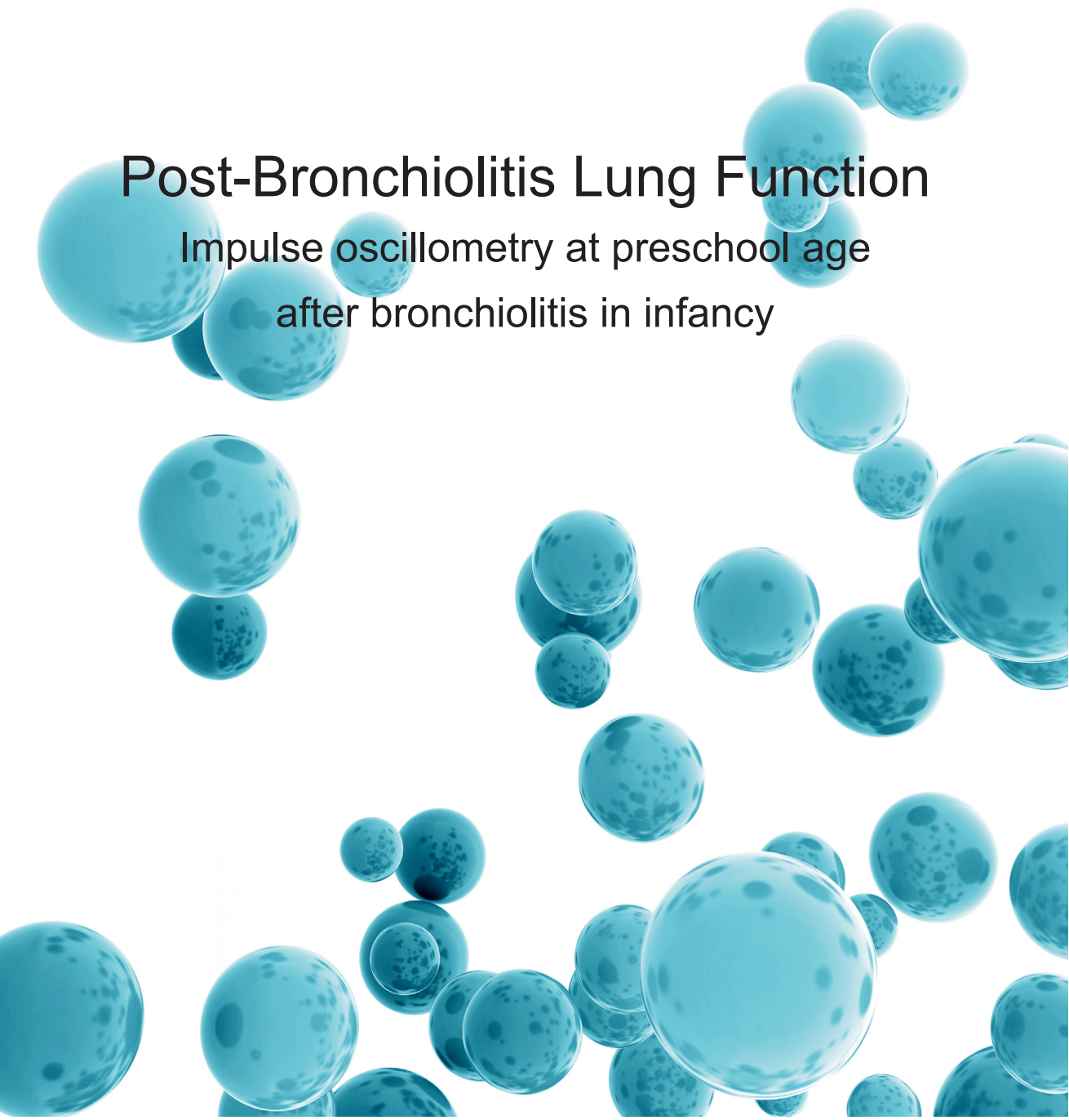
Acta Universitatis Tamperensis 2165

EERO LAUHKONEN

Post-Bronchiolitis Lung Function

Impulse oscillometry at preschool age after bronchiolitis in infancy



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ACADEMIC DISSERTATION
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UNIVERSITY OF TAMPERE

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Contents

List of original publications

This dissertation is based on the following five original publications:

I Lauhkonen E, Koponen P, Nuolivirta K, Paassilta M, Toikka J, Korppi M. Lung function by impulse oscillometry at age 5-7 years after bronchiolitis at age 0-6 months. *Pediatr Pulmonol. 2015 Apr;50(4):389-95*

II Lauhkonen E, Koponen P, Nuolivirta K, Paassilta M, Toikka J, Saari A, Korppi M. Obesity and bronchial obstruction in impulse oscillometry at age 5-7 years in a prospective post-bronchiolitis cohort. *Pediatr Pulmonol. 2015 Sep;50(9):908-14.*

III Lauhkonen E, Koponen P, Teräsjärvi J, Gröndahl-Yli-Hannuksela K, Vuononvirta J, Nuolivirta K, Toikka J, Helminen M, He Q, Korppi M. IL-10 gene polymorphisms are associated with post-bronchiolitis lung function abnormalities at six years of age. *PLoS One. 2015 Oct 16;10(10):e0140799.*

IV Lauhkonen E, Koponen P, Vuononvirta J, Teräsjärvi J, Nuolivirta K, Toikka J, Helminen M, He Q, Korppi M. Gene polymorphism of toll-like receptors and lung function at five to seven years of age after infant bronchiolitis. *PLoS One. 2016 Jan 7;11(1): e0146526.*

V Lauhkonen E, Koponen P, Nuolivirta K, Helminen M, Toikka J, Korppi M. Following Up Infant Bronchiolitis Patients Provided New Evidence For and Against the United Airway Disease Hypothesis. *Acta Paediatrica 2016 (Accepted for publication doi: 10.1111/apa.13537).*

The original publications are referred to by the numerals I–V, and they have been reprinted with the permission of the copyright holders.

Abbreviations

AAP = American Association of Pediatrics
ASM = Airway smooth muscle
ATS = American Thoracic Society
BD = Bronchodilator
BHR = Bronchial hyperreactivity
BN = Brown Norway
CAMP = Childhood Asthma Management Program
cAMP = cyclic adenosine monophosphate
COAST = Childhood Origins of Asthma
COPD = Chronic obstructive pulmonary disease
COPSAC = Copenhagen Prospective Studies on Asthma in Childhood
Crs = Respiratory system compliance
dRrs/df = Frequency dependency of resistance
ECT = Exercise challenge test
ERS = European Respiritory Society
ERV = Expiratory reserve volume
FEF = Forced expiratory flow
FEV_x = Forced expiratory volume in X seconds
FOT = Forced oscillations technique
FRC = Functional residual capacity
Fres = Resonant frequency
FVC = Forced vital capacity
FVS = Flow volume spirometry
ICAM = Intracellular adhesion molecule
ICS = Inhaled corticosteroid
IFN-γ = Interferon-gamma
IgE/G = Immunoglobulin E/G
IL = Interleukin
IOS = Impulse oscillometry
LPS = Lipopolysaccharides

LRTI = Lower respiratory tract infection
MAF = Minor allele frequencies
NICE = National Institute for Health and Care Excellence
NK = Natural killer
NPA = Nasopharyngeal aspirate
PAMP = Pathogen-associated molecular pattern
PCR = Polymerase chain reaction
Post-BD = Post-bronchodilator
PRR = Pattern recognition receptor
Raw = Airway resistance
Rint = Interrupter resistance
RBM = Reticular basement membrane
Rrs = Resistance of respiratory system
RSV = Respiratory syncytial virus
sGaw = Specific airway conductance
SNP = Single nucleotide polymorphism
SPT = Skin prick test
sRaw = Specific airway resistance
ssRNA = Single-strand RNA
TCRS = Tucson Children's Respiratory Study
Th-cell = T-helper cell
TLR = Toll-like receptor
Treg-cell = Regulatory T-cell
UAD = United airways disease
VEGF = Vascular endothelial growth factor
VmaxFRC = Maximal flow at FRC
WHISTLER = Dutch prospective birth cohort study
Xrs = Reactance of respiratory system
zBMI = Body mass-index Z-score
Zrs = Impedance of respiratory system

Abstract

Viral bronchiolitis is a common respiratory infection during infancy, but the severity of the disease varies greatly depending on inherited and environmental factors. Bronchiolitis in infancy has been associated with the risk of obstructive lung diseases. It is likely that the reduction in lung function seen in asthma or chronic obstructive pulmonary disease has its origin in early childhood, but scientific evidence on the reduction of lung function before school age is scarce in children with a history of bronchiolitis.

Healthy, term children with a hospitalisation due to bronchiolitis before the age of six months were enrolled and followed-up prospectively to the age of 5–7.5 years with aim of finding possible risk factors for lung dysfunction tested with impulse oscillometry (IOS). The virus aetiology of bronchiolitis was obtained from nasopharyngeal aspirates during hospitalisation, and was found to be predominated by respiratory syncytial virus (RSV) in 60% of the cases.

Of the 103 children with full data available, 20% had a pathological low lung function at baseline IOS and 16% showed abnormality in response to exercise or bronchodilation. Irreversible lung function pathology was seen in only one child. No significant differences were found in lung function or hyperreactivity results between bronchiolitis cases with different viral aetiologies. Baseline lung function did not show any associations with allergic rhinitis or skin prick test-positivity. The children with previous, remitted asthma showed the lowest lung function by reactance compared to children with current preschool asthma or without asthma and a subsequent wheezing history after bronchiolitis at the age of six to seven years. Some preliminary associations were found between interleukin-10- or toll like receptor-gene polymorphisms and lung function or hyperreactivity.

Our main results suggest that the reduction in lung function seen in children with a history of early viral bronchiolitis is characterised by obstruction of the small airways, which was mostly reversible with a bronchodilator, as assessed by IOS. In addition, obstructive findings in IOS may partly result from obesity at preschool age.

Tiivistelmä (Abstract in Finnish)

Viruksen aiheuttama ilmatiehyttulehdus eli bronkioliitti on yleinen infektio imeväisillä, joskin taudin vaikeusaste vaihtelee suuresti riippuen yksilön perimästä ja ympäristötekijöistä. Imeväisiän bronkioliitti on yhdistetty riskiin sairastua ahtauttaviin keuhkosairauksiin. On todennäköistä että keuhkojen toiminnan heikentyminen astmassa tai keuhkoahtaumataudissa alkaa jo varhaislapsuudessa, mutta tutkimusnäyttöä keuhkofunktio-ennusteesta ennen kouluikää on vain vähän.

Tutkimukseen otettiin terveet, täysiaikaisena syntyneet lapset, jotka joutuivat bronkioliitin vuoksi sairaalahoitoon ennen puolen vuoden ikää ja heitä seurattiin prospektiivisesti 5-7-vuotiaiksi etsien riskitekijöitä keuhkojen toimintahäiriölle arvioituna impussioskillometrian (IOS) avulla. Bronkioliitin aiheuttanut virus määritettiin nenänielu-imunäytteistä sairaalassa ja tärkein taudinaiheuttaja oli respiratory syncytial virus (RSV) 60%:ssa tapauksista.

Niistä 103:sta lapsesta, josta kaikki tutkimusdata oli saatavilla, 20%:lla oli patologinen perusvaiheen keuhkofunktio ja 16%:lle tuli merkittävä vaste rasitus- tai bronkodilataatio-kokeessa. Palautumaton keuhkofunktion alenema löytyi vain yhdeltä lapselta. Eri virusten aiheuttamien bronkioliittitapausten välillä ei löytynyt merkittäviä eroja keuhkofunktiossa tai hyper-reaktiviteetissa. Perusvaiheen keuhkofunktio ei ollut yhteydessä allergiseen nuhaan tai ihon prick testi-positiivisuuteen. Astmasta parantuneilla lapsilla oli matalin keuhkojen reaktanssi verrattuna lapsiin, joilla oli esikouluiän astma sekä niihin, joille ei ilmaantunut lainkaan astmaa tai bronkioliitin jälkeistä vinkunaa 5-7 vuoden ikään mennessä. Esitämme lisäksi alustavia tuloksia interleukiini 10- ja toll like receptor-geenien polymorfismien yhteydestä keuhkofunktioon ja hyper-reaktiviteettiin.

Tutkimuksen päätulokset viittavat siihen, että imeväisenä bronkioliitin sairastaneilla lapsilla esiintyy pieniin hengitysteihin painottuva keuhkofunktion häiriö 5-7-vuotiaana, joskin löydös on vielä palautuva mitattuna IOS:lla. Lisäksi lihavuus esikouluiässä on yhteydessä poikkeaviin IOS tuloksiin.

1 Introduction

Viral bronchiolitis is a common lower airway infection affecting children especially during the first six months of life, with the respiratory syncytial virus (RSV) accounting for most cases (1,2). RSV-associated lower airway infections are a significant cause of mortality in developing countries, but they also cause a substantial burden to children in industrialised countries (3,4). Roughly estimated, one third of all children experience such an infection during their first year of life (3,5). In a recent study from Finland, 3.7 per 100 children under the age of six months were annually admitted to the emergency department due to bronchiolitis, and 70% of them had to be hospitalised (6).

There is a link between bronchiolitis and asthma. One third of children develop recurrent wheezing symptoms after bronchiolitis that mostly resolves during first three years of life (7), but also approximately 40% of children with a history of bronchiolitis tend to develop persistent asthma (8). It is largely unknown if this is causal to the bronchiolitis, or if the bronchiolitis reveals individuals predisposed to wheezing or asthma (9). Several early-life environmental and genetic risk-factors have been identified in studies following the development of asthma after viral bronchiolitis (10-14), recently also from this cohort (15). However, it seems that the outcome after bronchiolitis is not only linked to asthma, and characterised by atopic inflammation and bronchial hyperreactivity, but also to non-atopic chronic airway disease that could manifest symptoms much later in life.

Lung function is a continuous trait from development in utero to the exponential development of the alveoli during the first life years and the linear growth of lung size from birth to early adolescence (16). There is increasing evidence that lung function in later life is developed already in utero and in early infancy, with several possible altering factors, such as obstacles to mechanical growth or exposure to smoking during pregnancy (17-19). Early-life bronchiolitis again has been proposed to play a dual role in this: children with congenitally small airways or increased airway tone might be prone to wheeze during lower airway infections, and bronchiolitis might damage the lung structure and/or the growth process (20). In addition, a continuous subsequent inflammatory process leading to lung function reduction, such as airway remodelling in atopic asthma, could explain

a part of the pathology (21). There is, however, limited evidence on the causal association of bronchiolitis with lung function decline in children.

It is important to note when studying factors altering lung function in children that despite the treatment of atopic inflammation in asthmatic airways with inhaled corticosteroids (ICSs), while often leading to the relief of symptoms and less hyperreactivity, the decline in lung function continues (22,23). This suggests that the processes controlling lung function may differ from the mechanisms responsible for hyperreactivity in atopic asthma. Another key finding is that the long-term outcome after early viral bronchiolitis is an irreversible lung obstruction in adulthood (24-26), suggesting that the similar lung function decline as seen in chronic obstructive pulmonary disease (COPD) has its onset already in infancy.

Reliable lung function testing in young children is challenging due to the varying level of co-operation and technical difficulties, often leading to unreliable, unrepeatable measurements. We have used a novel method of impulse oscillometry (IOS), a modification of the forced oscillations method (FOT)(27), in studying lung function during tidal breathing in a cohort of five- to seven-year-old children hospitalised for viral bronchiolitis before the age of six months. Prior to our study, the FOT method was applied only in two short-term post-bronchiolitis follow-up studies (28,29). IOS has only been used in one long-term follow-up comparable to our study; it was utilised in one selected cohort study of children with a high risk of asthma (30). In children with bronchiolitis at this age, no studies focusing on lung function before school age have been published.

With this study, we aimed to evaluate the overall lung function prognosis after early-life bronchiolitis, and to study possible factors predicting lung function decline. We focused on testing associations between the viral aetiology of bronchiolitis, current obesity, genetic factors of innate immunity, and also the development of the united airways disease (UAD) – consisting of atopic sensitisation, allergic rhinitis, and asthma – with lung function at the mean age of 6.3 years investigated by IOS after bronchiolitis before the age of six months.

2 Review of the literature

2.1 Viral bronchiolitis

2.1.1 Bronchiolitis: Definition, prevalence, and epidemiology

Bronchiolitis is the pathological description of the inflammation of the smallest airways, the bronchioles. Bronchiolitis is characterised by increased mucus production due to inflammation, oedema, and necrosis of the epithelial cells of the small airways. The diagnosis is clinical (31-33). The disease course often begins with a coryzal phase with nasal obstruction, progressing to lower respiratory tract symptoms, such as persistent cough, tachypnoea and/or chest recession, and wheezing or crackling in the breathing sounds; it is also often associated with mild fever and feeding difficulty (31-33).

There is a discrepancy in upper age limits in bronchiolitis guidelines and studies (34). Current US (AAP) and UK (NICE) guidelines take children under the age of 24 months into account (32,33), whereas in most European countries bronchiolitis is often considered a first wheezing episode before the age of 12 months (1,2). There are notable differences in viral aetiology and atopic characteristics of bronchiolitis affected by age and previous wheezing episodes (34,35), thus stricter age limits of less than 12 months have been proposed to distinguish children with bronchiolitis from children with recurrent virus-associated wheezing (15,36).

Bronchiolitis is a common respiratory infection in children, and approximately one in three suffer from it during their first year of life (3,5,7). However, only 1–3% of all infants during an epidemic have a more severe disease course leading to hospitalisation; these infants are often aged under six months (1,2,6).

2.2 Viruses causing early-life bronchiolitis

2.2.1 RSV bronchiolitis

RSV is a major pathogen causing acute respiratory infection in children during the first five years of life; it is associated with 3.4 million hospitalisations worldwide every year (4). RSV is a single-strand RNA virus (ssRNA) belonging to the *paramyxoviridae* family. Lower respiratory tract infections (LRTI) associated with RSV are a common cause of mortality in developing countries (4), but RSV also causes substantial morbidity in Western countries in both outpatient and inpatient settings (3).

Approximately 2–3% of children under the age of six months are hospitalised because of RSV bronchiolitis, and peak hospitalisation rate occurs in infants aged under three months (5). RSV is the most common causative virus in infant bronchiolitis, and hospital cohort studies have shown that 50–70% of children admitted to hospital due to bronchiolitis test RSV-positive (37-39).

Most infants with an RSV infection are previously healthy and born at term. Male sex, low socio-economic status, low birth weight, and young age in particular have been identified as risk-factors for RSV-associated hospitalisation (3,5). A congenital heart or neurological condition, prematurity, and chronic lung disease due to prematurity are known risk-factors for a severe disease course of bronchiolitis that often needs intensive care (40). The first encounter does not provide immunity, and re-infections are common despite the production of antigens to RSV (41). Mixed infections with respiratory picornaviruses (rhino- and enteroviruses) are observed in 10–20% of RSV bronchiolitis cases (39). Predictable seasonal epidemics are characteristic to RSV (42). In countries with a temperate climate, the annual epidemics usually occur in the winter months, but in subtropical areas, they occur in the cool, rainy season. Previously, two-phased RSV outbreaks were observed to take place every second year (39).

On the cellular level, the superficial epithelial cells of the upper airways, the epithelium of the bronchioles, and pneumocytes in the alveoli are infected with the RSV (43). In lung biopsies archived from fatal bronchiolitis cases, acute bronchiolitis was profound in the medium and small bronchioles, where the airways were covered with the mucus of cell debris plugs (43).

Toll like-receptors (TLRs) are pattern recognition receptors (PRRs) that have an important role in virus recognition in the airways, leading to onset of innate and

adaptive immune response (41,44). Chemokines and pro-inflammatory cytokines, such as interferons, are produced and large amounts of neutrophil leucocytes are attracted to the site of infection, as are eosinophils and lymphocytes to a lesser extent (41). In the early stage of infection, the IFN-γ produced by natural killer-lymphocytes is important in activating the antigen-presenting cells and priming the T-cells (41).

Susceptibility to severe RSV is thought to be partly genetic (41). Both Th1- and Th2-type responses are observed during RSV infection, and some evidence exists for a Th2-skewed allergic-type inflammation in infants with severe RSV bronchiolitis (45,46). The suggested principal innate immunity factors involved in the neonatal susceptibility to severe RSV infection are illustrated in Figure 1.

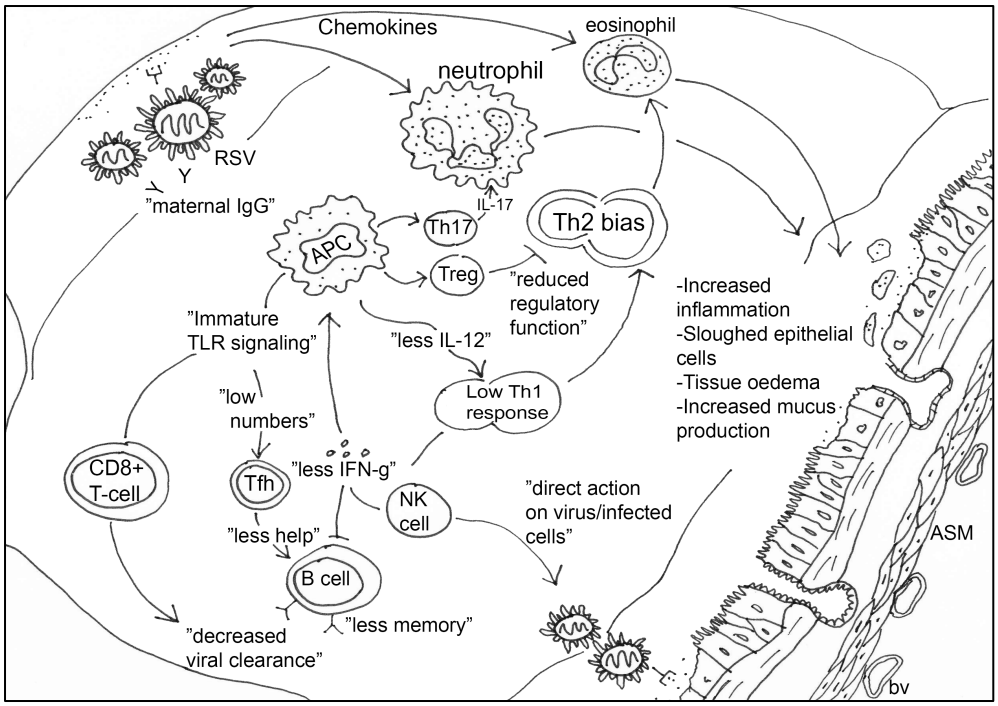

Figure 1. The "impaired" neonatal immune response to RSV infection. A schematic illustration, modified from (41). © Eero Lauhkonen

2.2.2 Rhinovirus and other non-RSV bronchiolitis

Rhinovirus infection is the dominant cause of the common cold in all age groups, but it is also the second most causative virus in infant bronchiolitis. Rhinovirus belongs to the *picornaviridae* family, and is also an ssRNA virus. Rhinovirus pathogenesis in bronchiolitis is less studied compared to RSV, partly because it was not until the development of a better identification method for rhinovirus by PCR that it was also increasingly noted as a lower airway pathogen (38,47).

Rhinovirus is a very common virus circulating in the population year-round, and non-symptomatic carriage of the virus is common. The prevalence of rhinovirus-associated wheezing increases with age and equals RSV prevalence in children at the age of 12 months (34). The overall prevalence of rhinovirus bronchiolitis is 20–40% (34,37,48), but in individuals with atopic predisposition and recurrent wheezing during the first year of life, the prevalence has been as high as 50–80% (49). In addition to age and atopy, low IFN-γ levels and IL-10 gene polymorphisms have been described as risk factors for rhinovirus (50,51). The clinical characteristics of rhinovirus bronchiolitis are similar to RSV, although recently rhinovirus aetiology was associated with a shorter length of hospital stay compared to RSV bronchiolitis (52).

The known 148 rhinovirus serotypes can be classified according to phylogenesis into groups A, B, and C (53). The majority belong to groups A and B and use ICAM-1 receptors when entering the epithelial cells of the airway, while group C uses LDL-receptors. In contrast to RSV, rhinovirus bronchiolitis does not cause significant cell destruction, but instead alters the epithelial barrier function, especially in the tight junctions, leading to vascular leaking and mucus production (54). Rhinovirus is detected by PRRs and importantly by TLRs, which in turn induce innate immunity cytokine expression, including interferons IL-8, IL-10, and a wide range of chemokines attracting neutrophils, eosinophils, and lymphocytes to the site (54).

Other respiratory viruses account for a minority of infant bronchiolitis cases and their role in the disease course is not yet fully established. Human metapneumovirus has been described in 4–7% of bronchiolitis cases in children under 24 months of age, parainfluenza 1, 2, and 3 viruses similarly in 4–7%, adenoviruses in 3%, and influenza viruses in 1–2% (39,52). These viruses, along with rhinovirus, are referred to in this dissertation under the term "non-RSV aetiology of bronchiolitis".

2.3 Cytokine dysregulation and rhinovirus *vs* RSV infections

The development of atopy is thought to be multifactorial, depending on genetic factors like cytokine dysregulation (Th1/Th2 imbalance) and environmental factors – such as viral infections – interacting at a susceptible age (9) (Fig. 2). In adults, rhinovirus infections in asthmatic subjects are strongly linked to increased Th2 and impaired Th1 and Treg (IL-10) responses (55).

The causality is unclear, as some studies have suggested that innate cytokine responses reflect the predisposal to early virus infections (56,57), and only a few studies have followed the development of innate immunity cytokine responses from birth over time.

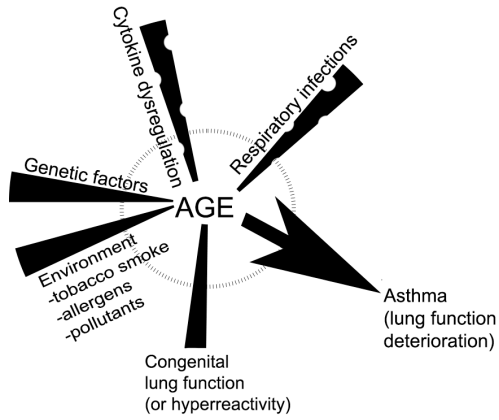

Figure 2. Schematic illustration of suggested factors contributing to the development of asthma. Modified from (9).

The innate immune response of the infant is primed to be Th2-oriented at birth (58). The innate Th2 cells produce IL-4, IL-5, IL-9, and IL-13, and the Th1-oriented response (production of IFN-γ, IL-2, IL-12) is decreased in neonates (58,59). According to the hygiene hypothesis (60), the increase in atopic disease, such as asthma, might be caused by inadequate environmental exposure to micro-organisms during a critical period in infancy that would normally develop the immune system towards a Th1/Th2 balance.

