout.

Lung function testing

At the time of pulmonary function testing, height and weight were measured. To assess reversibility, all lung function measurements were performed before and 15 min after inhalation of 200 μg salbutamol through a valve spacer device. The subjects were asked not to take bronchodilators for at least 24 h before the tests. All measurements were done in accordance with European Respiratory Society/American Thoracic Society (ERS/ATS) guidelines (161, 167, 195).

In connection with the visit for lung function testing a short questionnaire mainly focused on respiratory illness was completed by the parents/caregivers. Information on maternal smoking, heredity, allergy, transient or current wheezing symptoms, the use of inhaled corticosteroids and overall health during the first 12 years of life was collected.

Spirometry

Flow volume spirometry was performed using a Jaeger MasterScope (Erich Jaeger GmbH, Würzburg, Germany). The child was sitting in an upright position wearing a nose clip. By breathing into a mouthpiece which is connected to a spirometer, the child was asked to exhale as fast as they can after a full inhalation. Then exhalation continued until a plateau was reached in the flow-volume curve, which is at least 6

sec. Forced expiratory volume in 1 sec (FEV_1), forced vital capacity (FVC), mean forced expiratory flow between 25% and 75% of FVC (FEF_{25-75}) and slow vital capacity (VC) was measured and the ratio FEV_1/FVC was calculated. FEF_{25-75}/FVC and the dysanapsis ratio, calculated according to Duke et al (162), were used to describe the discrepancy in size between airways and lungs. In this age group, according to data from ERS Global Lung Initiative (196), the lower limit of normal (5^{th} centile, -1.64 SD) for spirometry measures corresponds to approximately 80% of predicted, except for FEF_{25-75} where the lower limit of normal corresponds to 70% of predicted. The lower limit of normal for FEV_1/FVC is approximately 0.75.

Body plethysmography

Static lung volumes, that is total lung capacity (TLC), and residual volume (RV) were measured by body plethysmography using a MasterScreen Body (Erich Jaeger GmbH, Würzburg, Germany). The ratio RV/TLC was calculated as a measure of air trapping.

Plethysmographic measurements were performed after stabilization of temperature and pressure with the child placed in the box, door closed. Sitting in an upright position, equipped with a nose clip the screening begins and the child starts to breath tidally through a mouthpiece. Flow and pressure were measured. By closing the shutter valve, lung volumes could be calculated. Thereafter, a slow full exhalation, followed by a full inhalation and immediately afterwards a forced expiratory manoeuvre was performed, and when the expiratory time exceeded 6 sec the measurements were finished.

Carbon monoxide diffusion capacity

Single breath (SB) determination of carbon monoxide (CO) uptake in the lung during a 10 sec breath-hold at maximal inspiration was done using the MasterScreen PFT equipment (Erich Jaeger GmbH, Würzburg, Germany). The accessible alveolar volume was simultaneously measured using methane (CH_4) as a biologically inert tracer gas. The child wore a nose clip, sat in an upright position and initially breathes tidally through the mouthpiece, followed by a slow full exhalation and directly afterwards a quick full inhalation. Diffusion capacity of the lung for CO (DL_{CO}) is the product of the rate constant for uptake of CO per unit barometric pressure (KCO) and alveolar volume (VA).

Impulse oscillometry

Impulse oscillometry (IOS) was performed using the Jaeger MasterScreen IOS (Erich Jaeger GmbH, Würzburg, Germany). Pressure impulses are sent through the

respiratory system at a spectrum of frequencies, and measurements provide information about the mechanical properties of large as well as small airways. Participants pressed their palms against their cheeks and wore a nose clip to avoid upper airway shunting. Resistance is estimated at 5 Hz (R_5, reflecting total resistance) and at 20 Hz (R_{20}, reflecting central resistance). If small airways are obstructed, resistance becomes frequency dependent, with higher resistance at low frequencies, causing an increase in R_5-R_{20} (R_5-R_{20} reflecting peripheral resistance).

Reactance of the lung is estimated at 5 Hz (X_5, reflecting elasticity in the peripheral airways), and resonant frequency (F_{res}) is the frequency where the inflation pressure and elastic recoil cancels out and reactance is zero. Together, these variables form the area under the reactance curve (AX) as measured from X_5 to Fres.

Multiple breath washout

Multiple breath washout (MBW) of N_2 during O_2 breathing was performed with an Exhalyzer D (Eco Medics, Duernten, Switzerland). The child first breathed at a normal rate. After a few tidal breaths at room air, the measurements started by an influx of 100% oxygen (O_2) into the mouthpiece. Inhaled and exhaled air is continuously analysed for concentrations of N_2, O_2 and CO_2. The measurements stop automatically when the concentration of the marker gas (N_2) reaches $1/40^{th}$ of the starting concentration. The number of lung volume turnovers required to lower the end-tidal N_2-concentration to 5% and 2.5% of the initial concentration is termed lung clearance index ($LCI_{5.0}$ and $LCI_{2.5}$ respectively), a measure of overall ventilation inhomogeneity. Analysis of the N_2 concentration during different phases of each breath provide more detailed information of where the inhomogeneity arises. S_{cond} and S_{acin} are indices of ventilation inhomogeneity in the conductive and intra-acinar airways, respectively. Higher values of LCI, S_{cond} and S_{acin} indicate more ventilation inhomogeneity.

Biomarkers

CC16

Sampling from blood, gastric fluid and tracheal aspirate. Blood samples were obtained from the umbilical cord at birth and through an arterial line, at 24 h of age. After centrifugation plasma samples were stored at -80°C until analysed.

Gastric fluid was collected within the first hour after birth by a routine insertion of a nasogastric tube, and before any enteral feeding. The aspirate was immediately frozen after transferred into a sterile tube. The samples were stored at -80°C until assayed.

Tracheal aspirate was collected exclusively in infants on mechanical ventilation during a routine tracheal suction procedure within the first 24 h of age. If surfactant had been administered, sampling was performed at the earliest 6 h after the instillation and aspirate was collected using a tracheal suction set attached to a sampling tube (Unomedical A/S, Lejre, Denmark). After instillation of 0.3 mL of sodium chloride (9 mg/mL) into the tracheal tube, continuous suctioning at a standardized pressure of -200 mm Hg, was performed. This pressure was applied while slowly retracting the suction catheter. Afterwards the catheter was flushed with an additional 1 mL of sodium chloride into the sampling tubes. The aspirate was centrifuged at 1200 rpm for 10 minutes and the supernatant was removed and stored at -80°C until analysis.

CC16 was measured using the Human Club Cell Protein ELISA kit from BioVendor (Modrice, Czech Republic). Each analysis was run in duplicate and the detection limit for CC16 was 0.020 ng/mL. Since levels of CC16 in tracheal aspirate was diluted and heterogenic, the samples were normalized to total protein content.

Cytokines

Paper II

Samples were collected from umbilical cord blood and from arterial blood at 6, 24, and 72 h after birth through an indwelling arterial catheter (paper II). Blood samples were collected in a vacutainer tube containing the anticoagulant EDTA. After centrifugation plasma was stored at -80°C until analysed.