In early infancy, viral infections might play a role in disturbing the maturation process of the developing immune system (61). As a marker of genetic

predisposition to virus infection, early viral bronchiolitis could serve as a trigger leading to Th2-dominated (and Th1-impaired/undeveloped) imbalance and increase in immunoglobulin-E (IgE) secretion and atopic inflammation, thus creating the basis of chronic airway disease and abnormal lung function development (1,8).

From the Childhood Origins of Asthma (COAST) cohort study (62), there is evidence that low IFN-γ cytokine levels at birth from cord blood show an inverse correlation with an increased number of viral infections at the age of one year, suggesting genetic predisposition to viral infections. There was a measurable effect of the increase in the IFN-γ responses associated with frequent infections and daycare attendance during the first year of life, suggesting that viral infections can influence IFN-γ responses (63). In the same cohort study, the IL-5 and IL-13 responses increased and were associated with blood eosinophilia and elevated IgE levels by the age of one year (64). In addition, reduced cord blood IL-10 and IFN-γ responses were associated with egg-specific IgE sensitization (64).

In a Finnish study, innate cytokine responses of type Th1, Th2, and Treg (IL-10) were compared during the acute and convalescent phases of rhinovirus or RSV infection in 3–36-month-old children and asymptomatic controls. The rhinovirus-infected children had higher Th2 (IL-5 and IL-13) cytokines but also higher Th1 (IFN-γ, IL-12) cytokine levels in their serum compared to the RSV cases. Furthermore, 2–3 weeks later, the rhinovirus patients had higher IFN-γ, IL-13, and IL-10 levels compared to the RSV patients, and higher IFN-γ and IL-2 levels compared to the controls (65). The authors conclude that rhinovirus and RSV differ from their early-age cytokine response profiles, which do not strictly follow the Th1/Th2 classification, and that the augmented Th2 cytokine levels in rhinovirus infection might reflect a genetic predisposal to atopy and vulnerability to rhinovirus (65).

Thus, the complex, non-exclusive relationship of pre-existing innate immunity as a predisposing factor to viruses and the possible alteration of Th1/Th2 balance by virus infections – and the possibility that the long-term sequelae afterwards is a result from this interaction – needs further clarification and confirmative studies.

2.4 Lung structure of the newborn infant and postnatal lung growth

In the fully developed lung, the conducting airways divide in 23 generations from the trachea to the alveoli. From the level of the 10th to 15th generations, the term bronchioles is used when the cartilaginous support of the airway wall diminishes and the diameter of the airway lumen is less than 1mm. In the 16th to 19th generations, the terminal bronchioles narrow to an average of 0.5mm, and the basal membrane is in direct connection with the lung parenchyma. The cilia and mucous glands disappear and the bronchioles interconnect with each other and form the end of the conducting airways before the respiratory zone (Fig. 3).

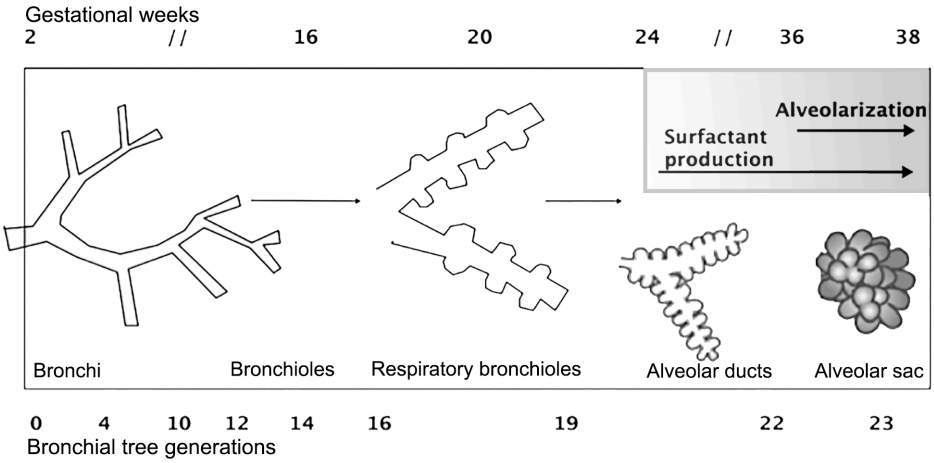

Figure 3. Prenatal bronchial tree development phases in utero (modified from www.embryology.ch, chapt. 18.1, Fig. 12).

Prenatally, the final generations of the airway start developing in the 24th to 25th gestational weeks, forming alveolar ducts and beginning surfactant production. The alveoli start developing in the 32nd to 36th gestational weeks, and it is estimated that some 15% of the alveoli are fully developed at birth (16). During this alveolarisation phase, the alveoli develop from the alveolar sacculi, grow larger, and form subdivided cavities with internal septae lined with a thin epithelium surrounded by a capillary network (16). In the first six months after birth, alveolarisation continues as the number of alveoli increases massively to the age of two to three years, and thereafter a slow increase continues during childhood into early adulthood (66-68).

From autopsy studies, it has been shown that compared to girls, boys have a greater number of alveoli shortly after birth as a consequence of larger lung volume and a slightly greater alveolar surface area in relation to body surface area (66). With increasing age, boys tend to have larger lungs per units of length (66). For both sexes, the diameter of the alveoli continues to increase by 30%, which approximately doubles the lung volume by the age of 13 years, thus halving the number of alveoli per unit volume (67,68).

Despite the larger lung size and greater number of alveoli, studies have suggested that in infancy boys tend to have lower lung function than girls (69), which is due to smaller airway calibre relative to lung size (70).

Known factors disturbing intrauterine lung growth include mechanical anatomical obstacles (chest wall anomalies, hernias, etc.), maternal hypoxia, toxic substances (alcohol, drugs), and exposure to maternal nicotine (16,17). As most of the alveoli develop in the post-natal period, several external factors including exposure to tobacco smoke, respiratory infections in infancy, and asthma have been suggested to inhibit lung growth also after birth (16,17).

2.5 Impact of bronchiolitis on lung function: Short-term sequelae

It can be hypothesised that lung injury due to bronchiolitis could result in decreased alveolarisation, but no firm evidence for this exists. There is also limited data on the functional changes of the lung during acute bronchiolitis and in the convalescent phase. Due to intraluminal obstruction in the small airways caused by mucus and cell debris, the functional residual capacity increases: the lungs become hyperinflated and the dynamic compliance falls. Air trapping leads to the collapse of the alveoli due to gas absorption, and further to localised atelectasis (2).

Most of children remain symptomatic shortly after bronchiolitis, and the most common short-term lung function abnormalities have been hyperinflation – lasting for the convalescent period in 77%, after three months in 43%, and after one year in 17% (28,29,71) – and hyperreactivity, reported in up to 75% shortly after bronchiolitis (72,73). Adenoviruses have been documented as a cause of post-infectious bronchiectasis, due to obliterating bronchiolitis (74).

2.6 Airway remodelling

In asthma and chronic obstructive pulmonary disease airway remodelling, a complex process of evolving structural airway changes in the bronchial wall is considered a characteristic pathological process (75-77). Although airway remodelling has been thought to be a consequence of chronic inflammation, non-inflammatory factors have gained increasing interest in recent research (78,79).

In asthma, thickening of the reticular basement membrane (RBM), increased large airway smooth muscle (ASM) mass, and eosinophilic inflammation with CD4+ helper T-cells are characteristic; as in COPD, small airway metaplasia, fibrosis, increased smooth muscle mass, and the infiltration of CD8+ cytotoxic T-cells are observed (21). The causal relationship between the inflammation and the structural changes is unclear, and it has been speculated that both the inflammation and the remodelling are outcomes from distinct processes seen in the same individual (78). Although anti-inflammatory treatment has a good effect on asthma symptoms, it is largely unknown how the remodelling of the airways is altered in children (80). In adult studies, some experimental evidence of steroidal treatment having a direct beneficial effect on remodelling processes exists (78), which is further supported by evidence of improving lung function by early-onset steroidal treatment (81,82).

In adults, the positive association of eosinophilia with RBM thickening has been shown (83), yet it is unclear when the structural changes seen in asthma first appear. In studies obtained with endoscopic biopsies, RBM thickening and eosinophilia are not yet present in symptomatic infants with reversible airway obstruction at the median age of 12 months (84), but they are demonstrable in preschool children at the median age of 29 months with a history of wheezing (85). It has been suggested that RBM thickness reaches its maximum at school age, where it is similar in children and adults with severe asthma (86).

In a recent follow-up study, the histologic findings of RBM thickness, ASM, and eosinophilia obtained in infancy did not correlate with asthma symptoms or lung function at eight years of age, but in the same cohort, low lung function measured in infancy was associated with reduced lung function and the use of asthma medication at school age (87). This suggests that the process of asthma onset in infancy differs from the pathology of the later established disease, thus perhaps requiring different treatment approaches to prevent the remodelling of the airways.

There is only limited evidence of early respiratory virus infections and their direct association with the growth factors involved in airway remodelling. Rhinovirus has been reported to enhance the airway epithelial cell production of Amphiregulin, vascular endothelial growth factor (VEGF), and Activin A in vitro, and to increase the VEGF protein measured from nasal wash in patients with a confirmed natural infection (88). RSV has also been associated with the upregulated production of VEGF in vitro (89).

In an animal model, Th2-skewed Brown Norway (BN) rats developed a chronic asthma phenotype with reversible airway obstruction associated with transepidermal growth factor (TGF-β) expression when exposed to parainfluenza virus, but the Th1-skewed line of rats did not (90,91). Further, the BN rats were observed to have an impaired IFN-γ response to virus infection (92), and the development of asthma was preventable with selective IFN-γ administration during the infection (93).

Repeated bronchoconstriction without additional inflammation was shown to lead to remodelling in adults with asthma (79). This suggests that prevention of the repeated airway narrowing could be a target in treating patients with asthma and also wheezing in children. However, there is no long-term evidence on this in children with repeated wheezing due to respiratory infections.

2.7 Exposure to smoking and childhood lung function

There is supporting evidence that prenatal and, in particular, exposure to maternal smoking in utero has adverse effects on infant lung function (94,95). In the majority of studies – but not all – reduced expiratory flow measurements (VmaxFRC) after birth are found in infants whose mothers reported smoking during pregnancy (96-98). There is, however, less evidence that prenatal smoking exposure increases airway reactivity in infancy (94,95). This could mean that prenatal exposure impacts lung development, resulting in diminished airway size, which is not due to increased smooth muscle tone.

Airway hyperreactivity is a more established finding accompanying lung function impairment associated with parental smoking exposure later in childhood and early adulthood (18,99,100). In a Finnish study, exposure to post-natal maternal smoking, which correlated well with measured cotinine levels, associated significantly with reduced lung function and increased FeNO levels in three- to seven-year-old children with multiple-trigger wheeze (100). In the Tucson

Children's Respiratory Study (TCRS) cohort, reported antenatal maternal smoking associated with the transient wheezing phenotype (11).

2.8 Birth cohort studies: Evidence of reduced lung function before any lower respiratory tract infection and follow-up thereafter

There is previous evidence that a proportion of term and otherwise healthy infants show premorbid lung function measurements already after birth, predisposing them to bronchiolitis and later wheezing symptoms and asthma (101-106).

Within a community-based birth cohort of the TCRS cohort (7), a subgroup of 124 children from 1,246 in total were tested for lung function shortly after birth (101). The risk of parent-reported wheezing at the age of 1.0 year was 3.7-fold in children with airway conductance in the lowest third compared to children with airway conductance in the upper two thirds. A 10-fold increase of parent-reported wheezing was observed in boys with initial conductance levels in the lowest third. In girls with a lung volume at the end of tidal expiration in the lowest third, a 16-fold increase in wheezing symptoms was seen.

In the same birth cohort study follow-up at six years of age, reduced lung function at birth by VmaxFRC (107) was associated with the transient wheezing phenotype (at least one parent reported LRTI before age <3 years, no wheezing at six years of age), presenting lowered lung function despite being symptom-free. The group of children with persistent wheezing symptoms beginning before the age of three years showed the lowest lung function measurements at the age of six, but normal values at birth (11) (Fig. 4).

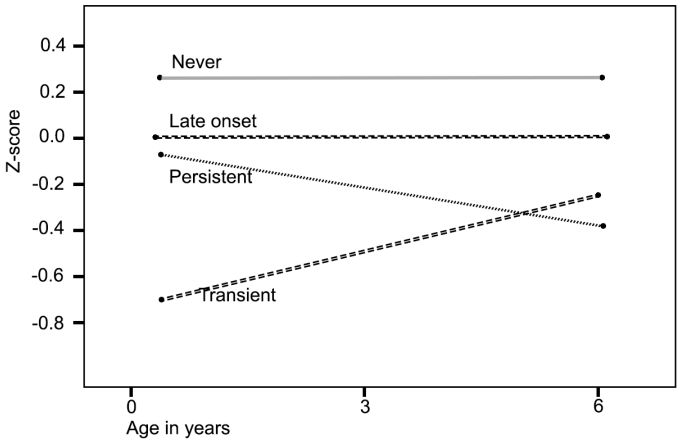

Figure 4. Lung function VmaxFRC Z-scores measured after birth and at the age of six years in TCRS subjects with different wheezing phenotypes. Modified from (11).

The TCRS also observed that the transient wheezing phenotype was associated with maternal smoking during pregnancy, but not with a family history of asthma or atopy, whereas the persistent wheezing phenotype was associated with higher IgE levels and also maternal asthma (11). There is further evidence from the TCRS that having a low lung function at birth predicts low lung function in adulthood (19).

A community-based birth cohort from Perth, Australia (108) investigated the lung function of 246 infants at the age of five, 27, and 55 weeks with a two-year follow-up of wheezing symptoms and bronchiolitis outcome; 17 of the children developed doctor-diagnosed bronchiolitis during the two years. There was a non-significant association of increased risk of bronchiolitis with lowered VmaxFRC at the age of five weeks (OR 3.16, 95%CI 0.87–11.6, $p<0.06$) during their first year of life, but no association during their second year of life (108). The same cohort study also found that cases with lowered lung function at five weeks of age and wheezing symptoms only during the first year of life also improved their lung function by the age of 12 months, but the cases with persistent wheezing symptoms during the first and second year also showed persistent lung function reduction both at five weeks and 12 months of age (109). Male gender and maternal smoking during pregnancy were associated with lower VmaxFRC values throughout the first year of life (110). In a long-term follow-up of this cohort, reduced lung function before and after bronchiolitis was maintained to the age of 11 years in 16 hospitalised children compared to 178 controls, and there were no differences in asthma, wheezing, or atopy between these groups (103).

In a study from Manchester, UK (111), a population-based birth cohort of over one thousand children and a subgroup of 69 children with atopic parents were recruited and studied for their lung function at the age of one month. In a one-year follow-up, parent-reported wheezing and cough were associated with reduced neonatal VmaxFRC (102). Decreased lung function shortly after birth was also associated with male gender, but no significant associations were seen with parental asthma or antenatal exposure to smoking (102). This birth cohort study on a wider scale followed the lung function as specific airway resistance (sRaw) measured by whole body plethysmography at the ages of three, five, eight, and 11 years, and demonstrated strongly that children with the persistent wheezing phenotype, frequent asthma exacerbations, and early atopy had consistently lower lung function and also a higher rate of lung function deterioration from the age of three onwards up to 11 years compared to children who never wheezed; these effects in lung function reduction were greater in boys (112).

In a Dutch prospective birth cohort study (WHISTLER), neonatal lung function was measured in 2,133 infants at a median age of two months. In a small subgroup of 18 hospitalised RSV-positive children, lowered neonatal lung function before the infection was associated with a more severe course of RSV bronchiolitis and post-RSV bronchiolitis wheezing compared to non-hospitalised RSV-positive children (105). In this study, decreased respiratory system compliance (Crs) in particular was associated with hospitalisation due to RSV bronchiolitis. From the same cohort, in 202 children with rhinovirus-associated wheezing in the first year of life, premorbid lung function was associated with the occurrence and duration of the wheezing episodes (113). Interestingly, in the neonatal lung function measurement, increased respiratory system resistance (Rrs) – but not Crs – was associated with wheezing. In addition, maternal smoking during pregnancy increased the risk of rhinovirus-associated wheezing. WHISTLER is the only birth cohort study addressing neonatal lung function before any lower respiratory tract infection and its association with either confirmed RSV bronchiolitis and post-bronchiolitis wheezing or rhinovirus-related wheezing.

In the WHISTLER cohort follow-up, a prospective cohort of 155 hospitalised RSV bronchiolitis patients from a previous study were compared to non-hospitalised healthy controls at the age of six years from the unselected birth cohort. Hospitalised patients had a lower mean FEV1, FVC, and FEV1/FVC ratio, and in addition interrupter resistance (Rint) was higher compared to the non-hospitalised children (114).

The most comprehensive prospective follow-up study addressing the relationship of reduced innate lung function and the progression of lung function deterioration later to school age is the Danish COPSAC study (106), where lung function in a high-risk birth cohort of 317 children with asthmatic mothers was followed to the age of seven years. Compared to children without asthma, the children who developed asthma by the age of seven years had a significantly lower neonatal FEF50% that progressed from Z-scores of -0.34 to -0.82 and a lower neonatal FEV0.5/FEV1-ratio Z-scores decreasing from -0.29 to -0.73 during childhood, respectively, suggesting that 40% of the lung function reduction seen by the age of seven is constituted by neonatal lung function reduction, and 60% is due to later disease (Fig. 5).

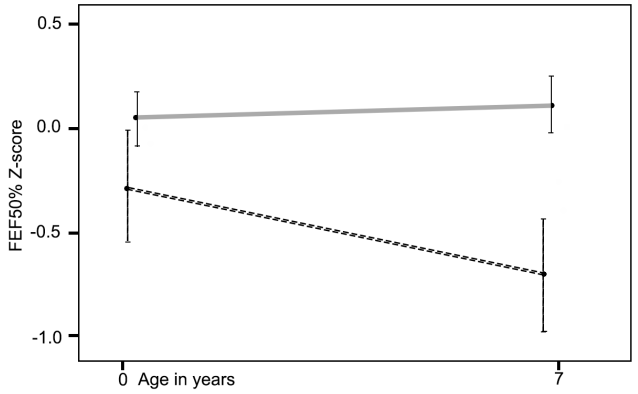

Figure 5. The COPSAC study observed the reduction of forced expiratory flow (FEF50%) rate from the age of one month (p=0.03) to seven years (p=0.001) in children without (solid line) and with (dashed line) current asthma. Modified from (106).

In the COPSAC study, the negative association of lung function growth with asthma was independent of atopy and aerogen allergen sensitisation at the age of six years (106), suggesting that in the development of lung function disorders, non-atopic factors also play a role. In addition, a sub-group of 34 children studied at one month of age who were later hospitalised due to severe bronchiolitis before the age of 24 months appeared to have increased neonatal hyperresponsiveness preceding hospitalisation compared to non-hospitalised children. No difference was, however, found in neonatal lung function between these groups (115).

In the COAST study (30) a high-risk selected cohort of 289 children with at least one atopic parent was followed after birth to the age of eight years. Outpatient visits with six months intervals and lung function testing annually from

the age of four to eight years with both FVS and IOS were performed. Although not addressing the preceding lung function in infancy, the study demonstrated that children wheezing with rhinovirus infections during the first three years have an impaired lung function at the age of eight years compared to children without RV wheezing, as measured by decreased FEV1 and FEV1/FVC ratio in spirometry and by a more negative Xrs5 and larger AX in IOS. Lung function in RSV wheezing was no different from the children not experiencing wheezing at all. In this study, asthma diagnosis at six or eight years of age was associated with lung function by spirometry as moderately lowered FEV0.5/FVC ratio and FEF25–75, but not with IOS indices (30).

2.9 Hospital-cohort studies: Lung function outcome after early bronchiolitis and recurrent wheezing episodes

Previous prospective hospital cohort studies have studied the long-term prognosis after viral bronchiolitis, pneumonia, or wheezing, but lung function outcome has not been addressed before school age.

In a Norwegian prospective cohort study, 57 bronchiolitis patients hospitalised under the age of 11 months were compared to age-matched controls at seven years of age. Reduced lung function by FVS was observed in the bronchiolitis patients compared to the controls, but no significant difference between the RSV and non-RSV groups was found (116).

In a Finnish prospective cohort of children hospitalised due to wheezing LRTI at the ages of 1–23 months, lung function was measured with spirometry at a median age of 7.2 years; 23% had a decreased lung function and 13% showed a rhinovirus-associated bronchial responsiveness to exercise (117). Later, from the same cohort at a median age of 11 years, virus-specific differences in bronchial responsiveness to exercise were absent, but RSV aetiology of early-life wheezing was associated with a restrictive pattern in lung function measured by FVS (118).

In a Swedish cohort study, children with a history of obstructive bronchitis during their first 24 (median 10.0) months of life were studied at the age of four to five years and revisited at the age of 10 years (119,120). Half of the children were symptom-free in the preschool follow-up, one third had mild asthma, and 15% had a moderate to difficult asthma (119); there was no neonatal or later lung function measurement at this study phase. At the age of ten years, 70% of the children were asthma-free, and persistent asthma was associated with an early start of wheezing,

marks of atopic predisposition other than asthma, and exposure to household smoking in infancy. RSV aetiology of bronchitis, present in 28% of cases, was not associated with persistent asthma (120). The children underwent histamine provocation that correlated well with persistent asthma, as 19% of the children had a mild, 12% a moderate, and 6% a severe reactivity to histamine (120). In a long-term follow up at 17–20 years, risk factors for asthma, present in 43%, were current allergy, female gender, and bronchial hyperreactivity (121). The subjects with a history of early wheezing had a significantly lower FEV1/FVC ratio and FEF50 compared to age-matched controls, both in pre- and post-bronchodilator measurements, but lowered baseline lung function was seen also in symptom-free subjects (121).

In another Swedish bronchiolitis cohort, 47 subjects, of which 43 were less than six months old during hospitalisation for RSV bronchiolitis, and 89 age-matched controls were prospectively followed to the mean age of 7.5 years. RSV aetiology had a significant association with atopic asthma, *i.e.* asthma with allergic sensitisation, compared to the control group (13). At the age of 13 years, similar associations with asthma and allergies persisted in children with a history of RSV bronchiolitis, and they also showed a lower FEV1/FVC ratio and FEF75 before and after bronchodilator compared to the controls (122). The asthma and allergy pattern persisted to the age of 18 years, when the RSV group also showed lower pre-bronchodilator FEV1, FEV1/FVC ratio, and FEF25–75 compared to the controls (25). In the dry air challenge, the fall in FEV1 was slightly greater in the RSV group, but in the post-bronchodilator measurement there was no difference in FEV1 between the RSV and control groups, though a statistically significant lower FEV1/FVC ratio and FEF25–75 were seen (25).

In the foremost long-term prospective bronchiolitis and pneumonia cohort from Finland (10), bronchial asthma was present by the age of eight years in 15% of bronchiolitis cases and in 2% of pneumonia cases. Decreased mid-expiratory flows were present in 29% of bronchiolitis patients and 21% of the pneumonia patients, and both groups had an increased bronchial response to methacholine. As young adults at 18–20 years of age, the bronchiolitis patients had lower baseline FEV1, FEV1/FVC, FEF50, and FEF25 compared to the controls, and the subgroup of 35 RSV-positive patients had lower FEV1 and FEF25 compared to the controls. Bronchial reactivity to methacholine was present in 48% of bronchiolitis cases and 41% of pneumonia cases vs 32% in the control subjects (123). Recently, in a long-term follow-up study of these former bronchiolitis and

pneumonia patients, 21% had an irreversible airway obstruction at the age of 28–31 years compared to the controls (26).

In conclusion, lung function abnormalities after bronchiolitis, defined by less strict age-limits compared to our study, have been documented in 23–29% of cases in studies with spirometry in seven- to eight-year-old children (10,117). Virus-specific differences in lung function outcome at this age have not been found (116,117), but some have suggested a rhinovirus-associated increase in bronchial hyperrreactivity (117). Lung function reduction at 11–13 years of age measured by FVS has been associated with the RSV-aetiology of bronchiolitis (118,122), persisting in two follow-up studies into early adulthood (25,123), but otherwise the long-term outcome of early bronchiolitis in terms of virus-specific lung function remains to be determined.

2.10 Lung function development and weight

It is often clinically suspected that obesity is the cause of dyspnoea and wheezing symptoms in children. In adults, evidence of an obesity-related asthma phenotype with increased bronchial hyperreactivity is controversial (124-126). It is worthwhile to investigate if such a phenotype exists in children, as it could be that obesity causes symptoms that only mimic asthma. A recent large-scale systematic review suggests that overweight BMI in childhood increases the risk of non-atopic asthma (127), and hypothetically it could be due to non-allergic inflammation or other metabolic abnormalities.

The studies on assessing the role of obesity in increased airway reactivity seem conflicting in children (128-130), but airway responsiveness to exercise rather than to methacholine has been increasingly associated with obesity (126). Somewhat more established is the finding of an association of obesity with reduced lung function by FVS in school-aged children (131-134). There is also data showing that obesity continuing from childhood to adulthood results in poorer lung function compared to normal-weight children who do not become obese until adulthood, although they also have a reduced lung function compared to normal-weight adults, addressing the importance of maintaining normal weight during growth (135).

Obesity has a negative effect on lung function in adults, as the obese tend to have an elevated diaphragm and increased extrathoracic compression of the thorax. This is thought to lead to airway narrowing due to reduced functional residual

capacity (FRC) and expiratory reserve volume (ERV) (136,137), as well as distal airway dysfunction and poorer bronchodilator response, as reflected by increased pre- and post-BD frequency dependency of resistance by IOS (137). In the only IOS study in unselected preschool-aged children, no association between increasing BMI and reduced lung function was found (138). Otherwise, the studies assessing obesity and pulmonary function have been done at school age; they show results mostly comparable to those in adults, i.e. negatively impacted lung function by FVS (131-134).

According to Barker's hypothesis (139), reduced intrauterine growth might result in increased cardio-pulmonary morbidity in later life. The effect of intrauterine insults on catch-up growth trajectories has been established in animal studies, but it has been mainly retrospectively observed in humans (140,141).

It is important to highlight the relationship of the body and airway growth: the airway structure develops by mid-pregnancy (Fig. 3), and after that the bronchial structure may not be able to catch up with other somatic growth, resulting in relatively small airways compared to body size. The imbalance with pulmonary and other somatic growth is referred to as "dysanapsis". Indirect evidence for this comes from a prospective cohort of 1,232 children in Chile, where increased gain in weight and length during infancy was associated with asthma symptoms at the ages of 23–29 years (142).

There is some evidence on the effect of low birth weight on increased asthma in adulthood, though a longitudinal aspect of post-natal rapid growth has shown more consistent results on the association with asthma in later life, with reduced lung function only in infancy, not in adulthood (143).

2.11 Genetics of bronchiolitis

In the neonatal period, the defence against infections is based on innate immunity and maternal inherited antibodies. Adaptive immunity takes several days to start before the clonal expansion of the lymphocytes and antigen-specific cell-mediated response take effect, and during that time any infection has to be controlled by the innate system. Genetic variance in innate immunity genes is common in different populations (144).

The epithelial cells and the overlying mucosal layer of the airway form a protective physical barrier for the first contact with inhaled foreign organisms (145). This serves as an important environment for adhesion of the microbe

surface structures or PAMPs (pathogen-associated molecular patterns), and as a response to that, direct antimicrobial compounds (lysozyme, lactoferrin) defend the host and many receptors signal to start a cascade leading to inflammatory response (145).

2.11.1 Toll-like receptors

Toll-like receptors (TLRs) are evolutionally conserved germ-line encoded molecular pattern recognition receptors (PRRs) of most crucial importance to the onset of innate immunity response and the following adaptive immunity (146). There are 10 known TLRs in humans that recognise a large number of different microbial components (TLRs 1, 2, 3, 6, and 10 encoded on chromosome 4, TLR4 on chromosome 9, TLRs 7 and 8 on the X chromosome, and TLR9 on chromosome 3).

TLRs are trans-membrane proteins; TLRs 1, 2, 4, 6, and 10 are located on the plasma membrane, while TLRs 3, 7, 8, and 9 are located in the endoplasmic reticulum, where they are transferred to lysosomes (145). Simplified, ligand attachment to TLRs starts a signalling cascade leading to the transcription of proinflammatory cytokines and type I interferons (147). According to the hygiene hypothesis, TLRs have been proposed to have a role in the development of Th1/Th2-imbalance, as the activation of TLRs leads to Th1-balanced cytokine production and might act as a counterbalance to Th2 cytokines (148). TLRs are able to distinguish host tissue from pathogens, but even though playing a vital part in the onset of acute inflammation, TLRs have been associated with chronic and autoimmune inflammation (149) and asthma (148). There are no previous post-bronchiolitis studies examining the associations of lung function and the genetic variation of TLRs.

Supporting the hygiene hypothesis, early childhood exposure to environmental house dust endotoxin has been associated with a decreased incidence of asthma in later life in several studies (150,151). TLR4 can recognise several unrelated structures like endotoxin (Lipopolysaccharides; LPS) and also the F protein of RSV (147). Originally, the *TLR4* rs4986790 (Asp299Gly) polymorphism was first associated with decreased airway reactivity to inhaled LPS in adult volunteers, and to a decreased TLR4 signalling cascade in vitro (152). This polymorphism has been confirmed to modify the effect of endotoxins in adults: when exposed to house dust endotoxin, carriers of at least one mutant allele were significantly less

asthmatic and airway reactivity was lower compared to the carriers of the wild-type allele (153).

During RSV infection, TLR4 is increasingly expressed in dendritic cells and macrophages. Mutation in the *TLR4* receptor gene has been shown to lead to attenuated signalling of the pathway and weak innate immune response to RSV (154). Studies have confirmed that the *TLR4* polymorphisms – and the hyporesponsiveness to endotoxin in particular – increase the risk of a severe RSV infection in infancy (155,156). However, some evidence suggests a RSV group and virulence dependency, since the association with a severe disease has varied between epidemics (157). It can be hypothesised that the *TLR4* polymorphism might serve as a risk factor for severe RSV infection in infancy, but, seen from a different point of view, also as a marker for a window of opportunity for environmental factors – such as endotoxins or viruses – to modulate the immune system away from the Th1/Th2-imbalance (148).

TLRs 1, 2, 6 and 10, the TLR2 subfamily, share a common signalling pathway (158). Signalling through TLR2 occurs as the heterodimers TLR2/TLR1, TLR2/TLR6, and TLR2/TLR10 (159). The TLR2 subfamily has a major role in the recognition of the lipoproteins of bacteria, viruses, and fungi (147). The genetic variation of the TLR2 subfamily has been associated with childhood asthma and allergies in several studies, though the direction of the association has varied (160-163). However, as the TLR2 subfamily is a heterodimeric group of different receptors, some studies have found associations with asthma only in haplotype-combinations within the TLR2 subfamily (164,165). Only *TLR6* rs5743810 (Ser249Pro) polymorphism has been linked to asthma in a few studies (162,166,167), and functionally this polymorphism alone has been linked to decreased IL-6 production in response to lipoproteins and mycobacterium (168). Increased IL-6 levels have been found in the lower airways of infants during RSV bronchiolitis (169). Earlier in this cohort, *TLR2* subfamily polymorphisms did not show significant associations with bronchiolitis severity or post-bronchiolitis wheezing (170), but at preschool age there was a significant association of atopic eczema with *TLR6* rs5743810 polymorphism and an increased risk of asthma associated with variant alleles in *TLR 1, 2*, and *6* haplotype analysis (171).

The TLRs 3, 7, 8, and 9 are specialised in recognising the nucleic acids of foreign DNA and RNA belonging to bacteria and viruses. To gain access to endosomal TLRs, the ligand has to be delivered into the cell by endocytosis (147). Concerning these TLRs, very little data has accumulated on associations with bronchiolitis or post-bronchiolitis sequelae, even though RSV and rhinoviruses

being single-strand RNA viruses plays an important role of these TLRs. Earlier from this cohort, *TLR3* variant polymorphism was associated with post-bronchiolitis wheezing (172). In vitro, TLR3 has been shown to be upregulated to ASM cells following contact with viral dsRNA (double-stranded RNA, TLR3 ligand) (173), but no studies focusing on childhood asthma or airway reactivity have been published.

In experimental animal studies with BN rats suffering from chronic asthma, a synthetic TLR7/8 ligand proved to have a potent anti-inflammatory effect and prevented airway remodelling (174). Only one study so far has linked the X-chromosomally located gene *TLR7* and *8* polymorphisms to childhood asthma (175); in another study no significant associations were found (162).

2.11.2 Interleukin-10

Interleukin-10 is a key cytokine having multiple regulatory effects on limiting the inflammatory response. IL-10 is produced in a variety of monocytes, macrophages, dendritic cells, and B-lymphocytes, but most importantly in regulatory naturally occurring Treg-cells and antigen-driven IL-10-secreting T-cells (176). IL-10 protects the host from an abnormally strong response to infections as well as autoimmune inflammation. The anti-inflammatory effects focus on activated macrophages/monocytes and dendritic cells, downregulating the production of IL-12, IL-18, IL-1, and TNF, reducing the function of Natural killer (NK) T-cells and the maturation of naive T-cells to Th1 cells (Fig 6).

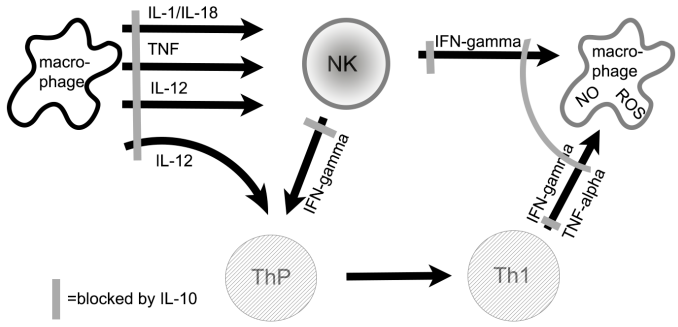

Figure 6. A schematic illustration of common sites for IL-10 in regulating innate and adaptive responses to infection. Modified from (177). © Eero Lauhkonen

In experimental studies, RSV infection has been shown to induce IL-10-producing CD4+/CD8+ T-cells in the lungs during the infection, and IL-10 deficiency in mice has been shown to lead to a more severe disease course and worse recovery, suggesting IL-10 has an inhibitory role controlling inflammation, especially during the recovery phase of the infection (178). In transgenic mice, an induced overexpression of the IL-10 gene and the presence of the IL-10 protein during the acute phase of the RSV infection attenuated the acute inflammation and also protected from late inflammatory changes (increased Th2 cytokine expression) (179). When the *IL-10* gene overexpression was delayed and artificially induced later, after the acute response to the RSV infection, the effect was more activating than inhibitory. In addition, in the mice without the RSV infection, increased *IL-10* expression led to Th2 cytokine and chemokine up-regulation (179). In vitro studies in children with RSV bronchiolitis have shown that IL-10 production is increased in the convalescent phase three to four weeks after the infection, and this was significantly associated with recurrent post-bronchiolitis wheezing (180). Thus, it could be that IL-10 has a dual role that can be either protective at the acute phase or pro-inflammatory in a later recovery phase of the infection, but this needs further clarification.

Interleukin-10 has become an interest in research also due to its role in the regulation of allergic inflammation, and experimental data suggest that in allergy, the regulatory T-cell populations might be impaired, perhaps leading to attenuated IL-10-mediated control of the allergic mechanisms (177,181).

In the highly polymorphic IL-10 promoter region, there are four functional single nucleotide polymorphisms (SNPs) influencing IL-10 production. In the proximal promoter area, three SNPs (–592 C/A, –819 C/T, and –1082 G/A) are in high linkage with each other. The common haplotypes GCC, ACC, and ATA have been recognised in Caucasian populations (182) and a fourth haplotype – GTA – in Asian populations (183). In vitro studies have suggested that the haplotype ATA is associated with low IL-10 production (184), or just the mutation at –1082 G/A regardless of the two other SNPs (182). Likewise, an SNP in the distal promoter region –3575 T/A has been associated with low IL-10 levels (185), but overall there is limited data concerning single-point mutations and the regulation of IL-10 production.

Two SNPs very close to our study at –1117 and –3585 have been previously associated with the severity of RSV bronchiolitis (186). From this cohort, severe rhinovirus bronchiolitis and preschool asthma were associated with the *IL-10* –1082 G/A polymorphism (51,187). In the only IL-10 study addressing lung

function in 518 asthmatic children at a mean-age of 8.1 years, the haplotype ATA was associated with a low level of FEV1 and the haplotype GCC with high FEV1 (188), *i.e.* the low-IL-10-producing haplotype was associated with low lung function. The polymorphism of –1082 G/A has been associated with adult asthma in a large meta-analysis of 11 studies (189). Supporting the significance of low IL-10 levels, at least in adult asthmatics, reduced IL-10 levels have been determined from bronchoalveolar fluid (190).

2.12 The united airways disease (UAD) hypothesis

According to the atopic march theory, atopic eczema, allergic rhinitis, and asthma share a common genetic background, but the disease manifestations and onset vary according to age (191). It is well-known that the first allergic sensitisation in life often concerns food allergens, commonly cow's milk allergy in infancy, often remitting by the age of two years. Thereafter, the IgE-mediated sensitisation to inhaled allergenes takes place, manifesting as allergic rhinitis. Childhood allergic rhinitis, airway hyperreactivity, and asthma have a well-known association (192-194).

Delineating the atopic march theory, the UAD hypothesis postulates that upper and lower airways diseases have a shared pathophysiological background, and that allergic rhinitis and asthma therefore develop jointly, co-exist, and have an effect on each other when manifested, and likewise respond the other way round when treated (195-197). Thus, according to the atopic march theory and the UAD concept, it can be hypothesised that lung function deterioration results from chronic atopic inflammation leading to airway remodelling changes. There is, however, limited evidence on this hypothesis contributing to lung function development.

2.13 Pulmonary function testing in young children

In pulmonary function testing, flow-volume spirometry (FVS) has been the standard in adults. However, this needs good patient compliance and skill in order to achieve deep inspiration and sufficiently strong expiratory blows. Preschool and school-aged children often show a poor success rate in spirometry (198,199) and

there is a high degree of variance in the results, especially in the parameters representing small airways (200).

In addition, spirometry measures only the air flow limitation, which is an end outcome of several factors involved in obstructive lung diseases. Alternative, non-invasive methods needing only little co-operation have been developed to obtain objective lung function measurements from young children (201). Direct measurement of airway resistance to airflow gives additional information on deeper lung mechanics and the surrounding thorax (202).

Common methods in clinical use measuring airway resistance are whole body plethysmography, deriving airway resistance (Raw), specific airway resistance (sRaw) and specific airway conductance (sGaw), the measurement of interrupter resistance (Rint), and the method of forced oscillations (FOT) (201).

2.13.1 Airway resistance in lung function

In obstructive lung diseases, the hallmark change in the airways is increased airflow resistance (202). This is determined by several factors, including length and lumen of the airway, gas density, and flow turbulence. It can be characterised by pressure change in relation to airflow, being inversely proportional to the fourth power of the radius of the airway lumen (r):

$$\frac{\Delta P}{\dot{V}} \sim \frac{1}{r^4}$$

In addition, the human airways are flexible and divided downwards into 23 generations (Fig. 3), so the total airway resistance is the reciprocal sum of the parts of the airways. In studies modelling the human lung, the airway resistance lowers in the trachea and the first four bronchial tree generations until it rises to its maximum level at 6–8 generations and starts to lower again continuously, being very low at the alveolar level (77). In all, the conducting airways up to the 16th generation of the bronchial tree (Fig. 3) account for the majority of airway resistance. Only a small part of the total lung resistance comes from the peripheral small airways (77) and as much as 40% comes from the surrounding lung tissue and thorax (203).

2.13.2 Airway responsiveness and bronchodilator response

The limited airflows in response to different external stimuli can be gathered under the umbrella term of airway hyperresponsiveness (204). The mechanisms leading to airway narrowing are not fully understood, but they probably consist of increased mass and constriction of airway smooth muscle (ASM), airway inflammation and remodelling, and neurogenic control of the airway tone (205).

External factors contributing to airway constriction can be divided into direct and indirect stimuli; direct mechanisms act through effector cells, such as ASM cells or mucus producing cells, and indirect mechanisms act through intermediary cells, such as mast cells or neuronal cells, which further stimulate the effector cells leading to bronchoconstriction (206). Methacholine and histamine inhalations are direct stimuli that can be used in bronchial provocation tests. Physical exercise and inhaled cold air are indirect stimuli that work by releasing inflammatory mediators and by neural activation in the bronchial provocation test. Administration of adenosine (cAMP) or bradykinin, for example, present pharmacologically induced indirect stimuli (206).

Bronchodilator responsiveness is defined as the reversibility of the airway obstruction achieved by bronchodilator administration. Such reversibility is an essential characteristic of asthma, as may also be bronchial hyperresponsiveness. Basically, bronchodilator responsiveness and bronchial hyperresponsiveness represent different physiological phenomena that often do not correlate, not even in all patients with asthma (207).

In this study, the term "bronchial hyperreactivity" (BHR) has been used in certain analyses combining exercise- and bronchodilator-induced responses, which differs from the exact use of the term BHR in previous literature.

2.14 Impulse oscillometry (IOS)

The use of the forced oscillations technique (FOT) in the measurement of airway impedance was introduced already in 1956 (27). The mechanical basis of oscillometry lies in external pressure signals conducted to the airways during the spontaneous breathing of the subject (208).

Impulse oscillometry (IOS) (209) is a quite recently introduced modification of FOT, where short time-discrete impulse-like external signals are conducted to the airways during tidal breathing through an open mouthpiece (208). The oscillation

amplitude and phase differences in the pressure and flow output signal are analysed to measure the total airway input impedance (Zrs) and further derive the resistance (Rrs) and reactance (Xrs) of the airways. Resistance reflects the energy loss due to resistive forces to the air flow in the airways. Reactance is an imaginary component, constituting the mass-inertia energy of the moving air column (inertance) and the elastic properties of the lung tissue and describing energy storage (capacitance). The resistance and reactance of the respiratory system change as a function of the oscillation frequency depending on standing height and age (210).

During the measurements, the child is seated and breathes normally, without crying or coughing. To prevent loss of pressure to the upper airways, a nose-clip is used and the child's cheeks are supported by the technician's hands. The input signal of harmonic oscillations at 1–35Hz is conducted through a mouthpiece to the bronchial tree as the child breaths normally. The oscillometric input impedance is recorded from the output airflow, and the resistance (Rrs) and reactance (Xrs) curves are calculated as a function of oscillation frequency (Fig. 7).

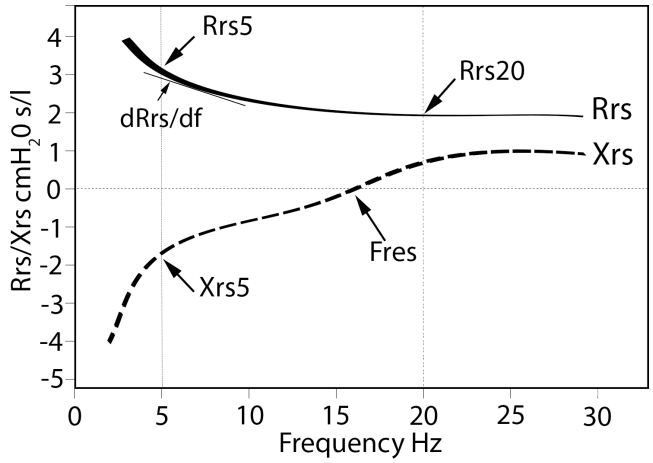

Figure 7. Illustration of the IOS curves as a function of oscillation frequency. dRrs/df=Frequency dependency of resistance; Fres=Resonant frequency, ©Eero Lauhkonen

Low frequencies (<15Hz) are thought to describe the peripheral (small) airways, and higher frequencies the central (larger bronchi) airway. Rrs increases as the airway lumen decreases in bronchoconstriction, inflammation, and oedema, resulting in increased (more negative slope) frequency dependency of resistance, dRrs/df. In asthmatic patients, bronchodilation may decrease Rrs in both high and low frequencies, but usually relatively more in low frequencies (small airways)

associated with a decrease in dRrs/df (208). The point at which reactance equals zero represents the equal magnitude of inertance and capacitance, and is called the resonant frequency (Fres). Reactance at low frequencies represents the capacitive properties of the peripheral lung, *i.e.* alveolar volume and compliance of the lung tissue including the thorax. Xrs decreases due to peripheral stiffening as a result of small airways obstruction, lung tissue fibrosis, or peripheral lung hyperinflation in emphysema (208).

2.14.1 Application of IOS

The method is non-invasive and relatively inexpensive, and one cycle of IOS measurement is relatively quick, which is a clear benefit in the paediatric setting, though often the measurements may take some time to practice and to ensure the patient's adequate co-operation. In contrast to spirometry, IOS is performed without deep inhalation and is therefore unlikely to alter the bronchial tone and confound the analysis (201,211).

IOS has been considered suitable for children as young as two to three years old (212-215). Finnish population-based references for two- to seven-year-old children are available, and the method can be used in analysing reactivity to exercise or bronchodilation (201,210,216). IOS is very sensitive to upper airway artefacts, such as glottis closure and the upper airway shunt, thus multiple measurements are needed to obtain reliable results (208).

The IOS method has been evaluated in multiple studies in comparison to other pulmonary function tests, and it has demonstrated high sensitivity to peripheral lung pathology, as in asthma or COPD (208). In a recent study with three- to 17-year-old asthmatic children and non-asthmatic controls, IOS was found to be superior in showing reversible obstruction compared to spirometry (217). IOS also classifies with an >80% accuracy an uncontrolled asthma status from asthmatic children and healthy controls with peripheral small airway parameters Rrs5–20 (difference in resistance from Rrs5 to Rrs20), Xrs5, Fres, and AX (low frequency reactance area) (218). Xrs5 has been shown to have a good diagnostic value in testing bronchial hyperresponsiveness with methacholine challenge in preschool-aged children and also in differentiating clinical severity of asthma in school-aged children (219,220). It has been suggested that reactance at low frequencies is a more sensitive marker of peripheral obstruction than respiratory system resistance (208).

In clinical studies, the use of IOS is not yet fully established, and it has been debated how the values correlate with the ones obtained with spirometry at the same age (30,221). In a high asthma-risk cohort study by Guilbert et al., asthma diagnosis at six or eight years of age was supported by moderately decreased lung function by FVS, but not by IOS measurements at the same timepoints (30). Thus, more comparable clinical studies are needed to clarify the relationship of flow-volume measurements by FVS and measurements of IOS.

In addition, the prognostic value of IOS lung function measurements in young children is not well known, though recently in asthmatic children IOS variables Rrs5 and dRrs/df at preschool age correlated with spirometric FEV1 at adolescence (222). Overall, the interpretation of IOS results needs careful consideration and more research is needed.

The normal limits of IOS values can be presented as height-adjusted Z-scores (210), with an upper reference limit (95th percentile) at +1.65, and the lower reference limit (5th percentile), respectively, at -1.65. In practice, a deviation of 1.65 above the predicted reflects a minor, >2.0 a moderate, and >4.0 a severe pathology in IOS parameters, *i.e.* in pathological conditions, the main parameter Rrs rises above +1.65 and Xrs decreases below -1.65 (208).

In the evaluation of bronchial reactivity, no clear consensus of the pathological limits exists. A Finnish study suggested a 35% limit for an Rrs5 increase after ECT (216). Furthermore, in the current Finnish IOS reference values, the decrease of 37% in Rrs5 was suggested as a pathological response in a post-bronchodilator test in two- to seven-year-old children (210). Others have suggested slightly stricter -40% to -42% limits as a pathological response in post-BD test Rrs5 in the same age group (223,224). In Denmark, a 37% increase in Rrs5 in a cold air challenge distinguished asthmatic and non-asthmatic two- to five-year-old children measured by IOS (225). The current ATS/ERS guidelines and Finnish current-care guidelines – as used in the present study – propose a minimum 35% change in resistance in response to exercise or bronchodilation to be suggestive of asthma (201).

3 Aims of the study

The principal aims of this study were to describe the lung function before school age in children hospitalised for bronchiolitis and to explore environmental and genetic factors possibly influencing the lung function outcome. To summarise, the more specific aims were:

- To evaluate lung function and bronchial reactivity using IOS at the age of five to seven years after hospitalisation for bronchiolitis at the age of less than six months.
- To assess the role of weight gain before school age as a risk factor for lung function reduction or bronchial hyperreactivity in post-bronchiolitis settings.
- To clarify the role of the polymorphisms of the four interleukin-10 encoding genes (rs1800896, rs1800871, rs1800872, and rs1800890) in post-bronchiolitis lung function and bronchial reactivity.
- To explore whether the polymorphisms of the nine toll-like receptor encoding genes (*TLRs 1, 2, 3, 4, 6, 7, 8, 9*, and *10*) are associated with post-bronchiolitis lung function or bronchial reactivity.
- To find evidence for or against the hypothesis of the united airways disease in this post-bronchiolitis cohort.

4 Materials and methods

4.1 Patient enrolment

At the Department of Paediatrics, Tampere University Hospital (Finland), 187 children were recruited during hospitalisation due to bronchiolitis. The enrolled children were previously healthy, born at term, and less than six months old. Bronchiolitis was diagnosed on the basis of history and physical examination of the infant as having LRTI with wheeze or inspiratory crackles, cough, tachypnoea, increased respiratory effort, and/or feeding problems (32). The children were enrolled during three subsequent epidemics between 1 December 2001 and 21 May 31 2004.

The aetiology of bronchiolitis was determined from nasopharyngeal aspirates analysed for antigen detection using indirect immunofluorescence and genome detection with reverse-transcriptase PCR for RSV, Influenza A and B virus, adenovirus, and parainfluenzaviruses 1 and 3. In addition, PCR covered human-metapneumovirus, rhinovirus, and bocavirus recognition, since there is no antigen detection in use for these. Altogether, combined results of nine different respiratory viruses were available, as was PCR for the bacterium *Bordetella pertussis*.

The participants' weight was measured during the hospital stay using a calibrated scale. The information on birth weight data was acquired from the hospital's register. Weight gain before hospitalisation was calculated as gram/week units. Parental history of smoking, asthma, and allergies were registered during hospitalisation.

4.2 Follow-up visit

A total of 127 children attended a follow-up visit at the age of 5–7.5 years. The control visits were organised outside the main pollen seasons in October 2008, January and March 2009, and October 2009. In case of a respiratory infection in the preceding two weeks, the follow-up was rescheduled.

Prior to the follow-up visit, the parents answered a structured questionnaire (published previously by Koponen P, Doctoral dissertation 2014, Appendix 1) to examine the child's previous wheezing episodes, prolonged cough (>4 weeks), or coughing during the night without having an infection, doctor-diagnosed asthma and the age of asthma-onset, as well as the use of bronchodilators and inhaled corticosteroids. In addition, data on atopic eczema in infancy and during the last 12 months, prolonged rhinitis or allergic conjunctivitis during the last 12 months, doctor-diagnosed parental asthma or allergy, maternal smoking during pregnancy, parental smoking before and after the age of one year, and the keeping of household pets was collected. The pre-filled questionnaire was revised together with the child and parents to avoid any misunderstandings and to add to the history of the child. Continuous asthma medication was not interrupted. The use of beta-agonists had to be stopped a minimum of 12 hours before the lung function testing.

4.3 Clinical data collection

At the control visit, all the children were physically examined by a paediatrician. Height and weight at the control visit were measured using standard techniques with scales and stadiometers.

In 124 children, data on skin prick test-sensitisation to cat and dog dander; birch, timothy grass, and mugwort pollens; house dust mites (*D.pteronyssimus* and *D.farinae*); and spores of the mould *Alternaria alternate* was available. The test was regarded as positive if the skin wheal increased to a diameter of 3mm and the negative control showed no reaction. Antihistamine medication had to be stopped for five days before the control visit.

Prolonged rhinitis was considered to be present if the child had experienced sneezing, rhinorrhoea, or a blocked nose outside infection in the preceding 12 months, with seasonal or animal contact onset, or if the child had been diagnosed with allergic rhinitis by a doctor.

In all, 107 children aged less than seven years performed lung function testing with impulse oscillometry (IOS, Master Screen IOS; Jaeger, Höchberg, Germany) followed by the exercise challenge test (ECT) and post-bronchodilator (post-BD) testing. Four were excluded from the lung function analysis due to following reasons: two refused to run on the ECT properly, a different IOS device was used in testing one child, and data from hospitalisation was missing in one case. Thus,

preschool-age follow-up data and complete lung function testing were available from 103 (55%) of the original study subjects.