Levels of pro-inflammatory (TNF-α, IFN-γ, IL-1β, IL-2, IL-6, IL-8, IL-12) and modulatory (IL-4, IL-10) cytokines in plasma as well as MMP-9 and MCP-1 were analysed using cytometric bead array and flow cytometry according to manufacturer's recommendation (Becton Dickinson, San Jose, CA, USA) (192). This assay is based on a mixture of six microbead populations with distinct separate

fluorescent intensities (FL-3) and it is coated with different capture antibodies specific for each cytokine in the sample. The technique uses the sensitivity of fluorescence detection by flow cytometry to measure soluble cytokines, and each individual bead provides a capture surface for a specific cytokine. Standard curves could be generated for each cytokine from given standard samples at 0-5000 pg/mL. Subsequently two-colour cytometric analysis was performed by using a FACSCalibur flow cytometer (BD Biosciences). Thereafter data was acquired and analysed by using Biosciences CBA software. To exclude sample particles other than the 7.5 micrometer polystyrene beads forward versus side scattering was used. Flow cytometric analysis was executed and analysed by a single operator. Concentrations of cytokines were determined based on the standard curves by CBA software. Different cytokines had various concentrations for lower limit of detection, but they all ranged from 2 to 10 pg/mL. For all cytokines, a level of < 0.1 pg/mL was regarded as non-detectable.

Paper I and IV

Plasma samples were obtained from the umbilical cord at birth and from an arterial line at 24 h of age. After centrifugation, plasma was stored at −80°C until analysed. Concentrations of TNF-α, IL-Iβ, IL-6, IL-10, MCP-1, and MMP-9 in plasma were measured in duplicate. Before analysis samples were diluted 1:4. Measurements were performed by a Bio-Plex Human Cytokine assay (Bio-Rad Laboratories, Hercules,CA) according to the manufacturer's protocol. Lowest levels of quantification were 0.42, 0.50, 0.38, 0.68, and 0.82 pg/mL for TNF-α, IL-Iβ, IL-6, IL-10, and MCP-1, respectively. The inter-assay coefficients of variation (CVs) were below 10%, and mean CVs between duplicates were below 7%. Plasma samples for MMP-9 analysis were diluted 1:20 and measured by Milliplex Human MMP panel (Merk Millopore, Darmstadt, Germany). Lowest level of quantification was 91.98 pg/mL. Inter-assay CVs were 9.93%,15.24%, and 27.45%, at 81546, 910, and 204 pg/mL, respectively. Mean CV between duplicates was 1.47%.

Tracheal aspirate was collected as described for CC16 within the first 24 h of age. In tracheal aspirate samples, total protein content was measured by the Bradford Protein Assay (Bio-Rad Laboratories) according to the manufacturer's protocol. After addition of bovine serum albumin (BSA) to tracheal aspirate samples (final concentration 0.5%), multiplex analyses were performed as described for plasma samples with minor modifications. Analyses of TNF-α, IL-Iβ, IL-6, IL-10, and MCP-1 were performed on undiluted samples. For MMP-9 analyses samples were diluted 1:20 in sodium chloride (0.9%) with BSA (0.5%). Lowest levels of quantifications of TNF-α, IL-Iβ, IL-6, IL-10, and MCP-1 were 0.11, 10.1, 0.45, 0.14, and 0.21 pg/mL, respectively, and 95 pg/mL for MMP-9. Mean CVs between duplicates were below 15%. Concentrations of respective tracheal aspirate marker were normalized total protein content.

Fractional exhaled NO

Fractional exhaled NO (FeNO) was measured using a NIOX Flex analyser (Aerocrine AB, Stockholm, Sweden) at flow rates of 50 (giving $FeNO_{50}$), 100, 200 and 300 mL/s (197). Estimation of alveolar NO concentration and bronchial NO flux were made using a two-compartment linear model (160). During testing procedure, the child inhales ambient air depleted of NO and exhales into a mouthpiece at a fixed flow rate. The test was performed four times per flow rate. The output of NO is plotted against the exhalation flow rate and the slope and intercept can be calculated. The parameters given are alveolar NO and bronchial flux of NO.

Statistics

Statistical analysis was performed using IBM® SPSS® Statistics version 20 to 28. Due to presence of asymmetric distribution in some variables even after logarithmical transformations, nonparametric tests were used. Spearman's rank test was used for correlation analyses, Mann-Whitney U-test was used for comparisons between two groups and Wilcoxon's signed-rank test was used for paired comparisons. For all correlations, associations caused by outliers were considered as non-significant. Linear or logistic regression was used to adjust for gestational age in analysis of neonatal morbidities. Levels of cytokines and MMP-9 were logarithmically transformed before statistical calculations. Chi-squared test or Fischer's exact test were used to evaluate relationships between categorical variables. For all studies, p-values < 0.05 were considered significant.

Ethical considerations

All studies were performed according to the declaration of Helsinki. Caregivers and children received both oral and written information about the studies and signed parental informed consents were obtained before inclusion.

All studies were reviewed and approved by the Regional Ethical Review Board in Lund, with the following diary number: no 423-99 with amendment jan-03, no 87-03 with amendment 428/2004, 270/2005 and no 2013/779 with amendment 2016/215 and no 42/2004.

Results

Paper I

In this study levels of CC16 from three different compartments (blood, gastric fluid and tracheal aspirate) in 64 very preterm infant (cohort 2) was measured and evaluated for pulmonary inflammation and need for respiratory support. CC16 in gastric fluid at birth increased with increasing gestational age and birth weight. There was a mutual relationship between the concentrations of CC16 from different compartments. Levels of CC16 in gastric fluid and in tracheal aspirate correlated with CC16 in umbilical cord plasma and with CC16 in plasma at 24 h (Figure 12).

For all figures in paper I, unfilled circles indicate infants who survived to 36 weeks gestational age and filled circles infants who died before that time point.

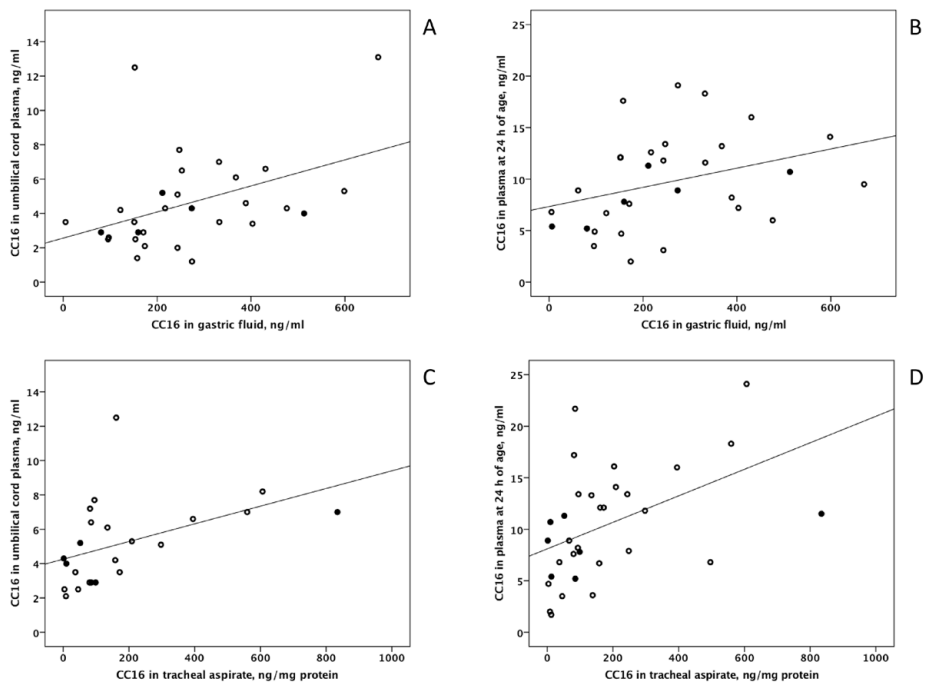

Figure 12. Scatter plot for relationship between CC16 in different compartments.

Low concentrations of CC16 in gastric fluid were associated with an increased requirement for both early and long-term respiratory support (Figure 13).

There were inverse correlations between CC16 in gastric fluid and concentrations of the pro-inflammatory cytokines IL-1β, TNF-α and IL-6 in tracheal aspirate. There was also an inverse correlation between CC16 in gastric fluid and the protease MMP-9 in tracheal aspirate (Figure 14).