Bronchial hyperreactivity (BHR) was considered present if the child had a 35% rise in response to exercise or a 35% decrease in response to bronchodilation in Rrs5, calculated as a change from the baseline Rrs5.

A new asthma diagnosis was made if a child had wheezing or prolonged cough (>4 weeks) or night cough apart from infection and simultaneous BHR documented in the IOS. One child had wheezing symptoms and a pathological ECT, and one child had prolonged cough symptoms and a pathological decrease in the BD test.

Current asthma was considered present when the child had been on continuous or intermittent ICS medication during the last 12 months or was newly diagnosed with asthma at the follow-up. Use of ICS medication for longer than three months before the preceding 12 months – but not during the 12 months – was classified as previous asthma.

4.4 Lung function testing by IOS

At the control visit, the children performed lung function measurement at the Department of Clinical Physiology, Tampere University Hospital. The tests were performed in October 2008 (27 children), January and March 2009 (16 children), and October 2009 (60 children). The mean outside temperature was +5 degrees Celsius, with a range from -8 to +17 degrees Celsius. The majority of the children (87/103) were tested in October in fair conditions. Some were tested during the winter months due to practical reasons. In all, 18/103 study subjects ran the ECT in a temperature below 0 degrees Celsius.

An experienced clinical physiologist judged the IOS curves to be graphically sound and artefact-free for the whole 30-second measurement time. Three acceptable IOS curves were demanded to accomplish each stage of the study protocol. The results were used to calculate the mean values and further the height-adjusted deviations as Z-scores for each parameter.

The ECT was performed after the baseline measurements. The children ran for 8 minutes outdoors reaching ≥90% of expected heart rate (205-age/2) for ≥2 min, which was monitored with a heart rate monitor (Polar Ltd, Kempele, Finland). Post-exercise IOS curves were measured straight after running and at 5-, 10-, and 15-minute timepoints after running. A clinical physiologist screened the appropriate measurements to be analysed, selecting three acceptable IOS curves, free from artefacts, mostly from the 5- or 10-minute timepoints after running.

The children then went through a modification of the post-bronchodilator (post-BD) test. After the ECT, the children were given 300μg salbutamol (Ventoline®, GSK) administered by inhalations through a spacer (Babyhaler®, GSK), and again, 15 minutes after the inhalation, three acceptable IOS curves were obtained, now representing the post-BD values.

The study protocol for each child was completed during one day. In all, baseline, post-exercise, and post-bronchodilator respiratory system impedance (Zrs), resistance (Rrs) and reactance (Xrs) at 5Hz, resonant frequency (Fres), and frequency dependency of resistance (dRs/df) measurements were acquired.

4.5 Genetic methods

Altogether, 135 whole blood samples were collected during hospitalisation and frozen for further DNA analysis. DNA was extracted from whole blood using a

commercial kit (QIAGEN Inc., USA) at the Department of Clinical Microbiology (Tampere University Hospital) and at the Department of Medical Microbiology and Immunology (Turku University Hospital).

4.6 Genotyping of *IL-10* gene promoter polymorphisms

PCR and allelic discrimination in genotyping of the *IL10* rs1800896 single nucleotide polymorphism (SNP) was carried out by using the ABI PRISM® 7000 Sequence Detection System (Applied Biosystems, CA, USA). A commercial kit (Assay On Demand, C_1747360_10 IL10) was used according to the manufacturer's protocol. The universal PCR thermal cycling conditions for ABI were as follows: 50°C for 2 min, then 95°C for 10 min, and then 40 cycles at 95°C for 15 s and 60°C for 1 min. The PCR reaction was performed in a 25 μl volume-containing TaqMan® Universal PCR Master Mix with AmpErase® UNG (ABI, CA, USA), 1x Assay Mix (primers and probes: ABI, CA, USA), and 10-100 ng of template DNA. The genotypes were selected manually from the allelic discrimination tab.

IL10 rs1800890 genotypes were determined by pyrosequencing (PSQTM 96MA Pyrosequencer, Biotage, Uppsala, Sweden) using a PSQTM 96 Pyro Gold Q96 reagent kit according to the manufacturer's protocol. Primers are designed to recognise the polymorphic site in the IL-10 promoter region (A changes to T).

The detection of *IL10* polymorphism sites rs1800871 and rs1800872 were performed using PCR and sequencing of the amplified region. The SNPs were detected simultaneously in one PCR reaction. The sequence of PCR primers were: forward: 5'-TAGGTCTCTGGGCCTTAGTT-3' and reverse: 5'-AAGGCCAATTTAATCCAAGGTT-3'. The correct PCR product size (440 bp) was verified with agarose gel electrophoresis. The forward primer was also used for the sequencing reaction. Sequencing reactions were performed at the Institute for Molecular Medicine Finland (FIMM, Helsinki). All PCR reactions were performed in the following conditions: initial denaturation at 95°C, denaturation at 95°C for 2 minutes, annealing at 60°C for 30 seconds, and extension at 72°C for 40 seconds. After 40 cycles, there was a final denaturation at 72°C for 7 minutes.

Data on the SNPs of *IL10* rs1800896, rs1800871, and rs1800872 were available for 99 of the children with IOS data, and data on the SNP *IL10* rs1800890 were available for 98 of the children with IOS data.

The individual haplotypes of rs1800896, rs1800871, and rs1800872 were counted from unphased genotype data using the Clark's algorithm (226), where all homozygote and single-site heterozygotes were identified and haplotypes resolved by direct counting. The SNPs rs1800871 and rs1800872 were co-segregating. In this way, 87% of the haplotypes were unambiguous, and the remaining 13% could be resolved using the haplotype frequencies. All four studied SNPs are highly linked (Fig. 8), but we were not able to resolve all four allele haplotypes, thus rs1800890 was omitted from the haplotype analyses.

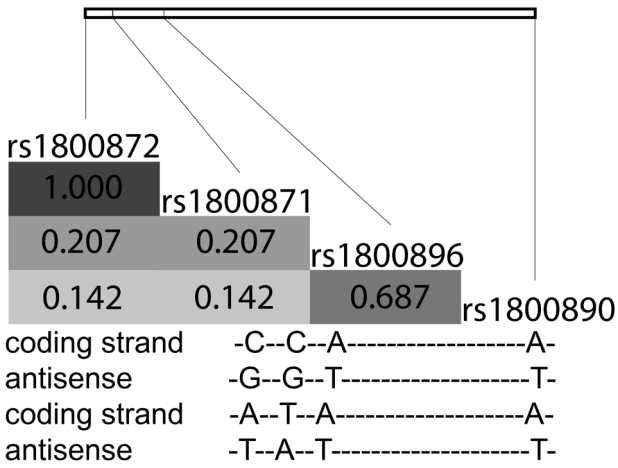

Figure 8. R2 values for the studied *IL-10* polymorphisms in Finnish population-based data. The formation of ACC(A)/ATA(A) genotype from the parental DNA strands are described as an example. Greyscale=R2-correlation, all pairs D´=1. Adapted from original article III.

4.7 Genotyping of the *TLR 1, 2, 3, 4, 6, 7, 8, 9*, and *10* polymorphisms

The genotyping of *TLR1* rs5743618, *TLR2* rs5743708, *TLR3* rs3775291, *TLR6* rs5743810, and *TLR8* rs2407992 was performed by pyrosequencing (PSQ™96MA Pyrosequencer, Biotage, Uppsala, Sweden). PCR products with potential SNPs were recognised as the template in the pyrosequencing reactions using a PSQ™96 Pyro Gold Q96 reagent kit according to the manufacturer's protocol.

The genotyping of *TLR4* rs4986790 was performed by pyrosequencing with the ABIPRISM 7000 Sequence Detection System (Applied Biosystems, CA),

supplemented later with pyrosequencing (PSQ™96MA Pyrosequencer, Biotage, Uppsala, Sweden) using a PSQ™96 Pyro Gold Q96 reagent kit.

For *TLR7* rs179008, the PCR products were first purified with a QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. PCR products with low DNA content were eluted to 30µl with elution buffer. After the purification, the PCR products were pipetted into a 96-well plate (5µl) together with *TLR7* rs179008 forward primer (1.6µl), and the 96-well plate was sent for full service sequencing at FIMM (Institute for Molecular Medicine) seqlab, Helsinki, Finland.

TLR9 rs187084 genotyping was performed by using BspTI restriction enzyme (ThermoFischer Scientific, Waltham, USA) for the digestion of PCR product. High resolution melting analysis (HRMA) (Roche Diagnostics Light Cycler 480, Basel, Switzerland) was used for the genotyping of *TLR10* rs4129009. HRMA PCR reactions were run at 95°C for 10 min followed by 45 cycles of amplification at 95°C for 10 s, then 59°C for 10s, and then 72°C for 15s. After the PCR process, the final melting cycle conditions were as outlined by the manufacturer: first heating to 95°C and holding for 1 min, followed by cooling to the pre-hold temperature (40°C) to make sure that all PCR products were re-associated and to encourage heteroduplex formation. The melting interval for collecting fluorescence was from 60°C to 95°C at a ramp rate of 0.02°C per second. In each run, known *TLR10* rs4129009 standards (wild type, heterozygote, and homozygote) were used. All the primers were purchased from SIGMA-ALDRICH, Finland.

The PCR and sequencing primers used for *TLR7* rs179008 and *TLR8* rs2407992 are located on the X chromosomes.

4.8 Statistical methods

The structured questionnaire data and clinical data including the viral aetiology of bronchiolitis, SPT results, genetic data, and measured IOS variables were collected on an Excel spreadsheet (Microsoft, Redmond, USA).

The three selected IOS measurement curves were used to calculate mean values, which were transformed into height-adjusted standard residuals (Z-scores) using a prediction equation from recently published Finnish population-based reference material (210). The upper reference limit for the Z-scores was set to +1.65 (95th percentile), and the lower reference limit, respectively, to -1.65 (5th percentile).

Bronchial reactivity was studied calculating exercise-induced changes and bronchodilator-induced changes in Zrs5, Rrs5, Xrs5, and Fres in continuous Z-score changes but also as the categorised pathological change of Rrs5. An exercise-induced increase of 35% from baseline Rrs5 and correspondingly a bronchodilator-induced decrease of 35% from baseline Rrs5 were considered pathological.

Using Finnish national population-based weight and height data as a reference (227), current weight and height data were transformed into age- and sex-specific height-related body mass index-for-age Z-scores (zBMI). The cut-off value of the zBMI for overweight was 1.16 for girls and 0.78 for boys (corresponding to a BMI of >25 kg/m2 in young adults) and the cut-off value of the zBMI for obesity was 2.11 for girls and 1.70 for boys (corresponding to a BMI of >30 kg/m2 in young adults).

The statistical significance of the differences were analysed using SPSS Statistical Package version 21 (IBM, NY, USA). A result was considered statistically significant if the *p*-value was less than 0.05. Analysis of variance (ANOVA) was used in the analyses of continuous data. The chi-squared and Fischer's exact tests were used in the analyses of categorised data. The analyses were adjusted for age, sex, maternal asthma, atopic eczema, RSV and non-RSV aetiology of bronchiolitis during hospitalisation, weight status, and maternal smoking during pregnancy, when appropriate. The results are represented as mean values, standard deviations (SD), and 95% confidence intervals (95%CI) for continuous variables, and as numbers and frequencies for categorised variables.

The correlation between weight status (zBMI) and IOS parameters was studied with multivariate linear regression, and the results are represented as Pearson's R, Pearson's R2, and adjusted *p*.

4.9 Ethics

The study was conducted according to the guidelines of the Finnish Advisory Board on Research Integrity and the Declaration of Helsinki. The study was approved by the Pirkanmaa Hospital District Ethics Committee (approval number R13025).

Study participation was approved by the parents, who gave written, informed consent. It was highlighted that participation was not mandatory and the families could decide to withdraw at any phase of the study. The families did not receive any payment for participating in the study.

As the children were five to seven years old, the parents received a written information letter before the control visit. Contact information for the study nurse was provided if they had any questions. The parents were asked to complete a structured questionnaire at home and to bring it with them or post it in case they were not able to participate any further. The children participated voluntarily at the control visit, where they were orally informed and written informed consent was obtained from the parents.

The use of the genetic data was limited to asthma research by the Ethics Committee permit. The blood samples were coded with numbers and analysed anonymously in the genetics laboratory. In practice, only the principal investigator had access to the personalised genetic data, which was stored separately from other data.

The study project did not receive any commercial funding associated with medical or pharmaceutical companies. The main funding was received from Finnish state research funding and non-profit research foundations. The researchers had no conflicts of interest during the study process or regarding the published scientific work.

5 Results

5.1 Background factors

Follow-up data and complete lung function data were available from 103 children at a median age of 6.3 years (Table 1).

Half of the study subjects were boys (49.5%). Parental smoking during the first 12 months of life was reported in 52.4% of cases, and maternal smoking during pregnancy in 20.6% of cases. Wheezing symptoms were reported in 22%, prolonged rhinitis in 26%, and atopic eczema in 30% of the study subjects in the preceding 12 months. Bronchodilator use was reported in 17.5% and 13 (12.6%) of the children had used inhaled corticosteroids during the 12 months prior to the follow-up visit. Three children received new diagnoses at the follow-up visit, one of them was symptomatic and already using ICS medication without a proper diagnosis, one had preceding wheezing symptoms and BHR in the ECT, and one had prolonged cough symptoms apart from infection and BHR documented in the post-BD test. In 15 children, current ongoing asthma was considered present, and a history of transient previous asthma was present in 12 children.

Table 1. Characteristics of study population.

No. of subjects	103
Male, n (%)	51 (49.5)
Female, n (%)	52 (50.5)
Age (y), mean (range)	6.3 (5.5–7.0)
Height (cm), mean (range)	120 (107–136)
Weight (kg), mean (range)	24 (16–39)
RSV bronchiolitis, n (%)	62 (60.2)
Rhinovirus bronchiolitis, n (%)	16 (15.5)
Non-RSV bronchiolitis, n (%)	41 (39.8)
-Including RV in 16 and influenza A in 5, parainfluenza in 5, metapneumovirus in 3, adenovirus in 2, *Bordetella pertussis* in 1, and negative in 9 cases	
Current asthma, n (%)	15 (14.6)
Previous transient asthma, n (%)	12 (11.7)
Regular use of ICS during the last 12 months, n (%)	13 (12.6)
Use of bronchodilators during the last 12 months, (%)	18 (17.5)
Wheezing symptoms during the last 12 months, n (%)	23 (22.3)
Prolonged rhinitis during the last 12 months, n (%)	27 (26.2)
Atopic eczema during the last 12 months, n (%)	31 (30.1)
SPT-positivity, n (%)	32 (31.1)
Maternal smoking during pregnancy, n (%)	19 (18.4)
Parental smoking during infancy (<12 months age), n (%)	54 (52.4)
Maternal asthma, n (%)	14 (13.6)
Paternal asthma, n (%)	5 (4.9)

RSV = Respiratory syncytial virus; ICS = Inhaled corticosteroids; SPT = Skin prick test

5.2 Clinical post-bronchiolitis lung function and airway reactivity data (I)

IOS results of the 103 children with complete data for the baseline, post-exercise, and post-bronchodilator (post-BD) measurements are published in original article I as absolute values (units kPa/l/s) and relative height-adjusted values (Z-scores). When compared to the baseline values, the post-BD values were significantly better, but there were no significant differences in the baseline and post-exercise measurements (I). Boys had lower post-exercise reactance values, but otherwise there were no sex-specific differences in the IOS values (I). Exposure to parental smoking during infancy was not associated with lung function, ECT, or post-BD IOS values. Maternal smoking during pregnancy or infancy was associated with less response in Xrs5 after bronchodilation (I).

One fifth (20.4%) of the study subjects had either pathological Rrs5 or Xrs5 in the baseline measurements. Xrs5 was pathological in 19 of the cases and Rrs5 was pathological in eight of the cases, and both were pathological in six children. After bronchodilator administration, all but one case returned to normal values (Table 2).

Table 2. Numbers of study subjects with increased airway resistance (Rrs5) and decreased airway reactance (Xrs5) at 5 Hz in baseline and post-bronchodilator values (Z-scores), and mean Rrs5 and Xrs5 values (Z-scores) in 103 former bronchiolitis patients at a mean age of 6.3 years.

Parameters*	Baseline values	Post-bronchodilator values
	n (%)	n(%)
Rrs5 ≥ +1.65 (pathologic)	8 (7.8)	0 (0)
Rrs5 < +1.65 (normal)	95 (92.2)	103 (100)
Xrs5 ≤ -1.65 (pathologic)	19 (18.4)	1 (1.0)
Xrs5 > -1.65 (normal)	84 (81.6)	102 (100)
Rrs5 or Xrs5 pathologic	21 (20.4)	1 (1.0)
Rrs5 and Xrs5 pathologic	6 (5.8)	0 (0)
Mean Rrs5 (95%CI)	-0.09 (-0.29–0.12)	-1.72 (-1.96–-1.56)
Mean Xrs5 (95%CI)	-0.74 (-0.98–-0.50)	0.33 (0.20–0.47)

Rrs = resistance; Xrs = reactance, *in Z-scores based on the Finnish reference material (210)

In Rrs5, a pathological ≥35% increase in response to exercise was present in five children and a significant decrease of 35% in response to bronchodilator was present in 11 children (Table 3).

Table 3. Exercise- and bronchodilator-induced changes in IOS (Z-scores), percentage and categorised Rrs5 change in 103 former bronchiolitis patients at the mean age of 6.3 years.

Parameter	Exercise-induced change	Bronchodilator-induced change
	Mean (95% CI)	Mean (95% CI)
ΔZrs5	0.66 (0.21, 1.10)	0.10 (-0.08, 0.28)
ΔRrs5	0.51 (0.16, 0.87)	0.12 (-0.05, 0.28)
ΔXrs5	-0.35 (-0.60, -0.11)	0.09 (-0.11, 0.30)
ΔFres	0.49 (0.07, 0.88)	-0.46 (-0.71, -0.21)
ΔRrs5 (%)	+4.9% (range -25.6% – 70.7%)	-21.7% (range 9.1% – -45.2%)
ΔRrs5 ≥35%	5 (4.9%)	11 (10.7%)

Zrs =Total impedance; Rrs=Resistance; Xrs=Reactance; Fres=Resonant frequency

When analysed as categorised variables, of the eight children with a pathological Rrs5 in baseline IOS, none had a pathological increase in Rrs5 in the ECT, and four (50.0%) had a pathological decrease in Rrs5 in the post-BD test (p=0.004, against those 95 (7.4%) with normal baseline Rrs5).

In the 19 children with a pathological Xrs5 in baseline IOS, none had a pathological increase in Rrs5 in the ECT, and similarly four (21.4%) had a 35% decrease of Rrs5 in the post-BD test (p=0.120 against those 84 (16.5%) with a normal baseline Xrs5).

5.3 Virus aetiology of bronchiolitis (I)

The aetiology of bronchiolitis was RSV in 60.2% (n=28 former RSV A and 34 RSV B) of the subjects. In five RSV cases, co-infections with other viruses were found, mainly with rhinoviruses. In the non-RSV cases, rhinovirus was positive in 16, influenza A in five, parainfluenza in five, adenovirus in two, and metapneumonvirus in three. In one case, *Bordetella pertussis* was found and in nine children the pathogen could not be detected. There were no significant differences in lung function or bronchial reactivity to ECT or in response to the post-BD test between RSV and non-RSV cases, or between RSV A and RSV B cases (I). Rhinovirus aetiology of bronchiolitis was associated with lower baseline resistance at 20 Hz compared to the RSV cases (I).

5.4 Asthma and the IOS outcome (I, V)

Current asthma (n=15) or previous transient asthma (n=12) did not associate significantly with baseline lung function (V) (Table 4). Between the current asthma, previous asthma, and no asthma groups, there was a non-significant trend towards increased Rrs5 and decreased Xrs5 in the children with a history of asthma, and the children with previous transient asthma in particular had the lowest reactance at 5Hz (Xrs5 -1.50 Z-scores) when compared to children with current asthma (Xrs5 -0.83, p=0.44), and no asthma (Xrs5 -0.60, p=0.04).

Current asthma was associated with BHR (V), as 38.5% of the cases with current asthma had pathological hyperreactivity in ECT or response in the post-BD test compared to 10.9% of children with no asthma (p=0.042). The children with previous asthma did not differ from those with no asthma regarding BHR (Table 4).

Table 4. Association of baseline impulse oscillometry and bronchial hyperreactivity with the presence of current asthma, previous asthma, and no asthma.

Asthma status	Baseline Rrs5 or Xrs5 pathological	*p*-value*	Bronchial hyperreactivity present	*p*-value*
Current asthma	4/15 (26.7%)	0.604	5/13** (38.5%)	0.042
Previous asthma***	4/12 (33.3%)	0.358	1/12 (8.3%)	1.00
No asthma	13/75 (17.3%)	---	8/75 (10.9%)	---

Rrs5 was pathological (>+1.65 SD) in eight and Xrs5 was pathological (<-1.65 SD) in 19 cases. BHR was defined as a >35% increase in resistance at 5Hz in the ECT (n=5) or a >35% decrease in the post-BD test (n=11).

* The *p*-values are calculated against the no asthma group with Bonferroni correction.

** The asthma diagnosis was partially based on BHR documented by IOS in two cases, and these cases are not included in the analyses between BHR and asthma.

*** Children with current asthma not included.

In the 13 former bronchiolitis patients who had used ICSs, post-exercise values in all IOS parameters were significantly higher (and Xrs and dRs/df lower) compared to those 90 who had not used ICSs during the preceding 12 months (I) (Table 5).

The use of ICSs during the last 12 months did not associate with baseline lung function or response to bronchodilation. ICS use was associated with pathological categorised ECT, as three out of five children with a positive ECT had ICS use (p=0.014). When exercise induced changes were analysed as continuous variables, the ICS medication was associated significantly with Rrs5 and Xrs5 compared to non-ICS users (I) (Table 5).

Table 5. Post-exercise IOS values (Z-scores) and exercise-induced changes (Z-scores and percentages) in the 13 former bronchiolitis patients who had used ICSs regularly during the preceding 12 months, compared to those 90 who had not used them.

Parameter	ICS use	p-value*	No ICS use
	Mean (SD)		Mean (SD)
Rrs5	0.92 (1.22)	<0.01	0.05 (1.11)
Xrs5	-2.36 (1.90)	<0.01	-0.90 (1.28)
Fres	2.94 (0.79)	<0.01	2.30 (0.84)
dRrs/df	-2.52 (1.36)	<0.01	-1.18 (1.29)
ΔRrs5	1.67 (2.79)	0.01	0.35 (1.59)
%ΔRrs5	+16.7 (25.8)	<0.01	+3.3 (12.7)
ΔXrs5	-1.65 (2.19)	<0.01	-0.16 (0.95)

Rrs = Resistance; Xrs = Reactance; Fres = Resonant frequency; dRrs/df = Frequency dependency of resistance, ICS = Inhaled corticosteroid, *adjusted for atopic eczema, maternal asthma, and RSV bronchiolitis

5.5 Atopic sensitisation, rhinitis, and respiratory allergy (V)

Skin prick tests (SPTs) were positive in 32 (31.4%) of the study subjects, and there was a significant association in both outdoor and indoor allergen SPT-positivity with current asthma and prolonged rhinitis, most commonly with birch or timothy pollen and cat or dog dander (V). However, only 62.9% of children with rhinitis were SPT-positive (Table 6).

Table 6. Skin prick test results in relation to current asthma and prolonged rhinitis in 102 former bronchiolitis patients at five to seven years of age.

Skin prick test results	Current asthma (n=15)	*p*-value *vs* no current asthma	Prolonged rhinitis (n=27)	*p*-value *vs* no rhinitis
Indoor allergens (n=18)	7 (46.7%)	0.005	10 (37.0%)	0.004
Cat dander (n=12)	6	0.002	8	0.002
Dog dander (n=13)	7	<0.001	8	0.005
Dust mites (n=4)*	0	0.542	1	0.715
Outdoor allergens (n=28)	8 (53.3%)	0.020	14 (51.9%)	0.001
Birch (n=20)	6	0.042	11	0.002
Timothy (n=19)	7	0.007	9	0.026
Mugwort (n=4)	2	0.102	2	0.285
Alternaria alternata (n=3)	2	0.056	2	0.170
Positive to indoor and outdoor allergens (n=14)	7 (46.7%)	0.001	9 (33.3%)	0.002
Positive to at least one indoor or outdoor allergen (n=32)	8 (53.3%)	0.049	17 (62.9%)	0.002

**D. farinae* in zero cases, *D. pteronyssimus* in four cases; Current asthma = Continuous or intermittent ICS medication during the last 12 months (n=13) or current symptoms and BHR (n=2).

SPT positivity was not associated with baseline lung function, but showed a significant association with hyperreactivity, as 56.3% of the 16 cases with BHR had at least one positive SPT (Table 7) (V). Prolonged rhinitis during the last 12 months, present in 27, was not associated with baseline lung function by IOS (V). Rhinitis was not associated with a pathological categorised ECT or post-BD test, (Table 7) but when analysed with continuous IOS variables, it showed a pathological trend in Rrs5 and Xrs5 in ECT, but not in the post-BD test (Unpublished data).

When combined with SPT-positivity, 31.3% of the hyperreactive cases had both rhinitis and SPT-positivity, but the association was not statistically significant (Table 7). However, 68.8% of the cases with BHR had either rhinitis or SPT positivity compared to 11.3% of the cases with no BHR (p=0.02) (Table 7).

Table 7. Baseline impulse oscillometry and bronchial hyperreactivity (BHR) in relation to prolonged rhinitis and/or skin prick test (SPT) positivity to inhaled allergens.

Rhinitis and/or SPT positivity	Baseline Rrs5 or Xrs5 pathological (n=21)	*p*-value *vs* baseline Rrs5 and Xrs5 normal	Bronchial hyperreactivity present (n=16)	*p*-value *vs* bronchial hyperreactivity absent
Prolonged rhinitis (n=27)	5 (23.8%)	0.498	7 (43.8%)	0.084
SPT-positivity (n=32)	6 (28.6%)	0.756	9 (56.3%)	0.020
Rhinitis and SPT-positivity (n=15)	3 (14.3%)	0.629	5 (31.3%)	0.057
Rhinitis or SPT-positivity (n=44)	8 (38.1%)	0.601	11 (68.8%)	0.024

Rrs5 was pathological (>+1.65 SD) in eight and Xrs5 was pathological (<-1.65 SD) in 19 cases. BHR was defined as a >35% increase Rrs5 in the ECT (n=5) or a >35% decrease in the BD test (n=11).