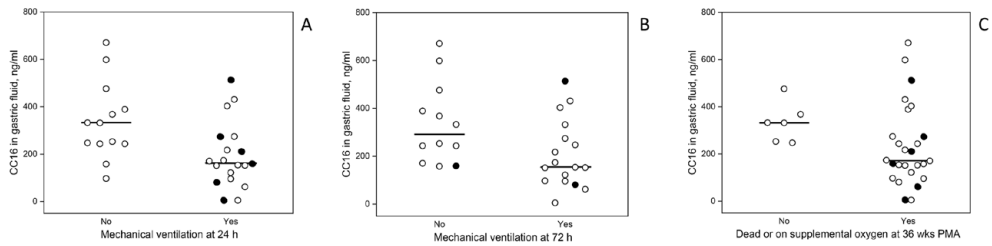

Figure 13. Boxplot for CC16 in gastric fluid and relationship to mechanical ventilation and BPD
Median and individual values for CC16 in gastric fluid at birth in infants with vs without mechanical ventilation at 24 h and at 72 h (A-B). Infants with no supplemental oxygen at 36 weeks postmenstrual age (PMA) vs infants with supplemental oxygen at 36 weeks PMA or death before 36 weeks PMA (C).

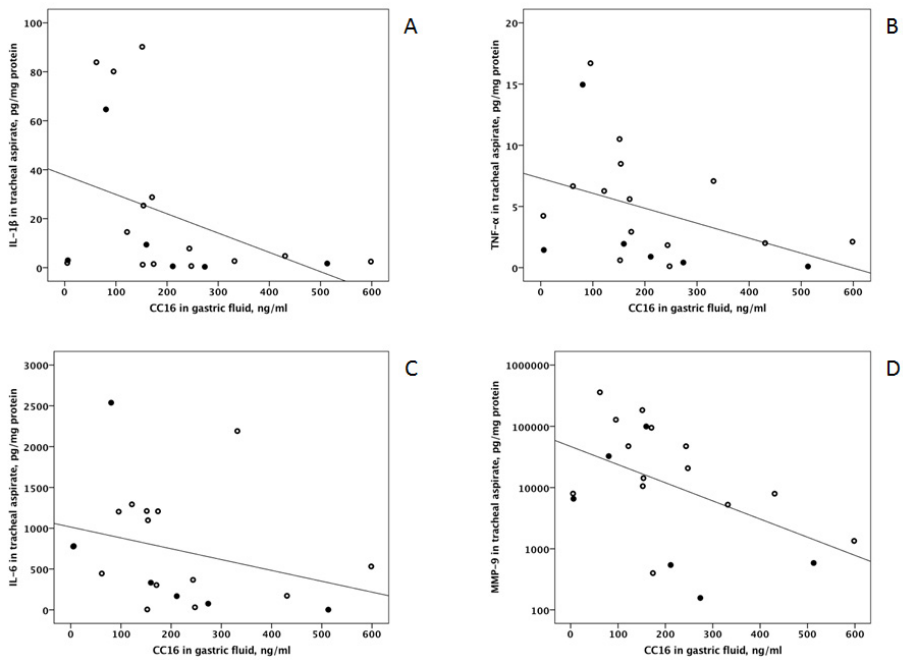

Figure 14. Scatter plot for individual values of CC16 in gastric fluid in relation to concentrations of IL-1β, TNF-α, IL-6 and MMP-9.

Lower concentrations of CC16 in gastric fluid at birth was associated with higher FiO_2 at 6 h of age, higher $PaCO_2$ at 24 h, more days on mechanical ventilation and more days with supplemental oxygen (Figure 15)

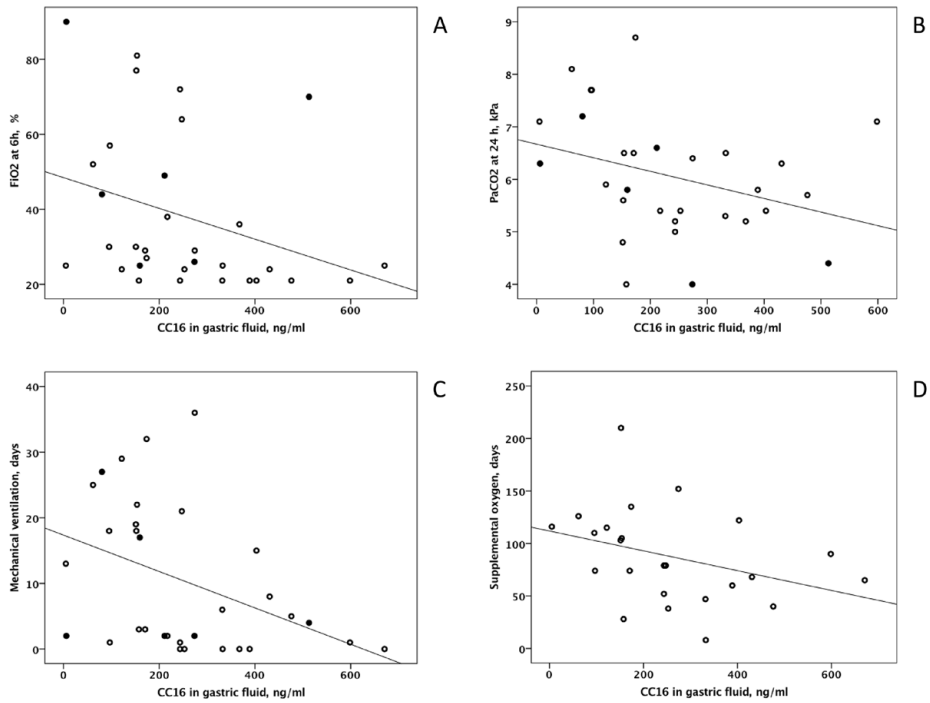

Figure 15. Scatter plot for individual values of CC16 in gastric fluid in relation to FiO_2 at 6 h, $PaCO_2$ at 24 h after birth (A-B) and days on mechanical ventilation and days with supplemental oxygen (C-D).

Paper II

Of the 71 survivors from cohort 1, 54 children came for lung function testing at 12 years of age. Infants with BPD had lower gestational age and lower birth weight in comparison to infants without BPD. A diagnosis of BPD was associated with more severe neonatal lung disease, reflected by higher need for surfactant, mechanical ventilation and postnatal corticosteroids.

Perinatal systemic inflammation, measured as a higher plasma cytokine burden (AUC from 0-72 h) was associated both with a more severe neonatal lung disease quantified as need for respiratory support and with a diagnosis of BPD. Infants in need of mechanical ventilation had higher levels of the pro-inflammatory cytokines IL-6, IL-8 and IL-10 (Figure 16).

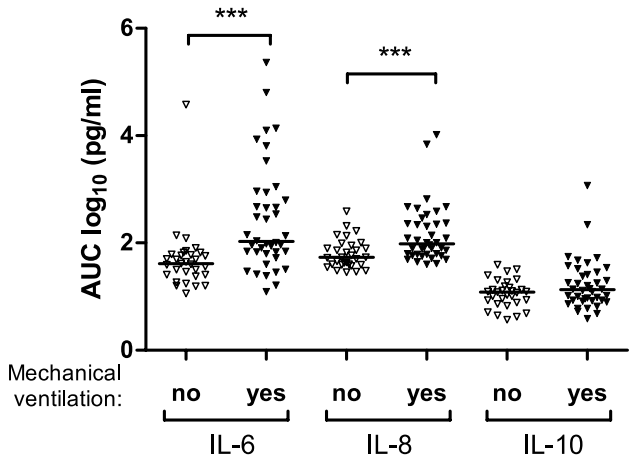

Figure 16. Individual area under the curve (AUC) values for IL-6, IL-8 and IL-10 in relation to need for mechanical ventilation.

Infants with a later diagnosis of BPD had higher plasma levels of IL-6, IL-8, and IL-10 in cord blood at birth and at most of the other sampling time points (Figure 17).

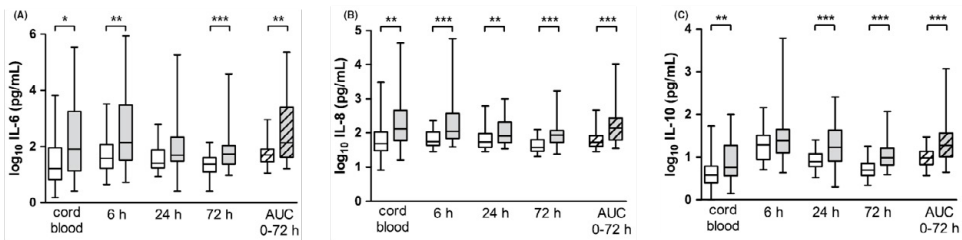

Figure 17. Plasma levels of IL-6 (A), IL-8 (B) and IL-10 (C) at four time points as well as AUCs for the three cytokines from birth to 72 h.
In each pair of boxes, the left (white) box shows infants without and the right (grey) box infants with BPD.

When evaluating a history of airway disease (history of wheezing, exercise-induced wheezing, sleep disturbed by wheezing and nocturnal cough), more children with a previous diagnosis of BPD had a diagnosis of asthma (44% vs. 53%, p=0.03) or a history of allergy (33% vs. 10%, p=0.04). Sixty percent of children with BPD vs. 41% of children without BPD reported at least one from a list of symptoms of airway obstruction, but that difference was not significant (p=0.17).

Lung function measurements showed that, values below the lower limit of normal were more common for tests reflecting airway obstruction than for lung volumes. Twenty-one percent of children had FEV_1/FVC <0.75, and 48% had FEF_{25-75} <70% of predicted. Lung volumes seemed to be unaffected. There was no significant difference in lung function between children with vs. without BPD during infancy. A higher plasma AUC for IL-6 from birth to 72 h of age was associated with lower FEV_1 and lower FEF_{25-75} at 12 years of age. (Figure 18).

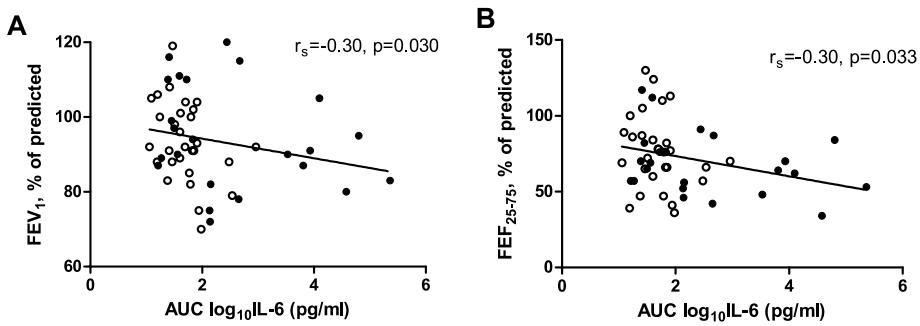

Figure 18. Individual values for FEV_1 and FEF_{25-75} as percent of predicted at 12 years of age.
Filled circles are children with and open circles children without BPD.

Paper III

Children from all the three cohorts participated in this study, that is 136 children born preterm and 56 children born at term. Among the children born preterm, 73% were born extremely preterm, *i.e.,* below 28 weeks of gestation and 62.5% had a diagnosis of BPD. Children with BPD had a lower gestational age at birth, a lower birth weight and were more often male.

Having had mechanical ventilation during the neonatal hospitalization was associated with increased airflow obstruction, lower diffusion capacity and lower KCO.

A history of wheezing and a diagnosis of asthma was more common in children born very preterm than in those born at term. Asthma was equally common in very preterm children with or without BPD.

Overall, children born very preterm had worse lung function at 12 years of age than children born at term. This was most evident before bronchodilator inhalation and was seen in most of the tests, both as absolute values and as percent of predicted normal values (Figure 19 A-F), that is reversibility occurred both in children born at term and in children born very preterm, but the effect was in general larger in the

very preterm group. This was distinct for FEV_1/FVC, FEF_{25-75}, R_{5-20}, X_5, AX, F_{res}, DL_{CO}, KCO, S_{cond} and S_{acin}.

In children born very preterm, spirometry showed significant airway obstruction, measured as reduction in FEV_1, FEV_1/FVC and FEF_{25-75}. In children born very preterm 23.5% had FEV_1 below the lower limit of normal (LLN), while in children born at term, the corresponding proportion was only 3.6%. For FEV_1/FVC the proportions were 25.0% vs 9.1% and for FEF_{25-75} 39.7% vs 10.9%. The ratio FEF_{25-75}/FVC was lower in children born very preterm, but no differences in dysanapsis ratio (discrepancy in size between airways and lungs) was shown.

For body pletysmography inspiratory, expiratory and total airway resistance were higher in children born very preterm compared to children born at term. Impulse oscillometry showed obstruction of small airways in children born very preterm, with a higher total resistance (R_5), more frequency dependence of resistance (higher R_5-R_{20}), lower reactance (X) at 5 Hz, a higher resonant frequency (F_{res}) and an increased area under the reactance curve (AX).

Diffusion capacity (DL_{CO} and KCO) were significantly lower in very preterm children than in children born at term.

When performing N_2-washout children born very preterm had significantly higher lung clearance index, both as $LCI_{2.5}$ and $LCI_{5.0}$, indicating an increased ventilation inhomogeneity in the peripheral airways.

Children born very preterm were more reversible in FEV_1/FVC and FEF_{25-75} compared to children born at term, as well as more reversible in total airway resistance, frequency dependence of resistance (R_5-R_{20}) and airway reactance (X_5, AX and F_{res}). DL_{CO} and KCO increased after bronchodilator inhalation but remained significantly lower than in term children, though almost all children had values within a normal range after the inhalation.

At 12 years of age, children born very preterm with a former diagnosis of BPD had more airflow obstruction, higher airway resistance and a lower diffusion capacity than preterm children without BPD. However, the proportion of children with lung function outside of the normal range was not significantly different between children with or without BPD for any of the tests performed.

In a subgroup analysis among the children born very preterm with vs without BPD, the bronchodilator response was found to be similar and with the same significance levels, for spirometry, body plethysmography and diffusion capacity. Dysanapsis ratio was lower in children with a history of BPD, particularly in boys, but responded significantly to bronchodilator inhalation, so that after treatment, there was no longer a difference in this ratio between children born very preterm vs. at term.