In the children with current asthma, we constructed four groups defined by the presence or absence of rhinitis and the presence or absence of SPT-positivity (Table 8). None of these groups showed significant associations with lung function. BHR was associated with the first group having the strictest definition (current asthma, rhinitis, and SPT-positivity present) and with two other groups having less strict definitions (Table 8)(V).

Table 8. Baseline impulse oscillometry and bronchial hyperreactivity in the groups constructed on the basis of respiratory allergy and current asthma.

Respiratory allergy group	Baseline Rrs5 or Xrs5 pathological	Baseline Rrs5 and Xrs5 normal	Bronchial hyperreactivity present**	Bronchial hyperreactivity absent**
	(n=20)*	(n=80)*	(n=14)*	(n=86)*
Current asthma plus rhinitis and SPT-positivity (n=6)	1 (5.0%) *p*=0.833	5 (6.3%)	3 (21.4%) *p*=0.034	3 (3.5%)
Current asthma plus rhinitis or SPT-positivity (n=8)	2 (10.0%) *p*=0.712	6 (7.5%)	3 (21.4%) *p*=0.081	5 (5.8%)
Current asthma or rhinitis and SPT-positivity (n=21)	5 (25.0%) *p*=0.623	16 (20.0%)	6 (42.9%) *p*=0.041	15 (17.4%)
Current asthma or rhinitis or SPT-positivity (n=48)	9 (45.0%) *p*=0.764	39 (48.8%)	12 (85.7%) *p*=0.002	36 (41.9%)

* The current asthma diagnosis was partially based on BHR documented by IOS in two cases, and these cases are not included in the analyses (n=100). ** For the definition, see the methods.

5.6 Weight gain: Effect on the IOS outcome (II)

For the 99 children with weight data available in full, at the control visit at the age of five to seven years, 22 (22.2%) children were overweight and seven (7.1%) were obese according to the newly published height-adjusted BMI Z-scores (Table 9).

Table 9. Weight parameters of 99 preschool children hospitalised for bronchiolitis in infancy.

Weight (kg), mean (median; range)	24 (24; 16–39)
zBMI (Z-score), mean (range)	0.39 (-2.2–2.5)
Overweight by zBMI, n (%)	22 (22.2)
Obese by zBMI, n (%)	7 (7.1)
Birth weight (kg), mean (range)	3.5 (2.3–4.6)
Age at hospitalisation (weeks), mean (range)	11 (1–24)
Weight at hospitalisation (kg), mean (range)	5.5 (2.8–9.7)
Weight gain before hospitalisation (g/week), mean (range)	190 (-150–580)

zBMI = Body mass index-for-age Z-score

There was no association in current weight status with baseline or post-exercise impulse oscillometry results, however in the seven obese children, the post-BD results remained statistically higher in Zrs5 (-0.94 Z-scores, p=0.03) and Rrs5 (-0.87, p=0.02) and lower in dRrs/df (-0.49, p=0.04) compared to normal-weight subjects (respectively -1.86, -1.83, and 0.14) (II). In multivariate linear regression analysis, there were statistically significant modest or low correlations between weight zBMI score and IOS parameters, showing a trend towards obstructive changes (II) (Table 10). There were no significant associations between birth weight and weight gain in infancy and lung function by IOS (II). Obesity or overweight did not associate significantly with BHR in the ECT or post-BD test (II).

Table 10. Impulse oscillometry vs weight by BMI Z-scores in 99 preschool children after bronchiolitis in infancy.

IOS parameter	Mean (SD)	Pearson's R	Pearson's R^2	Crude *p*	Adjusted *p**
Baseline					
Zrs5	-0.03 (1.15)	0.32	0.10	0.01	0.02
Rrs5	-0.10 (1.06)	0.33	0.11	0.01	0.01
Rrs20	-0.95 (1.14)	0.27	0.08	0.55	0.58
Xrs5	-0.70 (1.21)	-0.14	0.02	0.17	0.21
Fres	2.22 (0.83)	0.24	0.06	0.03	0.03
dRs/df	-1.00 (1.11)	-0.29	0.08	<0.01	<0.01
Post-exercise					
Zrs5	0.26 (1.27)	0.27	0.07	0.02	0.02
Rrs5	0.16 (1.16)	0.28	0.08	0.02	0.02
Rrs20	-0.91 (1.20)	0.29	0.08	0.63	0.70
Xrs5	-1.05 (1.45)	-0.21	0.04	0.05	<0.05
Fres	2.37 (0.87)	0.20	0.04	0.08	0.08
dRs/df	-1.34 (1.37)	-0.29	0.08	0.01	<0.01
Post-BD					
Zrs5	-1.77 (1.00)	0.20	0.04	0.06	0.07
Rrs5	-1.73 (0.99)	0.22	0.05	0.04	<0.05
Rrs20	-2.02 (1.18)	0.20	0.04	0.45	0.53
Xrs5	0.35 (0.65)	-0.13	0.02	0.23	0.26
Fres	0.85 (0.99)	0.23	0.05	0.04	0.04
dRs/df	0.05 (0.75)	-0.27	0.07	0.04	0.03

Zrs =Total impedance; Rrs=Resistance; Xrs=Reactance; Fres=Resonant frequency; dRrs/df=Frequency dependency of resistance *adjusted for atopic eczema, maternal asthma, and RSV-bronchiolitis. Correlations: R (R^2) > 0.60 (>0.36) or < –0.60 (>0.36) = strong; 0.3–0.6 or -0.3–0.6 (0.09–0.36) = modest; 0–0.3 (<0.09) or 0–-0.3 (<0.09) = low.

5.7 Innate immunity genetics and IOS outcome (III, IV)

Four functional point mutations in IL-10 (chromosome 1) and nine in a panel of TLR-encoding genes (*TLRs* 1, 2, 3, 6, and 10 on chromosome 4, *TLR4* in chromosome 9, *TLR*s 7 and 8 on the X chromosome, and *TLR9* on chromosome 3) were analysed against lung function and hyperreactivity measurements. All the SNPs were in Hardy-Weinberg equilibrium (HW p-value >0.05) and the genotype and minor allele frequencies (MAF) were comparable to Finnish population data in the 1000 genomes project (228).

5.7.1 Interleukin-10

The three adjacent (proximal) promoter polymorphisms were analysed as combined IL-10 rs1800896 (–1082G/A), rs1800871 (–819C/T), and rs1800872 (–592C/A) genotypes and haplotypes. The haplotype GCC (major alleles) was present in 71.7%, ACC was present in 59.6%, ATA (minor alleles) was present in 21.2%, and GTA was present in 10.1% (III) (Table 11). The haplotype GCC with major alleles was associated with normal IOS results both in genotype and haplotype analyses (III).

Table 11. The *IL-10* rs1800896, rs1800871, rs1800872, and rs1800890 polymorphism numbers, haplotype carrier rates, and frequencies in 99 cases hospitalised for bronchiolitis.

Genotype	**n (%)**
Combined *IL-10* rs1800896, rs1800871, and rs1800872	
GCC/GCC (GG+CC+CC)	16 (16.2)
GCC/GTA (GG+CT+CA)	7 (7.1)
GTA/GTA (GG+TT+AA)	3 (3.0)
GCC/ACC (GA+CC+CC)	35 (35.4)
GCC/ATA (GA+CT+CA)*	13 (13.1)
ACC/ACC (AA+CC+CC)	17 (17.2)
ACC/ATA (AA+CT+CA)	7 (7.1)
ATA/ATA (AA+TT+AA)	1 (1.0)
IL10 rs1800890	
A/A	35 (38.8)
A/T	50 (51.0)
T/T	13 (13.3)
Haplotype carrier rate	n (%)
Combined IL-10 rs1800896, rs1800871, and rs1800872:	
GCC	71 (71.7)
GTA	10 (10.1)
ACC	59 (59.6)
ATA	21 (21.2)
IL10 rs1800890	
A-allele	85 (86.7)
T-allele	63 (64.3)
Haplotype frequency	f
Combined IL-10 rs1800896, rs1800871, and rs1800872:	
GCC	0.44
GTA	0.07
ACC	0.39
ATA	0.11
IL10 rs1800890	
A-allele	0.61
T-allele	0.39

*GTA/ACC or ACC/GTA not present as estimated from the study population frequencies.

The genotype GTA/GTA (n=3) was associated with a lower baseline Xrs5 (-2.52, SD 1.42, p=0.01) and post-exercise Xrs5 (-3.15, SD 1.48, p=0.01) compared to other genotypes (-0.67, SD 1.17, and -1.00, SD 1.42, respectively). The haplotype GTA did not show any associations with decreased lung function (III).

At baseline, the IOS genotype ACC/ATA (n=7) was associated with a higher Rrs5 (0.72, SD 0.68, adjusted p=0.04) compared to other genotypes (-0.12, SD 1.05) in crude and adjusted analyses, and lower Xrs5 (-1.55, SD 2.30, adjusted p=0.03) compared to other genotypes (-0.66, SD 1.09), but not in the crude analyses (p=0.06) (III). The haplotype ACC was associated with normal IOS results, but the haplotype ATA with minor alleles showed a trend towards the highest baseline Rrs5 (0.29, SD 1.01, crude p=0.08, adjusted p=0.03) and the lowest Xrs5 (-1.08, SD 1.69, crude p=0.13, adjusted p=0.07) compared to other haplotypes (-0.16, SD 1.05 and -0.63, SD 1.05, respectively).

To conclude, in the analysis of the combined *IL-10* rs1800896, rs1800871, and rs1800872, the genotypes and the haplotype GCC with the major alleles showed normal baseline IOS results, while the genotypes and haplotype ATA with minor alleles showed some decreased baseline IOS results. There were no significant associations with BHR between any of these *IL-10* SNP combinations and IOS measurements (III).

In *IL10* rs1800890 (–3575A/T), the A/A-genotype was associated with lower baseline Xrs5 (-1.02, SD 1.25, crude p=0.04, adjusted p=0.03) compared to other genotypes (-0.52, SD 1.12). The carriage of the major allele A was associated with lower baseline Xrs5 (-0.79, SD 1.18, crude p=0.04, adjusted p=0.04) compared to the non-carriers (-0.06, SD 1.08). The carriage of the minor allele T was associated with higher Xrs5 (-0.52, SD 1.12, crude and adjusted p=0.04) compared to non-carriers (-1.02, SD 1.25). Carriers of the T-allele also showed lower baseline Rrs5 (-0.18, SD 1.04, adjusted p=0.04) and lower post-exercise Rrs5 (0.05, SD 1.13) compared to non-carriers of allele T (0.10, SD 1.03 and 0.37, SD 1.12, respectively) but only in the adjusted analysis (p=0.03) (III).

In conclusion, in *IL10* rs1800890 the genotype A/A and the major allele A were associated with decreased reactance in baseline IOS, but there were no significant associations with BHR (III).

5.7.2 Toll-like receptors

Analysis of TLR protein-encoding gene polymorphisms were done separately for each SNP, and in addition for the *TLR* 1, 2, 6, and 10 genotype combinations. The X chromosomal *TLR7* and *TLR8* SNPs were analysed separately for boys and girls. The genotype and minor allele distributions of the analysed *TLR* genes are presented in Table 12.

Table 12. Toll-like receptor 1, 2, 3, 4, 6, 7, 8, 9, and 10 genotype and minor allele frequencies in 98 children hospitalised for bronchiolitis and in the Finnish population data.

SNP (Major>Minor)	Major/Major	Major/Minor	Minor/Minor	MAF	FIN
	(Wild*)	(Variant*)	(Variant*)		
TLR1 rs5743618 (G>T)	0.78	0.18	0.04	0.13	0.17
TLR2 rs5743708 (C>T)	0.94	0.06	0	0.03	0.03
TLR3 rs3775291 (C>T)	0.49	0.41	0.10	0.31	0.33
TLR4 rs4986790 (A>G)	0.85	0.15	0	0.08	0.12
TLR6 rs5743810 (C>T)	0.31	0.45	0.24	0.47	0.42
TLR7 rs179008 (A>T)	0.62 (girls)	0.32 (girls)	0.06 (girls)	0.27 (all)	0.31
	0.81 (boys)	0	0.19 (boys)		
TLR8 rs2407992 (G>C)	0.38 (girls)	0.42 (girls)	0.20 (girls)	0.42 (all)	0.36
	0.56 (boys)	0	0.44 (boys)		
****TLR9** rs187084 (T>C)	0.30	0.44	0.26	0.48	0.45
****TLR10** rs4129009 (A>G)	0.85	0.14	0.01	0.08	0.08

*Wild genotype means that the minor allele is not presented and the variant genotype that minor allele presents is either heterozygous or homozygous, **n=97 for *TLR9* and *TLR10*; MAF=minor allele frequency, FIN=Finnish MAFs as in (228).

Toll-like receptor-encoding gene polymorphisms showed some preliminary associations with BHR in the ECT (IV). The *TLR4* rs4986790 wild A/A-genotype was associated with a greater increase in Rrs5 in response to ECT compared to the variant A/G genotype (Table 13a). In *TLR6* rs5743810, the variant genotype (CT or TT) was associated significantly with a greater increase in Rrs5 and a greater

decrease in Xrs5 compared to the wild CC-genotype (Table 13b). There were, however, no significant associations with post-BD test results (IV).

Table 13. *TLR4* rs4986790 genotypes (a) and *TLR6* rs5743810 genotypes (b), presented as wild/variant genotypes, and exercise-induced changes (Z-scores) in the 98 former bronchiolitis patients.

a)	**Wild genotype (n=83)**		**Variant genotype (n=15)**
Parameter	Mean (SD)	***p*-value***	Mean (SD)
ΔRrs5	0.72 (1.81)	0.03	-0.42 (1.84)
ΔXrs5	-0.39 (1.35)	0.40	-0.10 (0.90)
b)	**Wild genotype (n=30)**		**Variant genotype (n=68)**
Parameter	Mean (SD)	***p*-value***	Mean (SD)
ΔRrs5	-0.03 (1.61)	0.04	0.80 (1.90)
ΔXrs5	0.05 (0.98)	0.08	-0.52 (1.37)

Rrs5 = Resistance at 5 Hz; Xrs5 = Reactance at 5 Hz; Fres = Resonant frequency; dRrs/df = Frequency dependency of resistance, *adjusted for age, sex, RSV aetiology of bronchiolitis, BMI Z-score, atopic eczema, and use of inhaled corticosteroids during the preceding 12 months

In the joint analysis of *TLR1* rs5743618, *TLR2* rs5743708, *TLR6* rs5743810, and *TLR10* rs4129009, we found that 20 children had the combination of four wild homozygous genotypes, and this combination was associated with a smaller response to exercise in Rrs5 (-0.17 vs 0.73, p=0.049) compared to those 77 with a combination of one or more variant genotypes; it also showed a trend in improving Xrs5 (0.12 vs -0.48, p=0.07), but the association was not statistically significant. In addition, the combination that included the wild *TLR1* rs5743618, *TLR2* rs5743708, and *TLR10* rs4129009 genotypes, together with the variant *TLR6* rs5743810 genotype, was present in 50 children and was associated with a greater response to exercise in Rrs5 (0.91 vs 0.16, p=0.043) compared to those 47 children with other genotype combinations. Any other combination of genotypes, including one, two, three or four variant genotypes, did not show significant associations with IOS results (Table 14).

Table 14. The joint analyses of the *TLR1* rs5743618, *TLR2* rs5743708, *TLR6* rs5743810, and *TLR10* rs4129009 genotype combinations and baseline (Z-scores) and hyperreactivity IOS measurements (ΔZ-scores) in 97 children hospitalised for bronchiolitis.

TLR1/TLR2/TLR6/TLR10	n	Rrs5	Xrs5	Fres	dRrs/df	ΔRrs5	ΔXrs5
genotype combination		Mean	Mean	Mean	Mean	Mean	Mean
		(SD)	(SD)	(SD)	(SD)	(SD)	(SD)
wild/wild/wild/wild	20	0.14	-0.81	2.31	-1.22	**-0.17***	0.12
		(1.19)	(1.19)	(1.05)	(1.40)	(1.70)	(1.16)
variant/wild/wild/wild	3	-0.21	-0.64	2.12	-0.94	0.20	-0.21
		(0.77)	(0.39)	(0.12)	(0.31)	(1.07)	(0.68)
wild/wild/variant/wild	50	-0.26	-0.71	2.23	-1.01	**0.91****	-0.53
		(1.08)	(1.38)	(0.69)	(1.09)	(1.87)	(1.30)
variant/wild/variant/wild	3	0.86	-1.38	3.02	1.93	1.47	-1.44
		(1.07)	(0.75)	(0.93)	(0.80)	(2.83)	(3.44)
variant/wild/wild/variant	7	-0.31	-0.71	1.69	-0.35	0.25	-0.06
		(0.74)	(1.43)	(1.42)	(0.86)	(1.67)	(0.44)
wild/variant/variant/wild	5	0.64	-0.64	2.29	-0.89	-0.37	-0.17
		(0.41)	(0.91)	(0.50)	(1.18)	(0.98)	(0.87)
variant/wild/variant/variant	8	0.14	-0.52	2.28	-1.20	0.65	-0.48
		(0.93)	(0.94)	(0.72)	(0.95)	(2.42)	(1.36)
variant/variant/variant/wild	1	0.89	-0.85	2.59	-1.09	0.81	-0.78

Rrs5 = Resistance at 5 Hz; Xrs5 = Reactance at 5 Hz; Fres = Resonant frequency, dRs/df = Frequency dependency of resistance; *p=0.049 and **p=0.043 vs all other genotype combinations, as adjusted for age, sex, RSV aetiology of bronchiolitis, current BMI Z-score, atopic eczema, and use of inhaled corticosteroids during the preceding 12 months.

There were no significant associations with baseline lung function measurements by IOS in the *TLR*s 1, 2, 3, 4, 6, 8, 9, or 10; however, some associations were found in the X-chromosomally inherited *TLR7* rs179008 in girls, but not in boys (IV). The girls with the A/T genotype had the highest baseline Fres (adjusted p=0.02) and lowest dRrs/df (adjusted p=0.01) compared to other genotypes. The girls with the A allele had higher baseline Fres (2.28 vs 0.89 in non-carriers, adjusted p=0.01) and lower dRrs/df (-1.03 vs 0.61 in non-carriers, adjusted p=0.01).

In conclusion, no significant associations were seen in the analyses of *TLR* 1–4 and 6-10 polymorphisms with baseline lung function, except for minor changes in *TLR7* rs179008 in girls (IV). The *TLR4* rs4986790 and *TLR6* rs5743810 polymorphisms were associated with hyperreactivity in response to exercise, and the *TLR6* rs5743810 variant genotype in particular showed significant associations with ECT changes, including when analysed together with the *TLR2* subfamily polymorphisms (IV).

6 Discussion

6.1 Lung function outcome after viral bronchiolitis in infancy

In 103 children hospitalised at less than six months of age due to bronchiolitis caused predominantly by RSV, a pathological (>95th percentile) lung function decline measured by IOS was present in 20% at the age of five to seven years. In the baseline measurement, eight cases had an increased resistance and 19 cases a lowered reactance measured at a frequency of 5Hz. These results can be characterised as peripheral, small airway obstruction. In addition, this was mostly reversible by bronchodilation, as only one child with a pathological reactance did not return to normal values, at least as reflected by IOS measurements.

This is a preliminary study addressing lung function by IOS at preschool age, but the results seem comparable with previous studies using FVS. In Finland, abnormal baseline parameters have been found in 23–33% of slightly older children at the age of 8–11 years after bronchiolitis (10,118). In particular, the RSV aetiology of bronchiolitis has been associated with decreased lung function parameters in several studies at the age of 7–13 years (104,114,116,122,229), and although the symptoms remit by age, the loss of lung function after bronchiolitis persists into early adulthood (24,25,123).

Several studies have suggested that some children are predisposed to bronchiolitis already after birth due to pre-existing low lung function (101,103,105), but whether this is due to permanent small airway calibre or increased airway tone is not known because the reversibility of the bronchial constriction cannot be evaluated at this age. In addition, there is evidence that bronchial hyperresponsiveness, not low lung function, precedes severe bronchiolitis (115). This paradigm of predisposal is indirectly supported by studies on premature infants showing an increased risk of severe RSV infection (3,230). Our study design was not planned to evaluate this, and it is clear that neonatal lung function abnormality may contribute to the risk of hospitalisation and later lung function reduction also in term infants. The causality of this predisposition is not well established, as studies supporting this hypothesis appear to use experimental techniques as a measurement of infant lung function (101,108) or they have a very

low number of study subjects (103,105). Our current results challenge the idea of pre-existing small airways as a major causal factor leading to hospitalisation during bronchiolitis. In this RSV cohort consisting of children born at term, the lung function reduction was mostly reversible after bronchodilation, suggesting increased but reversible airway tone.

In the current study, bronchial responsiveness to exercise challenge or in response to bronchodilation was present in 16%, which is relatively little. The hyperreactivity to exercise challenge showed surprisingly low numbers (5%), and a pathological response to bronchodilation was more common (11%), reflecting increased baseline small airway obstruction in the children. These results are, however, well explainable with the different, stricter selection of our study participants compared to previous studies. Wheezing due to rhinovirus during the first two years of life has been connected increasingly to airway hyperreactivity at school age (30,117). In the COAST study children with atopic predisposition and outpatient rhinovirus-induced wheezing episodes during the first three years of life were associated with hyperreactivity and low lung function at the age of eight years, as RSV-induced wheezing did not show any significant differences compared to children without wheezing (30).

It seems that admission to hospital due to bronchiolitis and outpatient treatment due to early-life wheezing are somewhat separate entities with different predisposing factors, where age at disease onset plays a particular role (34,231). Previous hospital cohort studies on virus-induced wheezing often lack a strict definition of bronchiolitis, as studies have included children less than 12 to 24 months old, leading to the possibly confounded analysis of different wheezing phenotypes. In addition, birth-cohort studies have not been able to collect high enough numbers in hospitalised bronchiolitis cases within the birth cohort to compare these entities. In the Dutch WHISTLER cohort of 2,133 unselected children, only 18 were hospitalised due to confirmed RSV bronchiolitis before 12 months of age (105). In a follow-up study, 159 hospitalised RSV bronchiolitis cases, mostly from a previous hospital cohort, were compared to 549 healthy non-hospitalised unselected controls from the birth cohort, showing an impaired lung function at the age of six years associated with the RSV bronchiolitis in infancy (114). However, even then, the optimal study design comparing children hospitalised due to RSV or non-RSV bronchiolitis to non-hospitalised healthy controls within an unselected population was not achieved.

We were not able to confirm any significant differences in the lung function or ECT or post-BD test outcomes between RSV, non-RSV, or rhinovirus aetiologies

of bronchiolitis. This result parallels the study from Fjaerli (116), where no differences in lung function decline were observed between RSV and non-RSV cases in children hospitalised under 12 months of age, although hospitalisation for bronchiolitis in general was associated with lower lung function compared to non-hospitalised healthy controls at the age of seven years.

The reversibility of lung function parameters has not been studied before at this early age, but our finding seems to be in line with the Tucson cohort result, where reduced lung function by FVS after RSV-associated non-atopic wheezing was still reversible after the use of a bronchodilator at 11 years of age (12). However, a history of early RSV bronchiolitis has been associated with mild obstruction even after bronchodilation at the age of 13 years, suggesting this age is pivotal for the development of irreversible lung function changes (122). In children with persistent wheezing with an onset before the age of three years and likely development of atopic asthma, the lung function reduction has been demonstrable already at the age of six to seven years in the Tucson and Denmark birth cohorts (11,106), but neither of these studies assessed reversibility due to bronchodilation at this age, making comparison to our post-bronchiolitis results difficult.

6.2 Weight trajectories and obesity in the IOS analysis

We found that lung function by IOS associated with current obesity in an expected way, reflecting obstruction with emphasis on small airways by increased Rrs5 and frequency dependency of resistance, and increased resonant frequency. This finding is in line with the abnormalities of lung mechanics previously observed in association with obesity (136,137).

We did not find any associations between overweight or obesity at preschool age and airway reactivity measured by IOS, which does not support the association of obesity and asthma found by others (232,233). In baseline and post-exercise measurements, there were no differences between the obese, overweight, and normal-weight children, but obese children showed more obstructive values in IOS after bronchodilation. This suggests an irreversible element in the lung function measurement, and could be of mechanical basis, as in obese adults, lung volumes tend to be smaller due to microatelectasis in the peripheral lung (136,137). In line with this, in children at the age of 7.2 years studied with FVS, some reduced FEV1/FVC ratios associated with obesity have been found (133), and in clearly older children at a mean of 11.8 years of age, increasing weight has been

thoroughly assessed to be associated with impaired lung function as shown in a decreased FEV1/FVC ratio and lower end expiratory volumes measured with spirometry (134).

There is very little evidence comparable to our study, and thus far the only lung function study at preschool age on the effect of obesity by body mass-index on lung function measured with similar methods – but without adjusting the BMI for age – did not show any associations (138). In our study, lung function parameters were associated significantly with weight BMI Z-score in multi-adjusted analyses, though the correlations were mainly low, less than R^2=0.10. As both the IOS variables and the BMI-score were age- and height-adjusted, this should be a close estimate of the effect of weight on lung function at preschool age. Again, in adults, distribution of body fat, rather than BMI, has been associated with lung function parameters (136), so it can be speculated that our child population may differ in their body composition, confounding the results. According to our results, obesity, not overweight, at preschool age is a modest predictor of lung function, and without evidence of any association with hyperreactivity, it contributes to IOS measurements mainly by altered lung mechanics.

6.3 Genetic factors of innate immunity and lung function development

Our study was able to produce indirect evidence on the association of *IL-10* polymorphisms and lung function abnormalities in a post-bronchiolitis setting. We did not measure IL-10 *in vivo* but relied on previous literature on the functionality of studied polymorphisms. The phenotypes that were previously associated with low IL-10-production showed increased resistance and decreased reactance values in baseline IOS. This delineates the clinical findings from the CAMP birth cohort (188), where the low IL10-producing phenotype ATA had a negative association with FEV1 at school age, but since it was also associated with hyperreactivity to histamine, our results differ partly. However, previously from this cohort an association of the *IL10* rs1800896 G/A polymorphism with preschool asthma was found (187). Thus, it is possible that our present results might carry a risk of type-two error due to the somewhat low numbers in hyperreactivity.

We claim that the functional *IL-10* polymorphisms contribute to later lung function after viral bronchiolitis in infancy, but the causality cannot be clarified by our study. It could be that the IL-10-deficient individuals were susceptible to a

more severe course of bronchiolitis leading to excessive lung damage, or it could be the ability to produce IL-10 is associated with the reparative or harmful processes after the injury. We propose that IL-10 does not contribute to baseline lung function only by altering mechanisms behind atopic inflammation, though it has been suggested to be a major regulatory cytokine in allergy (181). It seems that our cohort derives from distinct phenotypes of post-bronchiolitis wheezing, which could equally have an impaired IL-10 production contributing to bronchiolitis admission, but a different outcome in later life. As the children with previous asthma have a low lung function, it could result from lung injury partly due to the impaired anti-inflammatory response. In turn, the children with persistent atopic asthma also seem to associate with low IL-10 production and have an obviously genetic atopic predisposition. This group might develop a loss of lung function later than at the age of six years due to chronic inflammation.

The analysis of Toll-like receptors' genetic associations with lung function was limited to one SNP in each studied *TLR*. There was no significant associations with baseline lung function in the majority of the studied SNPs, except in *TLR7* rs179008 in girls. The *TLR4* rs4986790 (A>G) and the *TLR6* rs5743810 (C>T) polymorphisms showed inverse associations with hyperreactivity parameters in response to exercise, which is interesting because activation with infectious ligands through both TLR4 and TLR6 receptors have been noted to induce somewhat similar cytokine profiles (234).

The RSV infection has been shown to upregulate the airway epithelial cells' TLR4 expression, thus sensitising the cells to endotoxin (235). The now studied TLR4 encoding *TLR4* rs4986790 (A>G) polymorphisms cause an Asp299Gly substitution that alters the extracellular domain of the receptor interfering with the signal cascade, resulting in airway hyporesponsiveness to environmental lipopolysaccharide (152). Thus, our finding that the variant allele in *TLR4* rs4986790 results in less airway reactivity in response to exercise suggests a similar phenomenon occurring in individuals hospitalised due to bronchiolitis. The biological basis for this would be that RSV infection increases hyperreactivity in the airways to environmental factors in those with a common receptor function, and the individuals with the receptor mutation show a hypo-responsiveness that could continue after the infection. Intriguingly, the Asp299Gly polymorphism has been associated with a more severe course of RSV bronchiolitis (154), which could mean that the children who have a less effective response to the infection and likely have a more severe course of the disease could be at risk from lung function

problems after bronchiolitis. Our study was, however, restricted in conducting more stratified analyses, so we cannot confirm this hypothesis.

Results from Swedish children with asthma seem to be in conflict with our results, as especially the school-aged children with atopic asthma had an association with the *TLR4* rs4986790 polymorphisms (236), though in this study the children with non-atopic asthma showed very low numbers, decreasing the comparability to our study population. On the other hand, in a large family-based cohort of 756 families from North America, *TLR4* polymorphisms showed no associations with the development of asthma or atopy in adults (237). As our analyses were robust to multi-adjustment, including current atopic eczema at preschool age, our results suggest that the *TLR4* rs4986790 polymorphism independently differentiates outcomes in terms of airway hyperreactivity after bronchiolitis in infancy.

The *TLR6* rs5743810 (C>T) polymorphism causes a Ser249Pro amino acid substitution in the DNA transcript, and has been associated in experimental studies with decreased interleukin-6 production, which can be caused by impaired ligand recognition directly in the receptor (168). The IL-6 is a pro-inflammatory cytokine that promotes the production of acute-phase proteins in the liver and neutrophils in the bone marrow during infection (176). Thus, it could be postulated that the variance in *TLR6* rs5743810 could decrease inflammatory response to infection, perhaps resulting in decreased viral clearance and a longer duration of infection, or perhaps a turn into a Th2-oriented imbalance. However, there is to our knowledge no data on how *TLR6* polymorphisms alter IL-6 levels during RSV infection, though this cytokine is important during bronchiolitis (169). It is therefore clear that we cannot draw strong conclusions on the now-observed increased hyperreactivity in the bronchiolitis cases with *TLR6* rs5743810 variant T-allele carriage. However, this association was also confirmed in joint analyses with other *TLR2* subfamily SNPs, increasing the reliability of our results.

Previous clinical associations between asthma and this polymorphism seem to be conflicting, as it has been found that the variant allele T was associated with a decreased risk of asthma in African-American adults (166). However, on the contrary, in a German population a preliminary association between the T-allele and childhood asthma was found (167). This population resembles the Finnish population much better, as the African-Americans showed a significantly lower minor allele frequency of 0.08 in asthmatics compared to our study population (0.47). Our results are preliminary observations in a bronchiolitis cohort, but seem to delineate the previous results regarding the increased risk of asthma in children.

6.4 Evidence for united airways disease after bronchiolitis

Our results suggest that current asthma, IgE-mediated SPT-positivity, and rhinitis develop jointly and have an association with airway hyperreactivity, supporting the UAD hypothesis in a post-bronchiolitis setting. In children with current asthma, 53.3% had prolonged rhinitis, and BHR was present in 38.5%, compared to 22.6% and 10.9% in the children with no asthma, respectively. The 12 children with previous transient asthma, now at least in remission, showed indirect evidence for the UAD hypothesis: none of the children were SPT-positive and only one of the 12 showed airway hyperreactivity. However, lung function measured by IOS showed no significant differences between the children with current asthma, previous asthma, and no asthma, suggesting that the development of lung function may not be driven by the same mechanisms as in the UAD concept, or that lung function abnormalities within the UAD develop later in life. The previous transient asthma cases showed the lowest values of baseline reactance compared to the current asthma and no asthma groups, suggesting this phenotype is a risk group for lung function abnormalities, though atopy and allergies did not develop and symptoms remitted before school age. These findings are in line with the paediatric transient asthma phenotype described in the Tucson cohort, but their observations in children with persistent wheezing and lung function reduction at the age of six could not be confirmed by our study (11).

In the Manchester birth cohort, multiple-trigger atopy showed a constant association with decline in lung function from the age of three to 11 years, especially in boys with persistent wheezing (112). However, in the CAMP-cohort, treatment of asthma symptoms with anti-inflammatory budesonide or nedocromil during the ages of five to 12 years was no better than placebo in terms of lung function outcome (238). Similarly, as seen in the PEAK project, a decline in lung function was not preventable after two years of ICS use in children with a high asthma-predictive index (23). In the Danish COPSAC cohort, lung function decline in asthmatic children continued from infancy to school age independent of atopy (106), suggesting that other mechanisms than atopic inflammation may play a role in the development of lung function reduction.

Ultimately, as our results suggest that lung function does not relate to the UAD concept, we should keep in mind that in a predominantly RSV-enriched and very young cohort such as ours, the patients tend to be highly selected and – according to our knowledge – likely not to be prone to develop atopy. Thus, our study might be underpowered to evaluate lung function development in patients with atopic

inheritance. This, however, gives an interesting view of the lung function reduction seen in our population, and thus we speculate that the mechanisms leading to RSV infection-associated lung function decrease, before or after bronchiolitis, should be actively studied, and the prevention of RSV infections in early life could play a role in reducing the burden of COPD in later life.

6.5 Methodological aspects of the study

Our study carries in general a risk of selection bias, as the children hospitalised due to bronchiolitis are likely – though born at term and considered otherwise healthy – high-risk individuals with genetic, structural, and different environmental predisposing factors compared to children not hospitalised due to bronchiolitis with a less serious course of the disease. Therefore, drawing conclusions on the risk factors affecting lung function outcome at preschool age in the general population requires careful consideration. As a significant shortcoming, we did not recruit control patients to our study population, but often in prospective studies control population selection bias can also be problem. We have tried to minimise the effects of these factors by comparing our outcome variables in lung function to recently published age- and height-specific Finnish reference data with healthy kindergarten children investigated with similar IOS methods, but without atopic predisposition or previous wheezing illnesses. This can be counted as a strength of this study, but the ideal study design was not met. It is clear our results need further confirmation from unselected birth cohort studies to evaluate the causal association of bronchiolitis with lung function, and the conclusions now drawn cannot be strongly generalised to the whole population.

The use of IOS is not well established, and as it is a sensitive method to artefacts and can show variance in the measurements, one must be careful not to over-estimate lung function or ECT or BD test findings by IOS. On the other hand, it seems that IOS parameters can provide a complex profile on deeper lung mechanics, and not enough is known about how they correlate with FVS and other lung function methods.

We used IOS in measuring post-exercise and post-bronchodilation values one test after another, which might have affected the latter phase results. However the post-BD values were in general significantly better than the baseline values, which suggests that the exercise challenge between these measurements did not markedly confound the analysis. In addition, the very short-term exercise-induced

bronchoconstriction, observed sometimes in children only straight after running, might not be recognised by our measurements, as the IOS curves often showed artefacts when obtained during the first minutes. This might partially explain our low numbers in response to ECT. Due to these somewhat low numbers in pathological exercise-induced changes and responses to the use of a bronchodilator, we combined these in certain analyses under the term "bronchial hyperreactivity", which differs from the exact use of this term in previous literature.

The ideal measurement of bronchial hyperresponsiveness would consist of using multiple provocations, and now only indirect provocation with an exercise challenge was applied. It is possible – though not very likely – that some reactive cases were not seen in the children by this method. In addition, a significant proportion of children ran the exercise challenge in temperatures below zero Celsius. Cold, dry air, to our understanding, should increase rather than decrease hyperresponsiveness in the ECT. Nevertheless, we acknowledge the possible sources of bias due to weather circumstances in exercise challenge testing.

For a genetic association study, our population was without a doubt small in numbers, increasing the likelihood of type-two errors in the statistical analysis. Our data on SNP polymorphisms was, however, comparable to the Finnish public in data for Finland from the 1000 genomes project, and the study design allowed multi-adjusted analysis, increasing the reliability of our results. As our analysis involved multiple SNPs, we acknowledge the risk of multiplicity and admit that most of our genetics results would not have sustained multiple-testing correction. However, our analysis on genetics and lung function can mostly be considered exploratory and rather hypothesis-generating than confirmatory. Our results call for further studies on genetic associations in immunity and lung function.

As the most significant strength compared to previous post-bronchiolitis studies, our study population was well characterised to have a virally confirmed "pure" bronchiolitis before the age of six months, increasing the homogeneity of the group. Using novel techniques, the causative virus was identified in 92% of cases, characterising our study group as an "RSV cohort", which also gave us the opportunity to evaluate the role of non-RSV viruses in lung function development. The IOS method is feasible in studying lung function in this age-group of children, and the measurements went through a highly professional analysis, as they were collected by experienced nurses and pre-analysed by a clinical physiologist, thus increasing the reliability of our lung function results. We limited the IOS analysis to

children less than 7.0 years old, which reduced the numbers of the study participants, but added to the homogeneity of the group.

As methodological strengths, the clinical data was in all cases collected with a structured questionnaire and further confirmed with a clinical interview. In the analysis with parent-reported prolonged rhinitis, objective evidence on atopic sensitisation from skin prick tests were included to assess the role of "allergic rhinitis". In addition, measurements of weight and new height- and gender-adjusted relative values by Z-scores were available, increasing the accuracy of our results.

The relatively long follow-up over five years after bronchiolitis in infancy to the crucial preschool-age control visit gives a good perspective for evaluating the outcomes after bronchiolitis in comparison to previous cohort studies. Although the number of drop-outs was rather high, we were able to obtain all data from 55% of the original bronchiolitis cases at the control visit, which is an acceptable figure in long-term follow-up studies. Nevertheless, the limited adherence of follow-up patients reduces the power to generalise from the results.

7 Conclusions

After a "pure" viral bronchiolitis in infancy caused predominantly by RSV leading to hospitalisation before the age of six months, one fifth of the children had a pathological baseline lung function and one sixth were reactive to ECT or post-BD test as measured by IOS and compared to reference data at the mean age of 6.3 years. This result is in line with previous numbers presented in post-bronchiolitis studies, though the prevalence of reactivity to exercise or responsiveness to bronchodilation were rather low. This might depend on the fact that previous bronchiolitis studies have included older children in their analyses, with an increased atopic predisposition confounding the true effect of bronchiolitis and showing higher numbers in hyperreactivity. Reduced baseline lung function in this study concentrated on lowered reactance and increased frequency dependency of resistance – thought to reflect small airways obstruction – that was mostly reversible to normal limits by IOS in response to bronchodilation. Thus, the changes seem to be caused by increased airway tone and not by permanently narrowed airways at this age.

As obesity and asthma are both increasing epidemics, it is important to know how they are related to each other at the level of objectively measured lung function in order to avoid over- and under-diagnosis of asthma in children presenting with wheezing symptoms and overweight. According to our results, hyperreactivity measured by IOS at preschool age is not associated with obesity, but obesity may contribute to the obstruction seen in lung function parameters, reflecting especially obstruction in the peripheral small airways. Thus, hyperreactivity, the hallmark of asthma, should not be considered a consequence of obesity; obese children likely might tend to mimic symptoms of asthma due to altered lung mechanics.

The genotypes formerly associated with normal production of IL-10 did not show any significant associations with lung function or hyperreactivity, which suggests IL-10 plays a protective role during bronchiolitis and also a potentially a modifying role in the development of post-bronchiolitis lung function. The predisposition to a more severe disease course could be due to an impairment in the IL-10 response. It remains to be determined how IL-10 contributes to

increased airway tone and reversible lung function decline after bronchiolitis. Most of the studied *TLR*s did not associate with lung function, though it has been proposed that TLRs play a pivotal role in the recognition of pathogens and the onset of innate immunity response to RSV infection. Therefore, it seems that airway TLR function does not determine the lung function outcome in a post-bronchiolitis setting. However, the *TLR4* and *TLR6* polymorphisms might contribute to the development of hyperreactivity, or reflect a predisposition already present during and modifying the course of the bronchiolitis. In conclusion, the genetic associations between *IL-10* polymorphisms and post-bronchiolitis lung function or *TLR4* and *TLR6* polymorphisms with airway hyperreactivity are preliminary and indirect findings, suggesting a genetic predisposition to impaired responses to viral bronchiolitis, but the causal associations with genetic factors leading to the loss of lung function need further confirmatory studies.

In addition, we found new evidence regarding the UAD hypothesis, giving new insight into the atopic march theory. According to our results, post-bronchiolitis lung function reduction might develop independently from atopy-related disorders. We were able to distinguish a group of children with previous transient childhood asthma and without atopy who showed the lowest lung reactance values compared to the other children. This result delineates previous findings that loss of lung function after bronchiolitis continues to adulthood, even though the symptoms might remit, and therefore the children with a history of early bronchiolitis should be considered a risk group for COPD. On the other hand, according to the UAD concept, the children with allergic rhinitis and skin test positivity should be actively investigated for asthma symptoms and airway hyperreactivity, and vice versa. The mechanisms behind post-bronchiolitis lung function deterioration by preschool age, however, should be investigated also apart from the presence of markers of atopy.

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References

1. Smyth RL, Openshaw PJ. Bronchiolitis. *Lancet* 2006; 368: 312-22.
2. Meissner HC. Viral bronchiolitis in children. *N Engl J Med* 2016; 374: 62-72.
3. Hall CB, Weinberg GA, Iwane MK, Blumkin AK, Edwards KM, Staat MA, et al. The burden of respiratory syncytial virus infection in young children. *N Engl J Med* 2009; 360: 588-98.
4. Nair H, Nokes DJ, Gessner BD, Dherani M, Madhi SA, Singleton RJ, et al. Global burden of acute lower respiratory infections due to respiratory syncytial virus in young children: A systematic review and meta-analysis. *Lancet* 2010; 375: 1545-55.
5. Stockman LJ, Curns AT, Anderson LJ, Fischer-Langley G. Respiratory syncytial virus-associated hospitalizations among infants and young children in the united states, 1997-2006. *Pediatr Infect Dis J* 2012; 31: 5-9.
6. Pruikkonen H, Uhari M, Dunder T, Pokka T, Renko M. Infants under 6 months with bronchiolitis are most likely to need major medical interventions in the 5 days after onset. *Acta Paediatr* 2014; 103: 1089-93.
7. Taussig LM, Wright AL, Holberg CJ, Halonen M, Morgan WJ, Martinez FD. Tucson children's respiratory study: 1980 to present. *J Allergy Clin Immunol* 2003; 111: 661,75; quiz 676.
8. Jartti T, Makela MJ, Vanto T, Ruuskanen O. The link between bronchiolitis and asthma. *Infect Dis Clin North Am* 2005; 19: 667-89.
9. Gern JE, Lemanske RF,Jr, Busse WW. Early life origins of asthma. *J Clin Invest* 1999; 104: 837-43.
10. Korppi M, Kuikka L, Reijonen T, Remes K, Juntunen-Backman K, Launiala K. Bronchial asthma and hyperreactivity after early childhood bronchiolitis or pneumonia. an 8-year follow-up study. *Arch Pediatr Adolesc Med* 1994; 148: 1079-84.
11. Martinez FD, Wright AL, Taussig LM, Holberg CJ, Halonen M, Morgan WJ. Asthma and wheezing in the first six years of life. the group health medical associates. *N Engl J Med* 1995; 332: 133-8.
12. Stein RT, Sherrill D, Morgan WJ, Holberg CJ, Halonen M, Taussig LM, et al. Respiratory syncytial virus in early life and risk of wheeze and allergy by age 13 years. *Lancet* 1999; 354: 541-5.
13. Sigurs N, Bjarnason R, Sigurbergsson F, Kjellman B. Respiratory syncytial virus bronchiolitis in infancy is an important risk factor for asthma and allergy at age 7. *Am J Respir Crit Care Med* 2000; 161: 1501-7.

14. Kotaniemi-Syrjanen A, Reijonen TM, Korhonen K, Korppi M. Wheezing requiring hospitalization in early childhood: Predictive factors for asthma in a six-year follow-up. *Pediatr Allergy Immunol* 2002; 13: 418-25.
15. Koponen P, Helminen M, Paassilta M, Luukkaala T, Korppi M. Preschool asthma after bronchiolitis in infancy. *Eur Respir J* 2012; 39: 76-80.
16. Merkus PJ, ten Have-Opbroek AA, Quanjer PH. Human lung growth: A review. *Pediatr Pulmonol* 1996; 21: 383-97.
17. Merkus PJ. Effects of childhood respiratory diseases on the anatomical and functional development of the respiratory system. *Paediatr Respir Rev* 2003; 4: 28-39.
18. Goksor E, Amark M, Alm B, Gustafsson PM, Wennergren G. The impact of pre- and post-natal smoke exposure on future asthma and bronchial hyper-responsiveness. *Acta Paediatr* 2007; 96: 1030-5.
19. Stern DA, Morgan WJ, Wright AL, Guerra S, Martinez FD. Poor airway function in early infancy and lung function by age 22 years: A non-selective longitudinal cohort study. *Lancet* 2007; 370: 758-64.
20. Gern JE, Rosenthal LA, Sorkness RL, Lemanske RF,Jr. Effects of viral respiratory infections on lung development and childhood asthma. *J Allergy Clin Immunol* 2005; 115: 668,74; quiz 675.
21. Jeffery PK. Remodeling in asthma and chronic obstructive lung disease. *Am J Respir Crit Care Med* 2001; 164: S28-38.
22. Long-term effects of budesonide or nedocromil in children with asthma. the childhood asthma management program research group. *N Engl J Med* 2000; 343: 1054-63.
23. Guilbert TW, Morgan WJ, Zeiger RS, Mauger DT, Boehmer SJ, Szefler SJ, et al. Long-term inhaled corticosteroids in preschool children at high risk for asthma. *N Engl J Med* 2006; 354: 1985-97.
24. Goksor E, Gustafsson PM, Alm B, Amark M, Wennergren G. Reduced airway function in early adulthood among subjects with wheezing disorder before two years of age. *Pediatr Pulmonol* 2008; 43: 396-403.
25. Sigurs N, Aljassim F, Kjellman B, Robinson PD, Sigurbergsson F, Bjarnason R, et al. Asthma and allergy patterns over 18 years after severe RSV bronchiolitis in the first year of life. *Thorax* 2010; 65: 1045-52.
26. Backman K, Piippo-Savolainen E, Ollikainen H, Koskela H, Korppi M. Irreversible airway obstruction in adulthood after bronchiolitis in infancy: Evidence from a 30-year follow-up study. *Respir Med* 2014; 108: 218-23.
27. Dubois AB, Brody AW, Lewis DH, Burgess BF,Jr. Oscillation mechanics of lungs and chest in man. *J Appl Physiol* 1956; 8: 587-94.
28. Stokes GM, Milner AD, Hodges IG, Groggins RC. Lung function abnormalities after acute bronchiolitis. *J Pediatr* 1981; 98: 871-4.
29. Henry RL, Milner AD, Stokes GM, Hodges IG, Groggins RC. Lung function after acute bronchiolitis. *Arch Dis Child* 1983; 58: 60-3.
30. Guilbert TW, Singh AM, Danov Z, Evans MD, Jackson DJ, Burton R, et al. Decreased lung function after preschool wheezing rhinovirus illnesses in children at risk to develop asthma. *J Allergy Clin Immunol* 2011; 128: 532,8.e1-10.

31. American Academy of Pediatrics Subcommittee on Diagnosis and Management of,Bronchiolitis. Diagnosis and management of bronchiolitis. *Pediatrics* 2006; 118: 1774-93.
32. Ralston SL, Lieberthal AS, Meissner HC, Alverson BK, Baley JE, Gadomski AM, et al. Clinical practice guideline: The diagnosis, management, and prevention of bronchiolitis. *Pediatrics* 2014; 134: e1474-502.
33. Ricci V, Delgado Nunes V, Murphy MS, Cunningham S, Guideline Development Group and Technical Team. Bronchiolitis in children: Summary of NICE guidance. *BMJ* 2015; 350: h2305.
34. Jartti T, Lehtinen P, Vuorinen T, Ruuskanen O. Bronchiolitis: Age and previous wheezing episodes are linked to viral etiology and atopic characteristics. *Pediatr Infect Dis J* 2009; 28: 311-7.
35. Korppi M, Kotaniemi-Syrjanen A, Waris M, Vainionpaa R, Reijonen TM. Rhinovirus-associated wheezing in infancy: Comparison with respiratory syncytial virus bronchiolitis. *Pediatr Infect Dis J* 2004; 23: 995-9.
36. Midulla F, Pierangeli A, Cangiano G, Bonci E, Salvadei S, Scagnolari C, et al. Rhinovirus bronchiolitis and recurrent wheezing: 1-year follow-up. *Eur Respir J* 2012; 39: 396-402.
37. Rakes GP, Arruda E, Ingram JM, Hoover GE, Zambrano JC, Hayden FG, et al. Rhinovirus and respiratory syncytial virus in wheezing children requiring emergency care. IgE and eosinophil analyses. *Am J Respir Crit Care Med* 1999; 159: 785-90.
38. Papadopoulos NG, Moustaki M, Tsolia M, Bossios A, Astra E, Prezerakou A, et al. Association of rhinovirus infection with increased disease severity in acute bronchiolitis. *Am J Respir Crit Care Med* 2002; 165: 1285-9.
39. Jartti T, Lehtinen P, Vuorinen T, Osterback R, van den Hoogen B, Osterhaus AD, et al. Respiratory picornaviruses and respiratory syncytial virus as causative agents of acute expiratory wheezing in children. *Emerg Infect Dis* 2004; 10: 1095-101.
40. Purcell K, Fergie J. Driscoll children's hospital respiratory syncytial virus database: Risk factors, treatment and hospital course in 3308 infants and young children, 1991 to 2002. *Pediatr Infect Dis J* 2004; 23: 418-23.
41. Lambert L, Sagfors AM, Openshaw PJ, Culley FJ. Immunity to RSV in early-life. *Front Immunol* 2014; 5: 466.
42. Williams JV, Harris PA, Tollefson SJ, Halburnt-Rush LL, Pingsterhaus JM, Edwards KM, et al. Human metapneumovirus and lower respiratory tract disease in otherwise healthy infants and children. *N Engl J Med* 2004; 350: 443-50.
43. Johnson JE, Gonzales RA, Olson SJ, Wright PF, Graham BS. The histopathology of fatal untreated human respiratory syncytial virus infection. *Mod Pathol* 2007; 20: 108-19.
44. Ramet M, Korppi M, Hallman M. Pattern recognition receptors and genetic risk for rsv infection: Value for clinical decision-making? *Pediatr Pulmonol* 2011; 46: 101-10.

45. Legg JP, Hussain IR, Warner JA, Johnston SL, Warner JO. Type 1 and type 2 cytokine imbalance in acute respiratory syncytial virus bronchiolitis. *Am J Respir Crit Care Med* 2003; 168: 633-9.
46. Kaiko GE, Phipps S, Angkasekwinai P, Dong C, Foster PS. NK cell deficiency predisposes to viral-induced Th2-type allergic inflammation via epithelial-derived IL-25. *J Immunol* 2010; 185: 4681-90.
47. Papadopoulos NG, Bates PJ, Bardin PG, Papi A, Leir SH, Fraenkel DJ, et al. Rhinoviruses infect the lower airways. *J Infect Dis* 2000; 181: 1875-84.
48. Kotaniemi-Syrjanen A, Vainionpaa R, Reijonen TM, Waris M, Korhonen K, Korppi M. Rhinovirus-induced wheezing in infancy--the first sign of childhood asthma? *J Allergy Clin Immunol* 2003; 111: 66-71.
49. Jartti T, Lee WM, Pappas T, Evans M, Lemanske RF,Jr, Gern JE. Serial viral infections in infants with recurrent respiratory illnesses. *Eur Respir J* 2008; 32: 314-20.
50. Copenhaver CC, Gern JE, Li Z, Shult PA, Rosenthal LA, Mikus LD, et al. Cytokine response patterns, exposure to viruses, and respiratory infections in the first year of life. *Am J Respir Crit Care Med* 2004; 170: 175-80.
51. Helminen M, Nuolivirta K, Virta M, Halkosalo A, Korppi M, Vesikari T, et al. IL-10 gene polymorphism at -1082 A/G is associated with severe rhinovirus bronchiolitis in infants. *Pediatr Pulmonol* 2008; 43: 391-5.
52. Jartti T, Aakula M, Mansbach JM, Piedra PA, Bergroth E, Koponen P, et al. Hospital length-of-stay is associated with rhinovirus etiology of bronchiolitis. *Pediatr Infect Dis J* 2014; 33: 829-34.
53. Palmenberg AC, Spiro D, Kuzmickas R, Wang S, Djikeng A, Rathe JA, et al. Sequencing and analyses of all known human rhinovirus genomes reveal structure and evolution. *Science* 2009; 324: 55-9.
54. Kennedy JL, Turner RB, Braciale T, Heymann PW, Borish L. Pathogenesis of rhinovirus infection. *Curr Opin Virol* 2012; 2: 287-93.
55. Message SD, Laza-Stanca V, Mallia P, Parker HL, Zhu J, Kebadze T, et al. Rhinovirus-induced lower respiratory illness is increased in asthma and related to virus load and Th1/2 cytokine and IL-10 production. *Proc Natl Acad Sci U S A* 2008; 105: 13562-7.
56. Papadopoulos NG, Stanciu LA, Papi A, Holgate ST, Johnston SL. A defective type 1 response to rhinovirus in atopic asthma. *Thorax* 2002; 57: 328-32.
57. Juntti H, Osterlund P, Kokkonen J, Dunder T, Renko M, Pokka T, et al. Cytokine responses in cord blood predict the severity of later respiratory syncytial virus infection. *J Allergy Clin Immunol* 2009; 124: 52,58.e1-2.
58. Prescott SL, Macaubas C, Holt BJ, Smallacombe TB, Loh R, Sly PD, et al. Transplacental priming of the human immune system to environmental allergens: Universal skewing of initial T cell responses toward the Th2 cytokine profile. *J Immunol* 1998; 160: 4730-7.
59. Marodi L. Down-regulation of Th1 responses in human neonates. *Clin Exp Immunol* 2002; 128: 1-2.
60. Wills-Karp M, Santeliz J, Karp CL. The germless theory of allergic disease: Revisiting the hygiene hypothesis. *Nat Rev Immunol* 2001; 1: 69-75.