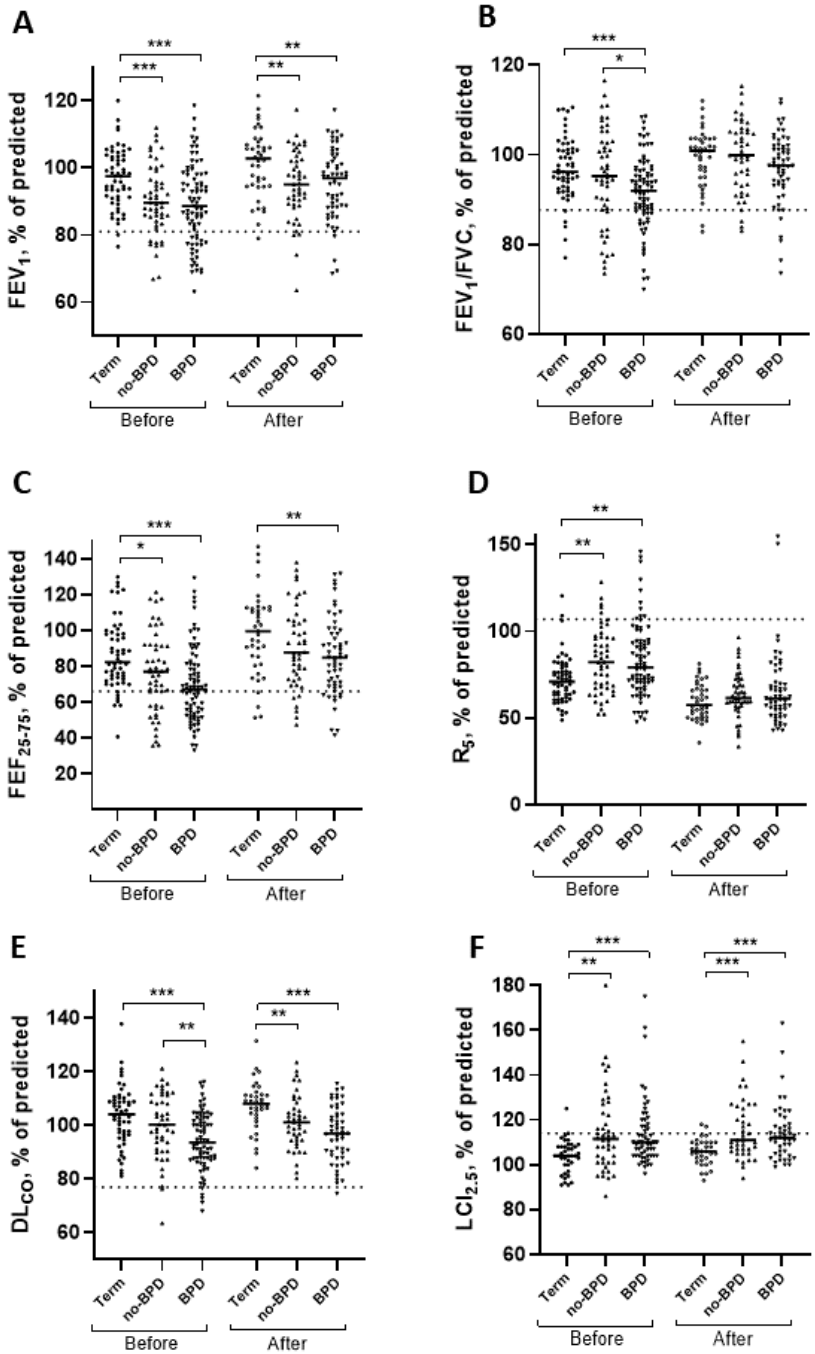

Figure 19. Individual boxplots for lung function tests in preterm children and children born at term, before and after bronchodilator and with and without a diagnosis of BPD.

During spirometry, boys born preterm had significantly worse results than both girls born preterm, and boys born at term, while girls born preterm did not differ significantly from girls born at term. However, during impulse oscillometry, a technique more sensitive in detecting dysfunction of small airways, values outside the normal range were equally common in preterm boys and girls, and both sexes had worse results than their counterpart.

Paper IV

In this study, CC16 levels have been measured in 41 of the 55 children that performed lung function testing at 12 years of age. The most common findings were airflow limitation and dysfunction of the peripheral airways. 20% of the children had expiratory flows below the lower limit of normal. When performing impulse oscillometry, 7-17% had R_5-R_{20}, F_{res} or AX above the 95th percentile, and 22% had X_5 below the 5th percentile, all findings are correlated as signs of small airway obstruction. Multiple breath washout showed ventilation inhomogeneity in the preterm children, while static lung volumes and diffusing capacity were within normal range. Lower levels of CC16 were associated with more airway obstruction reflected as a positive correlation between CC16 and the ratio FEV_1/FVC and an inverse correlation to the IOS parameters F_{res} and AX (Figure 20).

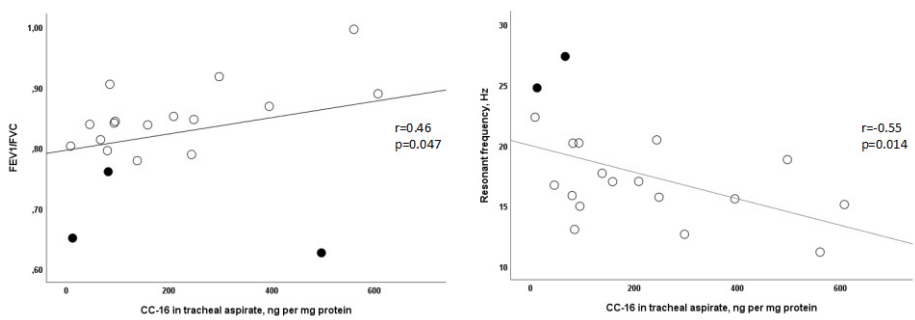

Figure 20. Association between CC16 in tracheal aspirate and parameters from spirometry and IOS. Open circles represent subjects with a lung function test within normal range, while filled circles are subjects with a lung function test outside the normal range.

The cytokine IL-1β in tracheal aspirate at birth was significantly higher in infants who later in childhood had clinically significantly airway obstruction defined as FEF_{25-75} < LLN, R_5 > 95th percentile, R_5-R_{20} > 95th percentile, X5 < 5th centile or F_{res} > 95th centile.

Ratios for IL-1β/CC16 and TNFα/CC16 were calculated as indices of inflammatory imbalance in tracheal aspirate. Higher values correlated with lower FEV_1, FEV_1/FVC, FEV_1/FVC, FEF_{25-75} and FEV_1/FVC and FEF_{25-75} both as percent of predicted. Also, higher values of these indices correlated with higher R_5-R_{20}, lower X_5, higher F_{res} and higher AX in impulse oscillometry as well as higher expiratory and total resistance during body plethysmography (Figure 21).

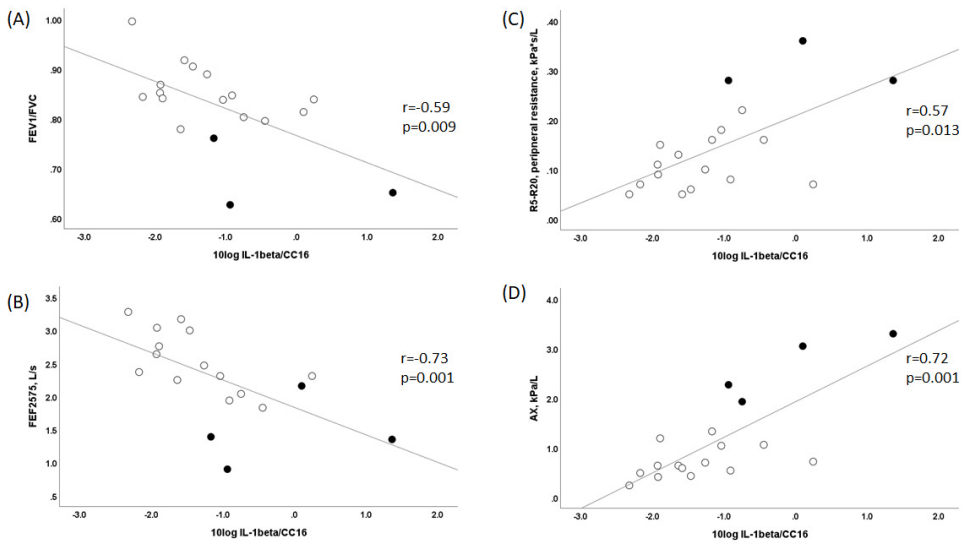

Figure 21. Scatterplot showing associations between the ratio of IL-1β/CC16 in tracheal aspirate after intubation in the first 24 h of life and measurements of airway obstruction. The ratio IL-1β/CC16 is presented as log 10 values. Open circles represent subjects with a lung function test within normal range, while filled circles are subjects with a lung function test outside the normal range.

Conclusions

Paper I

Low levels of CC16 in gastric fluid at birth was associated with increased inflammation in the trachea within the first 24 h of life and with more need for respiratory support in the neonatal period.

Paper II

Perinatal inflammation, quantified as AUC from 0 to 72 h for cytokines in plasma, was associated with BPD, and in the case of IL-6 also with airway obstruction at 12 years of age.

Paper III

At 12 years of age, children born very preterm had lower lung function than children born at term. Within the group of preterm born children, there was little difference between infants who had a previous history of BPD and those without such diagnosis. Obstruction of large as well as small airways improved markedly after bronchodilator inhalation.

Paper IV

An inflammatory imbalance in the trachea at birth, in very preterm infants, measured as levels of CC16 in relation to cytokines, may have consequences for airway function at mid-school age.

Discussion

During the process and work of this thesis, the complexity and multifactorial aspects of prematurity, BPD, lung function and future development towards a functional working pulmonary unit, complete or marked by the early-onset anatomical and physiologically changes evolved, have been more puzzling and challenging than first expected. Even though somehow everything seems to be connected and require a deeper and more thorough understanding, some pieces of the puzzle may be clearer, while others remain to be investigated.

Overall, children born preterm have developmental deficits in the lung and an increased risk of lung impairment during childhood, both regarding airway obstruction, but also in airway resistance, ventilation inhomogeneity and reduced diffusion capacity. The significance of BPD for future lung function seems to be of less importance regarding respiratory health, since lower gestational age and more pulmonary morbidity in children with BPD did not translate into a markedly more severe lung function impairment, though some differences remained, at school age (164, 166, 184). In this study, children with a history of BPD had more airway obstruction, measured as lower FEV_1/FVC, FEF_{25-75}, R_5-R_{20}, and a lower diffusion capacity than children without BPD, but these differences were smaller than the difference between the whole very preterm group and term-born controls. Also, the proportion of children with test results outside the normal range were not different between children with vs. without BPD for any of the tests performed.

The definition of BPD has changed over the past decades in an effort to maximize the possibility to predict future respiratory health and morbidity up to approximately two years of age, but the natural history of BPD later in life, both during childhood and adolescence and even up into adulthood is less well understood (19, 26, 170). The effects of prematurity per se may have a greater impact than previously assumed.

In paper II, more children with a previous diagnosis of BPD also had a diagnosis of asthma or a diagnosis of allergy, but the proportion of children reporting at least one in a list of symptoms of airway obstruction was not significantly different between those having or not having a diagnosis of BPD. Furthermore, a variety of airway symtoms have also been detected in children born moderately preterm without a diagnosis of BPD (198). Recent research also emphasize that BPD and asthma are two different entities with disparity in airflow and ongoing airway inflammation and

that different phenotypes affect the outcome and treatment strategies for these children (109).

Lung function impairment in children born preterm is often believed to be structurally determined and unaffected by treatment. There is insufficient evidence for reliable recommendations regarding routine management, long-term follow-up and a more aggressive approach for imaging and functional pulmonary function tests. Reversibility of lung function, after inhalation of a bronchodilator, in preterm children has previously mostly been studied for the effect of FEV_1 and to some extent even FEV_1/FVC and FEF_{25-75} (199). Our study confirms a significant reversibility for FEV_1, FEV_1/FVC, and FEF_{25-75}, but also for many other parameters, including resistance measured by body plethysmography, all parameters measured by impulse oscillometry and diffusion capacity. A substantial proportion of children born preterm normalized their airflow after a single dose bronchodilator inhalation, and for several parameters, especially those reflecting mainly central airway obstruction, the difference between children born preterm and at term was no longer significant after bronchodilator. These findings indicate that some children born very preterm may be undertreated. In a recent publication by Goulden et al (200), concluded that a combined treatment with inhaled corticosteroids (ICS) and long-acting β_2 agonists (LABA), is beneficial for lung impairment due to prematurity, in the ages 7-12 years. However, a substantial proportion of children in that study had FeNO >30 ppb, but was lower after treatment, indicating that eosinophilic inflammation may have been more common in that population than in ours (paper II).

Children born very preterm have been reported to have a small airway size in relation to lung size. This is often assumed to be a structural deficit related to a failure of airway growth, not responsive to treatment. We calculated dysanapsis ratio from spirometry data and age (162), but as all children were of the same age, we also used a simplified ratio, FEF_{25-75}/FVC. These two ratios were lower in children with a history of BPD, particularly in boys, but responded significantly to bronchodilator inhalation, so that after treatment, there was no longer a difference between children born very preterm vs. at term. Generally, the proportion of infants with irreversible airway obstruction is lower in our study than reported previously, not least for the children born at extremely low gestational ages. In the EPICure follow-up (110), 19% of children had irreversible airflow obstruction, defined as post-bronchodilator FEV_1/FVC below -1.96 z-scores, at 11 and at 19 years, which should be compared with only 8% (3/36) in our <26 weeks of gestation subgroup at 12 years. Our study thus gives a somewhat less dismal view of lung function in very preterm survivors than previously reported. The reasons for this are unclear but may perhaps be related to changing practices of delivery room care, less invasive respiratory support and better nutrition. Obviously, no conclusions can be drawn from measurements at a single time point, emphasizing the importance of follow-up of these well-studied cohorts, if possible until adulthood.

In our studies we could show that for different biomarkers, an inflammatory imbalance in the lung in the first day of life of very preterm infants may as well have consequences for airway function at school age. Several studies (10-13) have shown an association between elevated and in some cases depressed blood cytokines in the early life of preterm infants and a later diagnosis of BPD, but long-term outcome of the infants has not been reported. The cytokines associated with BPD, that is IL-6 and IL-10, showed an early elevation, whilst IL-8 displayed a more persistent elevation in these infants. IL-8 is a powerful attractor of neutrophils and is the cytokine most associated with BPD (201), whereas IL-6 participate in both the acute phase response and in the transition to chronic inflammation (202). Since cytokine concentrations change rapidly during the first hours and days of life, a measurement of cytokine burden, AUC, might be a more robust approach for an accurate reflection of changes in inflammatory status over a defined time period. The difficulty with many of the different cytokines are that they originate from different inflammatory cells. Systemic or circulatory signs of inflammation may not represent ongoing pulmonary inflammation, but we were able to evaluate and measure concentrations from different compartments in the preterm infant. Several studies have implicated a more severe development of BPD in preterm infants where an imbalance in inflammatory mediators could be measured (9, 14, 203).

For CC16, which is a highly lung specific protein, low levels in tracheal aspirate and/or in gastric fluid seem to predict respiratory outcome in a more precise manner compared to concentrations in plasma. Blood levels of CC16 reflects a diffusion along a concentration gradient from airways to serum, and may be a spill-over from a damaged alveoli-blood barrier (125). Guerra et al (131) demonstrated that low levels of CC16 in serum are a risk factor for lung function impairment in childhood and an accelerated decline in lung function in adults. All these samples were taken from blood in childhood and in adults. In our studies, where we had access to samples from different compartments early postnatally, we could show an association between long-term lung function outcome and CC16 in tracheal aspirate, but not in plasma. This might suggest that lung-specific levels of CC16 may be crucial for respiratory outcome in a more sensitive way. In gastric fluid, a low level of CC16, < 200 ng/mL, was associated with high inflammatory mediators in the lung but also with death and moderate to severe BPD.