61. Culley FJ, Pollott J, Openshaw PJ. Age at first viral infection determines the pattern of T cell-mediated disease during reinfection in adulthood. *J Exp Med* 2002; 196: 1381-6.
62. Friedlander SL, Jackson DJ, Gangnon RE, Evans MD, Li Z, Roberg KA, et al. Viral infections, cytokine dysregulation and the origins of childhood asthma and allergic diseases. *Pediatr Infect Dis J* 2005; 24: S170,6, discussion S174-5.
63. Copenhaver CC, Gern JE, Li Z, Shult PA, Rosenthal LA, Mikus LD, et al. Cytokine response patterns, exposure to viruses, and respiratory infections in the first year of life. *Am J Respir Crit Care Med* 2004; 170: 175-80.
64. Neaville WA, Tisler C, Bhattacharya A, Anklam K, Gilbertson-White S, Hamilton R, et al. Developmental cytokine response profiles and the clinical and immunologic expression of atopy during the first year of life. *J Allergy Clin Immunol* 2003; 112: 740-6.
65. Jartti T, Paul-Anttila M, Lehtinen P, Parikka V, Vuorinen T, Simell O, et al. Systemic T-helper and T-regulatory cell type cytokine responses in rhinovirus vs. respiratory syncytial virus induced early wheezing: An observational study. *Respir Res* 2009; 10: 85,9921-10-85.
66. Thurlbeck WM. Postnatal human lung growth. *Thorax* 1982; 37: 564-71.
67. Narayanan M, Owers-Bradley J, Beardsmore CS, Mada M, Ball I, Garipov R, et al. Alveolarization continues during childhood and adolescence: New evidence from helium-3 magnetic resonance. *Am J Respir Crit Care Med* 2012; 185: 186-91.
68. Herring MJ, Putney LF, Wyatt G, Finkbeiner WE, Hyde DM. Growth of alveoli during postnatal development in humans based on stereological estimation. *Am J Physiol Lung Cell Mol Physiol* 2014; 307: L338-44.
69. Hanrahan JP, Brown RW, Carey VJ, Castile RG, Speizer FE, Tager IB. Passive respiratory mechanics in healthy infants. effects of growth, gender, and smoking. *Am J Respir Crit Care Med* 1996; 154: 670-80.
70. Tepper RS, Morgan WJ, Cota K, Wright A, Taussig LM. Physiologic growth and development of the lung during the first year of life. *Am Rev Respir Dis* 1986; 134: 513-9.
71. Seidenberg J, Masters IB, Hudson I, Olinsky A, Phelan PD. Disturbance in respiratory mechanics in infants with bronchiolitis. *Thorax* 1989; 44: 660-7.
72. Price JF. Acute and long-term effects of viral bronchiolitis in infancy. *Lung* 1990; 168 Suppl: 414-21.
73. Dezateux C, Fletcher ME, Dundas I, Stocks J. Infant respiratory function after RSV-proven bronchiolitis. *Am J Respir Crit Care Med* 1997; 155: 1349-55.
74. Becroft DM. Bronchiolitis obliterans, bronchiectasis, and other sequelae of adenovirus type 21 infection in young children. *J Clin Pathol* 1971; 24: 72-82.
75. Fahy JV. Remodeling of the airway epithelium in asthma. *Am J Respir Crit Care Med* 2001; 164: S46-51.
76. Hashimoto M, Tanaka H, Abe S. Quantitative analysis of bronchial wall vascularity in the medium and small airways of patients with asthma and COPD. *Chest* 2005; 127: 965-72.

77. Bosse Y, Riesenfeld EP, Pare PD, Irvin CG. It's not all smooth muscle: Non-smooth-muscle elements in control of resistance to airflow. *Annu Rev Physiol* 2010; 72: 437-62.
78. Beckett PA, Howarth PH. Pharmacotherapy and airway remodelling in asthma? *Thorax* 2003; 58: 163-74.
79. Grainge CL, Lau LC, Ward JA, Dulay V, Lahiff G, Wilson S, et al. Effect of bronchoconstriction on airway remodeling in asthma. *N Engl J Med* 2011; 364: 2006-15.
80. Berair R, Brightling CE. Asthma therapy and its effect on airway remodelling. *Drugs* 2014; 74: 1345-69.
81. Haahtela T, Jarvinen M, Kava T, Kiviranta K, Koskinen S, Lehtonen K, et al. Comparison of a beta 2-agonist, terbutaline, with an inhaled corticosteroid, budesonide, in newly detected asthma. *N Engl J Med* 1991; 325: 388-92.
82. Haahtela T, Jarvinen M, Kava T, Kiviranta K, Koskinen S, Lehtonen K, et al. Effects of reducing or discontinuing inhaled budesonide in patients with mild asthma. *N Engl J Med* 1994; 331: 700-5.
83. Ward C, Reid DW, Orsida BE, Feltis B, Ryan VA, Johns DP, et al. Inter-relationships between airway inflammation, reticular basement membrane thickening and bronchial hyper-reactivity to methacholine in asthma; a systematic bronchoalveolar lavage and airway biopsy analysis. *Clin Exp Allergy* 2005; 35: 1565-71.
84. Saglani S, Malmstrom K, Pelkonen AS, Malmberg LP, Lindahl H, Kajosaari M, et al. Airway remodeling and inflammation in symptomatic infants with reversible airflow obstruction. *Am J Respir Crit Care Med* 2005; 171: 722-7.
85. Saglani S, Payne DN, Zhu J, Wang Z, Nicholson AG, Bush A, et al. Early detection of airway wall remodeling and eosinophilic inflammation in preschool wheezers. *Am J Respir Crit Care Med* 2007; 176: 858-64.
86. Payne DN, Rogers AV, Adelroth E, Bandi V, Guntupalli KK, Bush A, et al. Early thickening of the reticular basement membrane in children with difficult asthma. *Am J Respir Crit Care Med* 2003; 167: 78-82.
87. Malmstrom K, Malmberg LP, O'Reilly R, Lindahl H, Kajosaari M, Saarinen KM, et al. Lung function, airway remodeling, and inflammation in infants: Outcome at 8 years. *Ann Allergy Asthma Immunol* 2015; 114: 90-6.
88. Leigh R, Oyelusi W, Wiehler S, Koetzler R, Zaheer RS, Newton R, et al. Human rhinovirus infection enhances airway epithelial cell production of growth factors involved in airway remodeling. *J Allergy Clin Immunol* 2008; 121: 1238,1245.e4.
89. Lee CG, Yoon HJ, Zhu Z, Link H, Wang Z, Gwaltney JM, et al. Respiratory syncytial virus stimulation of vascular endothelial cell growth factor/vascular permeability factor. *Am J Respir Cell Mol Biol* 2000; 23: 662-9.
90. Uhl EW, Castleman WL, Sorkness RL, Busse WW, Lemanske RF,Jr, McAllister PK. Parainfluenza virus-induced persistence of airway inflammation, fibrosis, and dysfunction associated with TGF-beta 1 expression in brown norway rats. *Am J Respir Crit Care Med* 1996; 154: 1834-42.

91. Kumar A, Sorkness RL, Kaplan MR, Lemanske RF,Jr. Chronic, episodic, reversible airway obstruction after viral bronchiolitis in rats. *Am J Respir Crit Care Med* 1997; 155: 130-4.
92. Rosenthal LA, Mikus LD, Tuffaha A, Mosser AG, Sorkness RL, Lemanske RF,Jr. Attenuated innate mechanisms of interferon-gamma production in rats susceptible to postviral airway dysfunction. *Am J Respir Cell Mol Biol* 2004; 30: 702-9.
93. Sorkness RL, Castleman WL, Kumar A, Kaplan MR, Lemanske RF,Jr. Prevention of chronic postbronchiolitis airway sequelae with IFN-gamma treatment in rats. *Am J Respir Crit Care Med* 1999; 160: 705-10.
94. Stocks J, Dezateux C. The effect of parental smoking on lung function and development during infancy. *Respirology* 2003; 8: 266-85.
95. Milner AD, Rao H, Greenough A. The effects of antenatal smoking on lung function and respiratory symptoms in infants and children. *Early Hum Dev* 2007; 83: 707-11.
96. Hanrahan JP, Tager IB, Segal MR, Tosteson TD, Castile RG, Van Vunakis H, et al. The effect of maternal smoking during pregnancy on early infant lung function. *Am Rev Respir Dis* 1992; 145: 1129-35.
97. Brown RW, Hanrahan JP, Castile RG, Tager IB. Effect of maternal smoking during pregnancy on passive respiratory mechanics in early infancy. *Pediatr Pulmonol* 1995; 19: 23-8.
98. Tager IB, Ngo L, Hanrahan JP. Maternal smoking during pregnancy. effects on lung function during the first 18 months of life. *Am J Respir Crit Care Med* 1995; 152: 977-83.
99. Cook DG, Strachan DP. Parental smoking, bronchial reactivity and peak flow variability in children. *Thorax* 1998; 53: 295-301.
100. Kalliola S, Pelkonen AS, Malmberg LP, Sarna S, Hamalainen M, Mononen I, et al. Maternal smoking affects lung function and airway inflammation in young children with multiple-trigger wheeze. *J Allergy Clin Immunol* 2013; 131: 730-5.
101. Martinez FD, Morgan WJ, Wright AL, Holberg CJ, Taussig LM. Diminished lung function as a predisposing factor for wheezing respiratory illness in infants. *N Engl J Med* 1988; 319: 1112-7.
102. Murray CS, Pipis SD, McArdle EC, Lowe LA, Custovic A, Woodcock A, et al. Lung function at one month of age as a risk factor for infant respiratory symptoms in a high risk population. *Thorax* 2002; 57: 388-92.
103. Turner SW, Young S, Landau LI, Le Souef PN. Reduced lung function both before bronchiolitis and at 11 years. *Arch Dis Child* 2002; 87: 417-20.
104. Haland G, Carlsen KC, Sandvik L, Devulapalli CS, Munthe-Kaas MC, Pettersen M, et al. Reduced lung function at birth and the risk of asthma at 10 years of age. *N Engl J Med* 2006; 355: 1682-9.
105. Zomer-Kooijker K, Uiterwaal CS, van der Gugten AC, Wilbrink B, Bont LJ, van der Ent CK. Decreased lung function precedes severe respiratory syncytial virus infection and post-respiratory syncytial virus wheeze in term infants. *Eur Respir J* 2014; 44: 666-74.

106. Bisgaard H, Jensen SM, Bonnelykke K. Interaction between asthma and lung function growth in early life. *Am J Respir Crit Care Med* 2012; 185: 1183-9.
107. Taussig LM. Maximal expiratory flows at functional residual capacity: A test of lung function for young children. *Am Rev Respir Dis* 1977; 116: 1031-8.
108. Young S, O'Keeffe PT, Arnott J, Landau LI. Lung function, airway responsiveness, and respiratory symptoms before and after bronchiolitis. *Arch Dis Child* 1995; 72: 16-24.
109. Young S, Arnott J, O'Keeffe PT, Le Souef PN, Landau LI. The association between early life lung function and wheezing during the first 2 yrs of life. *Eur Respir J* 2000; 15: 151-7.
110. Young S, Sherrill DL, Arnott J, Diepeveen D, LeSouef PN, Landau LI. Parental factors affecting respiratory function during the first year of life. *Pediatr Pulmonol* 2000; 29: 331-40.
111. Custovic A, Simpson BM, Murray CS, Lowe L, Woodcock A, NAC Manchester Asthma and Allergy Study Group. The national asthma campaign manchester asthma and allergy study. *Pediatr Allergy Immunol* 2002; 13 Suppl 15: 32-7.
112. Belgrave DC, Buchan I, Bishop C, Lowe L, Simpson A, Custovic A. Trajectories of lung function during childhood. *Am J Respir Crit Care Med* 2014; 189: 1101-9.
113. van der Zalm MM, Uiterwaal CS, Wilbrink B, Koopman M, Verheij TJ, van der Ent CK. The influence of neonatal lung function on rhinovirus-associated wheeze. *Am J Respir Crit Care Med* 2011; 183: 262-7.
114. Zomer-Kooijker K, van der Ent CK, Ermers MJ, Uiterwaal CS, Rovers MM, Bont LJ, et al. Increased risk of wheeze and decreased lung function after respiratory syncytial virus infection. *PLoS One* 2014; 9: e87162.
115. Chawes BL, Poorisrisak P, Johnston SL, Bisgaard H. Neonatal bronchial hyperresponsiveness precedes acute severe viral bronchiolitis in infants. *J Allergy Clin Immunol* 2012; 130: 354,61.e3.
116. Fjaerli HO, Farstad T, Rod G, Ufert GK, Gulbrandsen P, Nakstad B. Acute bronchiolitis in infancy as risk factor for wheezing and reduced pulmonary function by seven years in akershus county, norway. *BMC Pediatr* 2005; 5: 31.
117. Kotaniemi-Syrjanen A, Reijonen TM, Korhonen K, Waris M, Vainionpaa R, Korppi M. Wheezing due to rhinovirus infection in infancy: Bronchial hyperresponsiveness at school age. *Pediatr Int* 2008; 50: 506-10.
118. Hyvarinen MK, Kotaniemi-Syrjanen A, Reijonen TM, Korhonen K, Korppi MO. Lung function and bronchial hyper-responsiveness 11 years after hospitalization for bronchiolitis. *Acta Paediatr* 2007; 96: 1464-9.
119. Wennergren G, Hansson S, Engstrom I, Jodal U, Amark M, Brolin I, et al. Characteristics and prognosis of hospital-treated obstructive bronchitis in children aged less than two years. *Acta Paediatr* 1992; 81: 40-5.
120. Wennergren G, Amark M, Amark K, Oskarsdottir S, Sten G, Redfors S. Wheezing bronchitis reinvestigated at the age of 10 years. *Acta Paediatr* 1997; 86: 351-5.
121. Goksor E, Amark M, Alm B, Gustafsson PM, Wennergren G. Asthma symptoms in early childhood--what happens then? *Acta Paediatr* 2006; 95: 471-8.

122. Sigurs N, Gustafsson PM, Bjarnason R, Lundberg F, Schmidt S, Sigurbergsson F, et al. Severe respiratory syncytial virus bronchiolitis in infancy and asthma and allergy at age 13. *Am J Respir Crit Care Med* 2005; 171: 137-41.
123. Piippo-Savolainen E, Remes S, Kannisto S, Korhonen K, Korppi M. Asthma and lung function 20 years after wheezing in infancy: Results from a prospective follow-up study. *Arch Pediatr Adolesc Med* 2004; 158: 1070-6.
124. Schachter LM, Salome CM, Peat JK, Woolcock AJ. Obesity is a risk for asthma and wheeze but not airway hyperresponsiveness. *Thorax* 2001; 56: 4-8.
125. Moore WC, Meyers DA, Wenzel SE, Teague WG, Li H, Li X, et al. Identification of asthma phenotypes using cluster analysis in the severe asthma research program. *Am J Respir Crit Care Med* 2010; 181: 315-23.
126. Shore SA. Obesity, airway hyperresponsiveness, and inflammation. *J Appl Physiol (1985)* 2010; 108: 735-43.
127. Papoutsakis C, Priftis KN, Drakouli M, Prifti S, Konstantaki E, Chondronikola M, et al. Childhood overweight/obesity and asthma: Is there a link? A systematic review of recent epidemiologic evidence. *J Acad Nutr Diet* 2013; 113: 77-105.
128. Schachter LM, Peat JK, Salome CM. Asthma and atopy in overweight children. *Thorax* 2003; 58: 1031-5.
129. Bibi H, Shoseyov D, Feigenbaum D, Genis M, Friger M, Peled R, et al. The relationship between asthma and obesity in children: Is it real or a case of over diagnosis? *J Asthma* 2004; 41: 403-10.
130. Scholtens S, Wijga AH, Seidell JC, Brunekreef B, de Jongste JC, Gehring U, et al. Overweight and changes in weight status during childhood in relation to asthma symptoms at 8 years of age. *J Allergy Clin Immunol* 2009; 123: 1312,8.e2.
131. Lazarus R, Colditz G, Berkey CS, Speizer FE. Effects of body fat on ventilatory function in children and adolescents: Cross-sectional findings from a random population sample of school children. *Pediatr Pulmonol* 1997; 24: 187-94.
132. Spathopoulos D, Paraskakis E, Trypsianis G, Tsalkidis A, Arvanitidou V, Emporiadou M, et al. The effect of obesity on pulmonary lung function of school aged children in greece. *Pediatr Pulmonol* 2009; 44: 273-80.
133. Sidoroff V, Hyvarinen M, Piippo-Savolainen E, Korppi M. Lung function and overweight in school aged children after early childhood wheezing. *Pediatr Pulmonol* 2010.
134. Davidson WJ, Mackenzie-Rife KA, Witmans MB, Montgomery MD, Ball GD, Egbogah S, et al. Obesity negatively impacts lung function in children and adolescents. *Pediatr Pulmonol* 2013.
135. Curry BA, Blizzard CL, Schmidt MD, Walters EH, Dwyer T, Venn AJ. Longitudinal associations of adiposity with adult lung function in the childhood determinants of adult health (CDAH) study. *Obesity (Silver Spring)* 2011; 19: 2069-75.
136. Littleton SW. Impact of obesity on respiratory function. *Respirology* 2012; 17: 43-9.
137. Oppenheimer BW, Berger KI, Segal LN, Stabile A, Coles KD, Parikh M, et al. Airway dysfunction in obesity: Response to voluntary restoration of end expiratory lung volume. *PLoS One* 2014; 9: e88015.

138. Kalhoff H, Breidenbach R, Smith HJ, Marek W. Impulse oscillometry in preschool children and association with body mass index. *Respirology* 2011; 16: 174-9.
139. Barker DJ. Fetal origins of cardiovascular disease. *Ann Med* 1999; 31 Suppl 1: 3-6.
140. Ay L, Van Houten VA, Steegers EA, Hofman A, Witteman JC, Jaddoe VW, et al. Fetal and postnatal growth and body composition at 6 months of age. *J Clin Endocrinol Metab* 2009; 94: 2023-30.
141. Remacle C, Bieswal F, Bol V, Reusens B. Developmental programming of adult obesity and cardiovascular disease in rodents by maternal nutrition imbalance. *Am J Clin Nutr* 2011; 94: 1846S-52S.
142. Rona RJ, Smeeton NC, Bustos P, Amigo H, Diaz PV. The early origins hypothesis with an emphasis on growth rate in the first year of life and asthma: A prospective study in chile. *Thorax* 2005; 60: 549-54.
143. Turner S. Perinatal programming of childhood asthma: Early fetal size, growth trajectory during infancy, and childhood asthma outcomes. *Clin Dev Immunol* 2012; 2012: 962923.
144. Lazarus R, Vercelli D, Palmer LJ, Klimecki WJ, Silverman EK, Richter B, et al. Single nucleotide polymorphisms in innate immunity genes: Abundant variation and potential role in complex human disease. *Immunol Rev* 2002; 190: 9-25.
145. Parker D, Prince A. Innate immunity in the respiratory epithelium. *Am J Respir Cell Mol Biol* 2011; 45: 189-201.
146. Hallman M, Ramet M, Ezekowitz RA. Toll-like receptors as sensors of pathogens. *Pediatr Res* 2001; 50: 315-21.
147. Akira S, Uematsu S, Takeuchi O. Pathogen recognition and innate immunity. *Cell* 2006; 124: 783-801.
148. Cook DN, Pisetsky DS, Schwartz DA. Toll-like receptors in the pathogenesis of human disease. *Nat Immunol* 2004; 5: 975-9.
149. Takeuchi O, Akira S. Pattern recognition receptors and inflammation. *Cell* 2010; 140: 805-20.
150. Braun-Fahrlander C, Riedler J, Herz U, Eder W, Waser M, Grize L, et al. Environmental exposure to endotoxin and its relation to asthma in school-age children. *N Engl J Med* 2002; 347: 869-77.
151. Gehring U, Bischof W, Fahlbusch B, Wichmann HE, Heinrich J. House dust endotoxin and allergic sensitization in children. *Am J Respir Crit Care Med* 2002; 166: 939-44.
152. Arbour NC, Lorenz E, Schutte BC, Zabner J, Kline JN, Jones M, et al. TLR4 mutations are associated with endotoxin hyporesponsiveness in humans. *Nat Genet* 2000; 25: 187-91.
153. Werner M, Topp R, Wimmer K, Richter K, Bischof W, Wjst M, et al. TLR4 gene variants modify endotoxin effects on asthma. *J Allergy Clin Immunol* 2003; 112: 323-30.
154. Tulic MK, Hurrelbrink RJ, Prele CM, Laing IA, Upham JW, Le Souef P, et al. TLR4 polymorphisms mediate impaired responses to respiratory syncytial virus and lipopolysaccharide. *J Immunol* 2007; 179: 132-40.

155. Tal G, Mandelberg A, Dalal I, Cesar K, Somekh E, Tal A, et al. Association between common toll-like receptor 4 mutations and severe respiratory syncytial virus disease. *J Infect Dis* 2004; 189: 2057-63.
156. Mandelberg A, Tal G, Naugolny L, Cesar K, Oron A, Houri S, et al. Lipopolysaccharide hyporesponsiveness as a risk factor for intensive care unit hospitalization in infants with respiratory syncitial virus bronchiolitis. *Clin Exp Immunol* 2006; 144: 48-52.
157. Lofgren J, Marttila R, Renko M, Ramet M, Hallman M. Toll-like receptor 4 Asp299Gly polymorphism in respiratory syncytial virus epidemics. *Pediatr Pulmonol* 2010; 45: 687-92.
158. Tapping RI, Omueti KO, Johnson CM. Genetic polymorphisms within the human toll-like receptor 2 subfamily. *Biochem Soc Trans* 2007; 35: 1445-8.
159. Oliveira-Nascimento L, Massari P, Wetzler LM. The role of TLR2 in infection and immunity. *Front Immunol* 2012; 3: 79.
160. Eder W, Klimecki W, Yu L, von Mutius E, Riedler J, Braun-Fahrlander C, et al. Toll-like receptor 2 as a major gene for asthma in children of european farmers. *J Allergy Clin Immunol* 2004; 113: 482-8.
161. Daley D, Park JE, He JQ, Yan J, Akhabir L, Stefanowicz D, et al. Associations and interactions of genetic polymorphisms in innate immunity genes with early viral infections and susceptibility to asthma and asthma-related phenotypes. *J Allergy Clin Immunol* 2012; 130: 1284-93.
162. Kormann MS, Depner M, Hartl D, Klopp N, Illig T, Adamski J, et al. Toll-like receptor heterodimer variants protect from childhood asthma. *J Allergy Clin Immunol* 2008; 122: 86,92, 92.e1-8.
163. Klaassen EM, Thonissen BE, van Eys G, Dompeling E, Jobsis Q. A systematic review of CD14 and toll-like receptors in relation to asthma in caucasian children. *Allergy Asthma Clin Immunol* 2013; 9: 10,1492-9-10.
164. Puthothu B, Heinzmann A. Is toll-like receptor 6 or toll-like receptor 10 involved in asthma genetics--or both? *Allergy* 2006; 61: 649-50.
165. Daley D, Lemire M, Akhabir L, Chan-Yeung M, He JQ, McDonald T, et al. Analyses of associations with asthma in four asthma population samples from canada and australia. *Hum Genet* 2009; 125: 445-59.
166. Tantisira K, Klimecki WT, Lazarus R, Palmer LJ, Raby BA, Kwiatkowski DJ, et al. Toll-like receptor 6 gene (TLR6): Single-nucleotide polymorphism frequencies and preliminary association with the diagnosis of asthma. *Genes Immun* 2004; 5: 343-6.
167. Hoffjan S, Stemmler S, Parwez Q, Petrasch-Parwez E, Arinir U, Rohde G, et al. Evaluation of the toll-like receptor 6 Ser249Pro polymorphism in patients with asthma, atopic dermatitis and chronic obstructive pulmonary disease. *BMC Med Genet* 2005; 6: 34.
168. Shey MS, Randhawa AK, Bowmaker M, Smith E, Scriba TJ, de Kock M, et al. Single nucleotide polymorphisms in toll-like receptor 6 are associated with altered lipopeptide- and mycobacteria-induced interleukin-6 secretion. *Genes Immun* 2010; 11: 561-72.