To further investigate the balance between pro-inflammatory and protective biomarkers we evaluated ratios of IL-1β/CC16 and TNF-α/CC16. We found that the inflammatory imbalances in tracheal aspirate after birth were associated with airflow limitations, small airway dysfunction and higher resistance in the airway at school age. This shows the significance of an abnormal inflammatory state at birth in the preterm infant, has impact on later lung function outcome.

An interesting study by Levine et al (140) showed that exogenous administration of CC16 intratracheally, could reduce neutrophil counts and protein concentrations in

tracheal aspirate as an indication of less acute lung injury in the preterm infant. The effect was not persistent since a rapid elimination could be shown which might suggest that repeated doses must be given to maintain relatively high levels of CC16 in the lung. One unexpected finding was that high doses of exogenous CC16 could down-regulate the endogenous gene expression, which should initiate further investigation. An optimal dosing regimen and the effect of treatment on the development of BPD and future long-term pulmonary outcome needs to be performed.

Overall, there are relatively few studies who reflect upon exposures or treatment that could potentially alter pulmonary outcome in very preterm infants and be important for lung function at school-age or into adolescence (66, 204). Since the trajectory of lung function through childhood, adolescence and into adulthood is largely unknown, long-term evaluation and follow-up seem vital for future respiratory health. Already during the neonatal hospitalization and beyond, both pulmonary testing as well as biomarkers from different compartments, is essential to establish guidelines on comprehensive strategies and management for treatment further on.

In conclusion it is vital to keep in mind that there are many perinatal risk factors that affect the immature and developing lung, but it is also obvious that many postnatal exposures may have an impact over the years. It is probably a variety of factors that influence individual outcome, in this vulnerable population.

Future perspectives

During the process and work of this thesis many reflections have been made and questions have been raised. Further and new aspects of lung function in preterm survivors have become apparent, which need to be evaluated.

- The trajectory of lung function in children born very preterm, from early childhood to adolescence and adulthood, is not completely understood. We hope to contribute by comparing lung function data at 6 and 12 years of age in the cohort of children born before 27 weeks of gestation.
- There is also insufficient data comparing trajectories of lung function at later ages in individuals born preterm vs. at term. We therefore hope to be able to repeat a comprehensive lung function test in late adolescence or early adulthood in some of the children now examined at 12 years of age, preferably together with term controls.
- In my study, the amount of difference in lung function between boys and girls and the amount of reversibility was unexpected. It has been reported that the male disadvantage is turned into an advantage after puberty, but this needs to be confirmed and more thoroughly examined, and we hope to be able to contribute to this.
- The reversibility in lung function demonstrated in this study indicates that these infants may be undertreated. We would like to perform a randomized follow-up trial to measure effects of different medication, like bronchodilators, inhaled steroids and antileukotrienes for both short- and long-term pulmonary outcomes?
- We are planning to start using a new technique for 'Airway Dimension Assessment' (AiDA) to determine distal airspace radius. This may be a new way to assess structural damage and developmental arrest in children born very preterm, with or without BPD.
- There is a great need for guidelines to determine which very preterm infants should be included in specialized follow-up programmes to maintain optimal development of lung function and respiratory health. This thesis will hopefully contribute to an evidence base for such follow-up.

Populärvetenskaplig sammanfattning

Att födas för tidigt, prematurt, dvs före 37 fulla graviditetsveckor, kan vara förenat med olika följd- och resttillstånd senare i livet. I Sverige föds ca 900 barn per år mycket för tidigt, före 32 graviditetsveckor, och denna sköra kategori av barn utsätts tidigt för skadliga stimuli från en miljö som de mognadsmässigt inte är helt redo för. Överlevnaden för barn som föds för tidigt har kontinuerligt ökat. Detta beror till stor del på förbättrad förlossningsvård med administrering av kortison till mamman vid hotande för tidig förlossning, vilket både påverkar produktionen i lungorna av det vattenavstötande ämnet surfaktant (smörjmedel i lungorna) och påskyndar den allmänna lungutmognaden, men också på grund av att den neonatala intensivvården har utvecklats snabbt de senaste 35–40 åren. Förbättrad näringstillförsel, avancerat andningsstöd, anpassad dosering av vätska, syrgas och läkemedel samt en individualiserad, utvecklingsstödjande vård har lett till bättre förutsättningar, både under neonatalperioden och under barndomen, för dessa barn.

Lungsjukdom till följd av prematuritet är vanligt och bronkopulmonell dysplasi (BPD) är den vanligaste komplikationen efter extremt prematur födsel. I en nationell svensk studie på barn födda före 27 graviditetsveckor drabbades 62% av BPD, varav svår BPD hos 14%. BPD karakteriseras av en avstannad utveckling av lungblåsor (alveoler) och kärl i den omogna lungan och leder till ett långvarigt behov av extra syrgas och en nedsatt lungfunktion. Orsaken är multifaktoriell där inflammation, tillväxthämning, ärftlighet, miljö, genetiska faktorer, skadefaktorer efter födelsen och störd kärltillväxt har associerats med BPD-utveckling, men där de exakta mekanismerna inte är helt kända (205). Effektiva strategier för att förebygga och/eller behandla BPD saknas i nuläget. Flera studier har visat att för tidigt födda barn löper en ökad risk för bestående lungfunktionspåverkan upp i vuxen ålder (169). Man har börjat använda en nyare term, post-prematurity respiratory disease (PPRD), som är ett samlingsbegrepp för att poängtera att även barn som inte fått diagnosen BPD kan ha en påverkad lungfunktion efter neonatalperioden och under uppväxten (30).

För att identifiera barn med lungfunktionspåverkan rekommenderas en tidig uppföljning av lungfunktionen. Från 3–4 års ålder kan man göra en impulsoscillometri (IOS), där man får information om barnet har trånga luftvägar och om andningen kan förbättras av luftrörsvidgande medicin. Detta kan göras i öppenvården och kräver inte att barnet behöver göra något annat än att andas som vanligt genom ett munstycke. Spirometri, den vanligaste av alla

lungfunktionsundersökningar, mäter förmågan att andas ut en stor volym så snabbt som möjligt, vilket barn brukar kunna medverka till vid omkring 5-7 års ålder. Många av de för tidigt födda barnen har symtom på överkänsliga (hyperreaktiva) luftrör trots en i övrigt relativt normal lungfunktion. De kan behöva testas vidare med andra alternativa metoder såsom ansträngningsprovokation. Ju äldre barnen blir desto fler lungfunktionstester finns, som i mer detalj kan avgöra var i luftvägsträdet som de största problemen för det enskilda barnet finns, dvs perifert eller centralt i luftvägarna. Det har tidigare saknats riktlinjer för hur för tidigt födda barn, med eller utan BPD, ska behandlas efter nyföddhetsperioden. European Respiratory Society, en internationell medicinsk organisation där man tar fram riktlinjer för olika typer av andningsrelaterade besvär och sjukdomar, har i vissa utvalda fall, och på individuell basis rekommenderat inhalationskortison och/eller bronkvidgande medicin (18). Syftet med behandling är att minska symtom på överkänsligheten i luftrören samt få muskulaturen i de trånga luftrören att slappna av, vilket ger barnen möjlighet att vara aktiva och delta i dagliga fysiska aktiviteter, utan symtomgivande andningsbesvär. Det är viktigt att tidigt identifiera och vid behov behandla dessa barn så att ett regelbundet fysiskt aktivitetsmönster kan grundläggas tidigt i livet.

I denna avhandling har jag kartlagt så kallade biomarkörer, vilket i detta fall betyder mätbara substanser som kan ge information om individens nuvarande eller framtida hälsotillstånd, under nyföddhetsperioden och relaterat dessa till lungfunktionen hos de för tidigt födda barnen vid 12 års ålder. Jag har varit särskilt intresserade av att se vilka effekter en obalans mellan inflammationspådrivande och inflammationshämmande markörer kan ha på kort och lång sikt för lungutvecklingen. Barnen i mina studier kommer från tre olika grupper av barn födda i Lund mer än 8 veckor för tidigt under åren 2001 till 2007. De har initialt ingått i olika neonatala studier med syfte att undersöka inflammatoriska parametrar, tillväxtfaktorer, olika typer av samsjuklighet till prematuritet såsom hjärnblödningar, ögonförändringar (ROP-retinopathy of prematurity) och BPD. Barnen bjöds in med sina föräldrar till en omfattande lungfunktionsundersökning vid 12 års ålder, där det också ingick ett frågeformulär om allmän hälsa, välmående, symtom från luftvägarna, astmadiagnos, allergi och eventuell medicinering för detta. Jag har kallat min studie LUFF – lungfunktionsundersökning av för tidigt födda barn.

CC16 är ett lungskyddande äggviteämne som bildas i luftvägarna och som kan finnas i otillräcklig mängd hos mycket för tidigt födda barn. Vätska från lungorna sväljs under fosterlivet ner i magen, och koncentrationen av CC16 i maginnehåll vid födelsen återspeglar barnets förmåga att bilda ämnet. I mitt första arbete kunde jag hos mycket för tidigt födda barn påvisa ett samband mellan lågt CC16 i magsaft, tidig inflammation i lungorna och mer andningsbesvär hos det nyfödda barnet.

I mitt andra arbete kunde jag visa att en högre mängd inflammationspådrivande ämnen (så kallade cytokiner) i blodet de första tre levnadsdygnen hade samband

med ett ökat behov av respiratorvård och en ökad risk för BPD, här definierat som fortsatt behov av extra syrgas fortfarande vid en mognadsgrad motsvarande 36 graviditetsveckor, dessutom en ökad täthet i luftrören vid 12 års ålder.

I mitt tredje arbete sammanställde jag resultat av en omfattande lungfunktionsundersökning vid 12 års ålder hos 192 barn, 56 födda i rätt tid och 136 födda mer än 8 veckor för tidigt, 85 med och 51 utan BPD. Jämfört med de fullgångna barnen hade barnen som fötts för tidigt sämre resultat på ett flertal lungfunktionsundersökningar, särskilt sådana som påvisar trånga förhållanden i stora och små luftrör och en ojämn fördelning av ventilationen i lungorna. De för tidigt födda barnen hade också fått diagnosen astma oftare än de barn som var födda i normal tid. Skillnaderna mellan mycket för tidigt födda barn med och utan BPD var inte lika stora, varken avseende lungfunktion eller symtom från luftvägarna. Däremot hade för tidigt födda pojkar en större påverkan på lungorna jämfört med flickorna. Glädjande nog svarade de för tidigt födda barnen bra på inhalation av luftrörsvidgande medicin med en betydande förbättring av lungfunktionen.

I ett fjärde arbete studerade jag balansen mellan inflammationspådrivande (cytokiner) och inflammationshämmande (CC16) ämnen i luftvägssekret från för tidigt födda barn som behövde respiratorvård första dygnet. Det fanns också ett samband mellan låga nivåer av CC16 och höga koncentrationer av en specifik cytokin, IL-1β, i luftstrupen och täthet i de små luftvägarna vid lungfunktionsundersökningarna vid 12 års ålder.

Sammanfattningsvis visar mina studier att det finns ett samband mellan nivåerna av inflammationspådrivande i förhållande till inflammationshämmande ämnen hos för tidigt födda barn redan kort efter födelsen och senare lungpåverkan både i ett kort- och långsiktigt perspektiv.

Målsättningen har varit att få en djupare förståelse för hur tidig inflammation hos prematurfödda barn kan påverka lungfunktionen när de blir äldre, men också försöka förstå hur man skall kunna identifiera de neonatala riskbarnen tidigare och kunna planera en strategi för utredning och behandling baserat på den unika individens förutsättningar.

Acknowledgements

This thesis would not have been possible without the work, support and encourage from many people. I would like to express my gratitude to some of all these amazing individuals.

First, I would like to thank all the preterm children and children born at term for taking their time to participate in the studies and their parents who supported the participation. Without all of you this thesis would never have been written.

I have had the privilege of having three outstanding supervisors *Ellen Tufvesson, Ingrid Hansen-Pupp and Lars Björklund.* Thank you for believing in me and provide me with this opportunity, for always standing beside me, motivate and support me during this process of work.

Ellen, in the beginning we did not know each other, but I liked you instantly. I could see your enthusiasm and curiosity for research and that was contagious. Over the years you have inspired me with your constant optimism, your ability to see possibilities instead of obstacles, your immense knowledge and to always asking the right questions. Sometimes I just needed to talk and you to listen, thank you for supporting me during difficult times, giving me of your time and for always being there during all these years. I actually think you made me a researcher and for that I am truly grateful.

Ingrid, this was your idea to begin with - to encourage me to write about the immature lung, to dare to step out into the research world, and you have been by my side all the way. Throughout this work you have supported and challenged me, helped me with wise discussions and pushed me forward when needed. Thank you for believing in me and never give up hope.

Lars, you are my true role model, as a doctor, a researcher and as a human. Your knowledge, humility and your ability to be in the moment. I always say that what you do not know in the field of neonatology, it is not worth knowing. You always manage to provide new angles and insightful suggestions. Your competence is an inspiration. Thank you for taking the time to help me and find the right way.

I owe my deepest gratitude to *Ann-Cathrine Berg* and *Eva Hammarstrand* for excellent help with retrieving clinical data, to *Ida Åberg* for help with analyzes of CC16 and to *Jonas Olsson* and *Anna Sikesjö* for lung function testing of the children. A special thanks to *Gunnel Hellgren* who participated in paper I.

I want to express my sincere gratitude to:

All my colleagues at the Pediatric Pulmonary Department, *Christine Hansen, Kristján Dereksson, Stephanie Diemer, Gunilla Garbov, Sandra Wallsten, Kristel Björkman, Sara Hedén, Marianne Wik, Karolina Wallin, Huda Abdulahi, Elin Wennerberg, Mikael Nilsson, Moa Eriksson* and *Helga Elidottir.* Thank you for your understanding during my long periods of absence in fulfilling this thesis and for your knowledge, friendship and warmth.

All my former and present colleagues at the Neonatal Department, for being my "home" for many years. Thanks for allowing me to work in the most educating and humble environment, for developing and changing me to be a better doctor, for all the laughter and encouragement and for growing as a human.

All amazing past and present staff at the "62an" and "mottagningen" – thanks for making every day a joy.

All my colleagues at the Pediatric Department in Ystad, *Björn Jönsson, Linda Resman, Anna Malmer, Djula Berisha* and all absolute wonderful staff who welcomed me with open arms when beginning as a paediatrician all over again.

All staff at the Respiratory Medicine and Allergology Department, for interesting discussions, encouragement, laughter, inspiration and always being welcoming.

Professor emeritus *Leif Bjermer,* for believing in this project and sharing your lifelong wisdom in pulmonary medicine.

Bengt Andreasson, for introducing me to the world of neonatology.

My dear family,

My mother, *Else-Britt,* for believing in me from the very beginning and always being supportive.

My father, *Lennarth,* for encouragement, support and a lot of invigorative talks.

My sister, *Katja,* my best friend and dearest sister. Thanks for always being there, believing in me when I didn't, for all our daily talks about everything and nothing. Love you sis!

The most important persons in the world, my wonderful sons, *Truls* and *Måns.* You are the light and joy in my life, the reason making everything worthwhile and the greatest inspiration. I have learned so much from you. I love you endlessly.

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