169. McNamara PS, Flanagan BF, Selby AM, Hart CA, Smyth RL. Pro- and anti-inflammatory responses in respiratory syncytial virus bronchiolitis. *Eur Respir J* 2004; 23: 106-12.
170. Nuolivirta K, Vuononvirta J, Peltola V, Koponen P, Helminen M, He Q, et al. Toll-like receptor 2 subfamily genotypes are not associated with severity of bronchiolitis or postbronchiolitis wheezing in infants. *Acta Paediatr* 2013; 102: 1160-4.
171. Koponen P, Vuononvirta J, Nuolivirta K, Helminen M, He Q, Korppi M. The association of genetic variants in toll-like receptor 2 subfamily with allergy and asthma after hospitalization for bronchiolitis in infancy. *Pediatr Infect Dis J* 2014; 33: 463-6.
172. Nuolivirta K, He Q, Vuononvirta J, Koponen P, Helminen M, Korppi M. Toll-like receptor 3 L412F polymorphisms in infants with bronchiolitis and postbronchiolitis wheezing. *Pediatr Infect Dis J* 2012; 31: 920-3.
173. Sukkar MB, Xie S, Khorasani NM, Kon OM, Stanbridge R, Issa R, et al. Toll-like receptor 2, 3, and 4 expression and function in human airway smooth muscle. *J Allergy Clin Immunol* 2006; 118: 641-8.
174. Camateros P, Tamaoka M, Hassan M, Marino R, Moisan J, Marion D, et al. Chronic asthma-induced airway remodeling is prevented by toll-like receptor-7/8 ligand S28463. *Am J Respir Crit Care Med* 2007; 175: 1241-9.
175. Moller-Larsen S, Nyegaard M, Haagerup A, Vestbo J, Kruse TA, Borglum AD. Association analysis identifies TLR7 and TLR8 as novel risk genes in asthma and related disorders. *Thorax* 2008; 63: 1064-9.
176. Akdis M, Burgler S, Crameri R, Eiwegger T, Fujita H, Gomez E, et al. Interleukins, from 1 to 37, and interferon-gamma: Receptors, functions, and roles in diseases. *J Allergy Clin Immunol* 2011; 127: 701,21.e1-70.
177. Moore KW, de Waal Malefyt R, Coffman RL, O'Garra A. Interleukin-10 and the interleukin-10 receptor. *Annu Rev Immunol* 2001; 19: 683-765.
178. Loebbermann J, Schnoeller C, Thornton H, Durant L, Sweeney NP, Schuijs M, et al. IL-10 regulates viral lung immunopathology during acute respiratory syncytial virus infection in mice. *PLoS One* 2012; 7: e32371.
179. Sun L, Cornell TT, LeVine A, Berlin AA, Hinkovska-Galcheva V, Fleszar AJ, et al. Dual role of interleukin-10 in the regulation of respiratory syncitial virus (RSV)-induced lung inflammation. *Clin Exp Immunol* 2013; 172: 263-79.
180. Bont L, Heijnen CJ, Kavelaars A, van Aalderen WM, Brus F, Draaisma JT, et al. Monocyte IL-10 production during respiratory syncytial virus bronchiolitis is associated with recurrent wheezing in a one-year follow-up study. *Am J Respir Crit Care Med* 2000; 161: 1518-23.
181. Hawrylowicz CM, O'Garra A. Potential role of interleukin-10-secreting regulatory T cells in allergy and asthma. *Nat Rev Immunol* 2005; 5: 271-83.
182. Turner DM, Williams DM, Sankaran D, Lazarus M, Sinnott PJ, Hutchinson IV. An investigation of polymorphism in the interleukin-10 gene promoter. *Eur J Immunogenet* 1997; 24: 1-8.

183. Mok CC, Lanchbury JS, Chan DW, Lau CS. Interleukin-10 promoter polymorphisms in southern chinese patients with systemic lupus erythematosus. *Arthritis Rheum* 1998; 41: 1090-5.
184. Crawley E, Kay R, Sillibourne J, Patel P, Hutchinson I, Woo P. Polymorphic haplotypes of the interleukin-10 5' flanking region determine variable interleukin-10 transcription and are associated with particular phenotypes of juvenile rheumatoid arthritis. *Arthritis Rheum* 1999; 42: 1101-8.
185. Gibson AW, Edberg JC, Wu J, Westendorp RG, Huizinga TW, Kimberly RP. Novel single nucleotide polymorphisms in the distal IL-10 promoter affect IL-10 production and enhance the risk of systemic lupus erythematosus. *J Immunol* 2001; 166: 3915-22.
186. Wilson J, Rowlands K, Rockett K, Moore C, Lockhart E, Sharland M, et al. Genetic variation at the IL10 gene locus is associated with severity of respiratory syncytial virus bronchiolitis. *J Infect Dis* 2005; 191: 1705-9.
187. Koponen P, Nuolivirta K, Virta M, Helminen M, Hurme M, Korppi M. Polymorphism of the rs1800896 IL10 promoter gene protects children from post-bronchiolitis asthma. *Pediatr Pulmonol* 2014; 49: 800-6.
188. Lyon H, Lange C, Lake S, Silverman EK, Randolph AG, Kwiatkowski D, et al. IL10 gene polymorphisms are associated with asthma phenotypes in children. *Genet Epidemiol* 2004; 26: 155-65.
189. Hyun MH, Lee CH, Kang MH, Park BK, Lee YH. Interleukin-10 promoter gene polymorphisms and susceptibility to asthma: A meta-analysis. *PLoS One* 2013; 8: e53758.
190. Borish L, Aarons A, Rumbyrt J, Cvietusa P, Negri J, Wenzel S. Interleukin-10 regulation in normal subjects and patients with asthma. *J Allergy Clin Immunol* 1996; 97: 1288-96.
191. Shaker M. New insights into the allergic march. *Curr Opin Pediatr* 2014; 26: 516-20.
192. Bousquet J, Van Cauwenberge P, Khaltaev N, Aria Workshop Group, World Health Organization. Allergic rhinitis and its impact on asthma. *J Allergy Clin Immunol* 2001; 108: S147-334.
193. Bousquet J, Schunemann HJ, Samolinski B, Demoly P, Baena-Cagnani CE, Bachert C, et al. Allergic rhinitis and its impact on asthma (ARIA): Achievements in 10 years and future needs. *J Allergy Clin Immunol* 2012; 130: 1049-62.
194. Cruz AA, Popov T, Pawankar R, Annesi-Maesano I, Fokkens W, Kemp J, et al. Common characteristics of upper and lower airways in rhinitis and asthma: ARIA update, in collaboration with GA(2)LEN. *Allergy* 2007; 62 Suppl 84: 1-41.
195. Ciprandi G, Caimmi D, Miraglia Del Giudice M, La Rosa M, Salpietro C, Marseglia GL. Recent developments in united airways disease. *Allergy Asthma Immunol Res* 2012; 4: 171-7.
196. Rimmer J, Ruhno JW. 6: Rhinitis and asthma: United airway disease. *Med J Aust* 2006; 185: 565-71.
197. Garcia-Marcos L, Rubi Ruiz T, Garcia-Hernandez G, Suarez-Varela MM, Valverde-Molina J, Sanchez-Solis M. Asthma and rhinoconjunctivitis

comorbidity: United airway disease or inherited target organs? *Pediatr Allergy Immunol* 2010; 21: e142-8.
198. Arets HG, Brackel HJ, van der Ent CK. Forced expiratory manoeuvres in children: Do they meet ATS and ERS criteria for spirometry?. 2001; 18: 655-60.
199. Aurora P, Stocks J, Oliver C, Saunders C, Castle R, Chaziparasidis G, et al. Quality control for spirometry in preschool children with and without lung disease. 2004; 169: 1152-9.
200. Studnicka M, Frischer T, Neumann M. Determinants of reproducibility of lung function tests in children aged 7 to 10 years. *Pediatr Pulmonol* 1998; 25: 238-43.
201. Beydon N, Davis SD, Lombardi E, Allen JL, Arets HG, Aurora P, et al. An official american thoracic society/european respiratory society statement: Pulmonary function testing in preschool children. *Am J Respir Crit Care Med* 2007; 175: 1304-45.
202. Kaminsky DA. What does airway resistance tell us about lung function? *Respir Care* 2012; 57: 85,96; discussion 96-9.
203. Kaczka DW, Ingenito EP, Suki B, Lutchen KR. Partitioning airway and lung tissue resistances in humans: Effects of bronchoconstriction. *J Appl Physiol (1985)* 1997; 82: 1531-41.
204. Sterk PJ, Fabbri LM, Quanjer PH, Cockcroft DW, O'Byrne PM, Anderson SD, et al. Airway responsiveness. standardized challenge testing with pharmacological, physical and sensitizing stimuli in adults. report working party standardization of lung function tests, european community for steel and coal. official statement of the european respiratory society. *Eur Respir J Suppl* 1993; 16: 53-83.
205. Berend N, Salome CM, King GG. Mechanisms of airway hyperresponsiveness in asthma. *Respirology* 2008; 13: 624-31.
206. Van Schoor J, Joos GF, Pauwels RA. Indirect bronchial hyperresponsiveness in asthma: Mechanisms, pharmacology and implications for clinical research. *Eur Respir J* 2000; 16: 514-33.
207. Suh DI, Lee JK, Kim CK, Koh YY. Bronchial hyperresponsiveness to methacholine/AMP and the bronchodilator response in asthmatic children. *Eur Respir J* 2011; 37: 800-5.
208. Smith H, Reinhold P, Goldman M. Chapter 5. Forced oscillation technique and impulse oscillometry. *European Respiratory Society Monograph* 2005; 31: 75-105.
209. Vogel J, Smidt U. Impulse oscillometry – analysis of lung mechanics in general practice and the clinic, epidemiological and experimental research. *Frankfurt, Pmi Verlagsgruppe GmbH* 1994.
210. Malmberg LP, Pelkonen A, Poussa T, Pohianpalo A, Haahtela T, Turpeinen M. Determinants of respiratory system input impedance and bronchodilator response in healthy finnish preschool children. *Clin Physiol Funct Imaging* 2002; 22: 64-71.
211. Oostveen E, MacLeod D, Lorino H, Farre R, Hantos Z, Desager K, et al. The forced oscillation technique in clinical practice: Methodology, recommendations and future developments. *Eur Respir J* 2003; 22: 1026-41.
212. Bisgaard H, Klug B. Lung function measurement in awake young children. *Eur Respir J* 1995; 8: 2067-75.

213. Ducharme FM, Davis GM. Measurement of respiratory resistance in the emergency department: Feasibility in young children with acute asthma. *Chest* 1997; 111: 1519-25.
214. Delacourt C, Lorino H, Herve-Guillot M, Reinert P, Harf A, Housset B. Use of the forced oscillation technique to assess airway obstruction and reversibility in children. *Am J Respir Crit Care Med* 2000; 161: 730-6.
215. Marotta A, Klinnert MD, Price MR, Larsen GL, Liu AH. Impulse oscillometry provides an effective measure of lung dysfunction in 4-year-old children at risk for persistent asthma. *J Allergy Clin Immunol* 2003; 112: 317-22.
216. Malmberg LP, Makela MJ, Mattila PS, Hammaren-Malmi S, Pelkonen AS. Exercise-induced changes in respiratory impedance in young wheezy children and nonatopic controls. *Pediatr Pulmonol* 2008; 43: 538-44.
217. Komarow HD, Skinner J, Young M, Gaskins D, Nelson C, Gergen PJ, et al. A study of the use of impulse oscillometry in the evaluation of children with asthma: Analysis of lung parameters, order effect, and utility compared with spirometry. *Pediatr Pulmonol* 2012; 47: 18-26.
218. Shi Y, Aledia AS, Tatavoosian AV, Vijayalakshmi S, Galant SP, George SC. Relating small airways to asthma control by using impulse oscillometry in children. *J Allergy Clin Immunol* 2012; 129: 671-8.
219. Jee HM, Kwak JH, Jung da W, Han MY. Useful parameters of bronchial hyperresponsiveness measured with an impulse oscillation technique in preschool children. *J Asthma* 2010; 47: 227-32.
220. Shin YH, Yoon JW, Choi SH, Baek JH, Kim HY, Jee HM, et al. Use of impulse oscillometry system in assessment of asthma severity for preschool children. *J Asthma* 2013; 50: 198-203.
221. Song TW, Kim KW, Kim ES, Kim KE, Sohn MH. Correlation between spirometry and impulse oscillometry in children with asthma. 2008; 97: 51-4.
222. Knihtila H, Kotaniemi-Syrjanen A, Makela MJ, Bondestam J, Pelkonen AS, Malmberg LP. Preschool oscillometry and lung function at adolescence in asthmatic children. *Pediatr Pulmonol* 2015.
223. Hellinckx J, De Boeck K, Bande-Knops J, van der Poel M, Demedts M. Bronchodilator response in 3-6.5 years old healthy and stable asthmatic children. *Eur Respir J* 1998; 12: 438-43.
224. Thamrin C, Gangell CL, Udomittipong K, Kusel MM, Patterson H, Fukushima T, et al. Assessment of bronchodilator responsiveness in preschool children using forced oscillations. *Thorax* 2007; 62: 814-9.
225. Nielsen KG, Bisgaard H. Lung function response to cold air challenge in asthmatic and healthy children of 2-5 years of age. *Am J Respir Crit Care Med* 2000; 161: 1805-9.
226. Clark AG. Inference of haplotypes from PCR-amplified samples of diploid populations. *Mol Biol Evol* 1990; 7: 111-22.
227. Saari A, Sankilampi U, Hannila ML, Kiviniemi V, Kesseli K, Dunkel L. New finnish growth references for children and adolescents aged 0 to 20 years: Length/height-for-age, weight-for-length/height, and body mass index-for-age. *Ann Med* 2011; 43: 235-48.

228. 1000 Genomes Project Consortium, Abecasis GR, Auton A, Brooks LD, DePristo MA, Durbin RM, et al. An integrated map of genetic variation from 1,092 human genomes. *Nature* 2012; 491: 56-65.
229. Noble V, Murray M, Webb MS, Alexander J, Swarbrick AS, Milner AD. Respiratory status and allergy nine to 10 years after acute bronchiolitis. *Arch Dis Child* 1997; 76: 315-9.
230. Broughton S, Bhat R, Roberts A, Zuckerman M, Rafferty G, Greenough A. Diminished lung function, RSV infection, and respiratory morbidity in prematurely born infants. *Arch Dis Child* 2006; 91: 26-30.
231. Jartti T, Korppi M. Rhinovirus-induced bronchiolitis and asthma development. *Pediatr Allergy Immunol* 2011; 22: 350-5.
232. Visness CM, London SJ, Daniels JL, Kaufman JS, Yeatts KB, Siega-Riz AM, et al. Association of childhood obesity with atopic and nonatopic asthma: Results from the national health and nutrition examination survey 1999-2006. *J Asthma* 2010; 47: 822-9.
233. Holguin F, Bleecker ER, Busse WW, Calhoun WJ, Castro M, Erzurum SC, et al. Obesity and asthma: An association modified by age of asthma onset. *J Allergy Clin Immunol* 2011; 127: 1486,93.e2.
234. Hume DA, Underhill DM, Sweet MJ, Ozinsky AO, Liew FY, Aderem A. Macrophages exposed continuously to lipopolysaccharide and other agonists that act via toll-like receptors exhibit a sustained and additive activation state. *BMC Immunol* 2001; 2: 11.
235. Monick MM, Yarovinsky TO, Powers LS, Butler NS, Carter AB, Gudmundsson G, et al. Respiratory syncytial virus up-regulates TLR4 and sensitizes airway epithelial cells to endotoxin. *J Biol Chem* 2003; 278: 53035-44.
236. Fageras Bottcher M, Hmani-Aifa M, Lindstrom A, Jenmalm MC, Mai XM, Nilsson L, et al. A TLR4 polymorphism is associated with asthma and reduced lipopolysaccharide-induced interleukin-12(p70) responses in swedish children. *J Allergy Clin Immunol* 2004; 114: 561-7.
237. Raby BA, Klimecki WT, Laprise C, Renaud Y, Faith J, Lemire M, et al. Polymorphisms in toll-like receptor 4 are not associated with asthma or atopy-related phenotypes. *Am J Respir Crit Care Med* 2002; 166: 1449-56.
238. Long-term effects of budesonide or nedocromil in children with asthma. *N Engl J Med* 2000; 343: 1054-63.

List of original publications

I Lauhkonen E, Koponen P, Nuolivirta K, Paassilta M, Toikka J, Korppi M. Lung function by impulse oscillometry at age 5-7 years after bronchiolitis at age 0-6 months. *Pediatr Pulmonol. 2015 Apr;50(4):389-95*

II Lauhkonen E, Koponen P, Nuolivirta K, Paassilta M, Toikka J, Saari A, Korppi M. Obesity and bronchial obstruction in impulse oscillometry at age 5-7 years in a prospective post-bronchiolitis cohort. *Pediatr Pulmonol. 2015 Sep;50(9):908-14.*

III Lauhkonen E, Koponen P, Teräsjärvi J, Gröndahl-Yli-Hannuksela K, Vuononvirta J, Nuolivirta K, Toikka J, Helminen M, He Q, Korppi M. IL-10 gene polymorphisms are associated with post-bronchiolitis lung function abnormalities at six years of age. *PLoS One. 2015 Oct 16;10(10):e0140799.*

IV Lauhkonen E, Koponen P, Vuononvirta J, Teräsjärvi J, Nuolivirta K, Toikka J, Helminen M, He Q, Korppi M. Gene polymorphism of toll-like receptors and lung function at five to seven years of age after infant bronchiolitis. *PLoS One. 2016 Jan 7;11(1): e0146526.*

V Lauhkonen E, Koponen P, Nuolivirta K, Helminen M, Toikka J, Korppi M. Following Up Infant Bronchiolitis Patients Provided New Evidence For and Against the United Airway Disease Hypothesis. *Acta Paediatrica 2016 (Accepted for publication doi: 10.1111/apa.13537).*

Lung Function by Impulse Oscillometry at age 5–7 Years After Bronchiolitis at Age 0–6 Months

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Summary. Background: Viral bronchiolitis in infancy has been associated with increased bronchial reactivity and reduced lung function in later childhood and even in adulthood. However, lung function at preschool age is less studied, mainly due to technical difficulties. The purpose of the study was to evaluate lung function and bronchial reactivity at preschool age in children who were hospitalized for bronchiolitis in early infancy. Subjects and methods: Airway resistance and reactance, and bronchial reactivity to exercise were studied with impulse oscillometry (IOS) at the mean age of 6.3 years in 103 children hospitalized for bronchiolitis at less than 6 months of age. Results: In baseline lung-function measurement, resistance (n = 8; 7.8%) or reactance (19; 18.4%) at 5 Hz were pathological in 20% of children compared to Finnish population-based height-adjusted reference values. Increased bronchial reactivity by exercise challenge (5; 4.9%) or bronchodilatation (11; 10.7%) tests was present in 16%. Irreversible changes were revealed in only one case. Conclusions: Though reduced lung function and increased airway reactivity were rather common, evidence for persistent lung function reduction was rare, less than 1%, at preschool age in children hospitalized for bronchiolitis caused mainly by respiratory syncytial virus at age less than 6 months. **Pediatr Pulmonol.**

Key words: pulmonary function testing; bronchial hyper-reactivity; viral bronchiolitis; asthma & early wheeze.

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INTRODUCTION

An increased risk of asthma in childhood and even in adulthood after bronchiolitis in infancy is well-documented.[1–3] In addition, increasing evidence has been accumulated that bronchiolitis in early life is associated with reduced lung function and increased airway reactivity at school age [4–6] and even in adulthood.[7,8] However, less data are available from lung function in preschool-aged children, mainly due to difficulties in measuring lung function at that age. For example, measurements with flow–volume spirometry require maximal blows to get forced expiratory flows necessary for diagnostic interpretations.

We have prospectively followed-up until the age of 5–7 years 166 children, who were hospitalized for bronchiolitis at less than 6 months of age.[9] Out of them, 127 attended the clinical follow-up study at the mean age of 6.3 years. Current asthma was present in 21 (12.7%) children: in 8.2% of the 110 former respiratory syncytial virus (RSV) versus in 24% of the former non-RSV patients. Atopic dermatitis, non-RSV bronchiolitis, and maternal asthma were independently significant early-life risk factors for asthma in adjusted analyses.[9]

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Impulse oscillometry (IOS), an application of the forced oscillation technique,[10] is a quite recently introduced promising method for lung function measurement during tidal breathing.[11,12] The method has been successfully used from the age of 2–3 years onwards. Population-based reference values are available for Finnish 2–7 years old children.[13]

At the post-bronchiolitis control visit at the mean age of 6.3 years, lung function was studied with IOS in those who were less than 7 years old. Both baseline, post-exercise, and post-bronchodilator values were measured. There are no previous studies focusing on lung function at preschool age in children with bronchiolitis at less than 6 months of age. Likewise, an application of forced oscillations has been used in only two post-bronchiolitis follow-up studies before school age.[14,15]

The purpose of the present study was to evaluate lung function, with a special focus on airway resistance, reactance, and reactivity to exercise by IOS at the age of 5–7 years in children hospitalized for bronchiolitis at less than 6 months of age, and to study if there are early-life factors involving with later lung function. Our hypothesis was that airway resistance and airway reactivity may be increased, and airway reactance decreased in the former bronchiolitis patients compared to the Finnish population-based reference values.

MATERIALS AND METHODS

Design and Background Factors

In all, 187 healthy, full-term infants were hospitalized for bronchiolitis at less than 6 months of age in Tampere University Children's Hospital, between December 1, 2001, and May 31, 2002, and between October 28, 2002, and May 31, 2004. Bronchiolitis was defined as lower respiratory tract infection with rhinitis, cough, and diffuse wheezes or crackles. The aetiology of bronchiolitis was assessed in nasopharyngeal aspirates by immunofluorescence for seven viruses including RSV and by polymerase chain reaction (PCR) for nine viruses, including RSV and rhinoviruses, and in addition, by PCR for the bacterium *Bordetella pertussis*.[16]

In 2008–2009, 127 children attended a clinical control visit and 39 were interviewed by phone at the mean age of 6.5 years, as published recently.[9]

Lung function was measured by IOS in 107 children who were less than 7 years old. Four children were excluded; two refused, one child was studied with a different IOS device than others, and in one case, other data were missing. Thus, altogether 103 children form the subjects of the present IOS study.

Impulse Oscillometry

IOS is measured during quiet tidal breathing when the child is sitting still at upright position. The nose is sealed with a clip and the cheeks are supported with hands by the technician to minimize pressure loss through the upper airway shunt. The input oscillatory signal containing harmonics at 5–35 Hz is conducted into the airways, and output pressure, and flow signals are analyzed for their amplitude and phase differences to determine the resistance (Rrs) and reactance (Xrs) of the airways, both being components of the total airway impedance (Zrs).[12,17]

Both Rrs and Xrs are dependent on height of the child and frequency of the signal. Low frequencies (<15 Hz) are considered to describe the small, peripheral airways, and higher frequencies (>20 Hz) the larger, more central airway.[18] In proximal or peripheral obstruction, Rrs increases above normal values. In peripheral obstruction, as in asthma, Rrs tends to rise in low frequencies; the frequency dependency of resistance strengthens and dRs/df shows more negative values. Xrs decreases as a result of peripheral restriction due to fibrosis or hyperinflation.[17] Resonant frequency (Fres) represents the point where the Xrs equals zero and increases both in restrictive and obstructive conditions.[17]

Measurements

At baseline measurements, IOS (Master Screen IOS; Jaeger, Hochberg, Germany) was repeated until three acceptable IOS curves were obtained. The IOS results were pre-analyzed by an experienced clinical physiologist (JT), and the IOS curves had to be graphically appropriate and free from artefacts for the whole 30 sec measurement time. The curves with the best coherence (threshold >0.6 at 5 Hz and >0.9 at 10 Hz) [19] were obtained.

After baseline measurements, the children performed exercise-challenge test (ECT) including 8 min free running outdoors at $\geq$90% of expected heart rate (205 minus age divided by 2) for $\geq$2 min using a heart rate monitor (Polar Ltd., Kempele, Finland). Similarly to the baseline measurements, post-exercise IOS curves were measured 5–10 min after running until three representative curves were obtained.

ABBREVIATIONS:

IOS	Impulse oscillometry
RSV	Respiratory syncytial virus
PCR	Polymerase chain reaction
BHR	Bronchial hyper-responsiveness
ECT	Exercise challenge test
SD	Standard deviation
CI	Confidence interval
ICS	Inhaled corticosteroid
Rrs	Resistance
Xrs	Reactance
Fres	Resonant frequency
dRs/df	Frequency dependency of resistance

Thereafter, children received 300 μg salbutamol (Ventoline®, GSK, Brentford, UK) administered by inhalation through a spacer (Babyhaler®, GSK, Brentford, UK), and again three good quality IOS curves were collected 15 min after the inhalation. These values are used as post-bronchodilator values. This modification of the bronchodilatation test allowed the completion of the study protocol for each child in 1 day.

Thus, baseline (pre-exercise), post-exercise, and post-bronchodilator values were measured, and among them Rrs, Zrs, and Xrs at 5 and 20 Hz, Fres and dRrs/df were recorded.

Since national, height-adjusted reference values were available,[13] the measured mean crude values were transformed using a prediction equation [13] to standard residuals (z-score) describing the deviation from the height-adjusted predicted values. For the standard residual (z-score), the upper reference limit (95th percentile) is at +1.65, and the lower reference limit (5th percentile), respectively, at −1.65.

Bronchial reactivity was studied calculating exercise-induced changes and bronchodilator-induced changes in Zrs, Rrs, and Xrs at 5 Hz, and resonant frequency z-score changes.

Rrs at 5 Hz was categorized as pathological if exercise-induced change in mean crude values was +35% or more,[20] or if bronchodilator-induced change in mean crude values was −35% or more.[19,20]

Statistics

SPSS® Statistical Package Version 21 (IBM®, Helsinki, Finland) was used in the statistical analyses of the data. The results are expressed as means, standard deviations (SD), and 95% confidence intervals (95% CI) for continuous variables, and numbers, and frequencies for categorized variables. Analysis of variance, adjusted for maternal asthma, atopic eczema in infancy, and RSV etiology of bronchiolitis during hospitalization when appropriate, was used in the analyses of continuous and Chi square and Fisher's exact tests in the analyses of categorized data.

Ethics

The Ethics Committee of the Tampere University Hospital District approved the study. Informed consent was obtained from parents both before enrolling the children in infancy and at the clinical control visit.

RESULTS

Background Factors

The mean age of the study subjects was 6.3 years (5.5–7.0 years), and 49.5% were boys. Thirteen (12.6%) study subjects were on continuous medication for asthma with ICS. The aetiology of bronchiolitis in infancy had been RSV in 60.2%. There were 28 former RSV A and 34 former RSV B cases. Over half (52.4%) had been exposed to parental smoking during infancy and 20.6% to maternal smoking during pregnancy (Table 1).

Lung Function

When baseline and post-exercise values were compared, there were no significant differences (Table 2). When baseline and post-bronchodilator values were compared, post-bronchodilator values were significantly better than baseline values (Table 2). The results were similar irrespective whether expressed as absolute or relative values.

Post-exercise Xrs at 5 Hz was lower in boys (−1.39, SD 1.64; $P = 0.03$) than in girls (−0.78, 1.16). There were no significant differences in IOS parameters between boys and girls in baseline or post-bronchodilator values (data not shown).

There were no significant differences in baseline, post-exercise, or post-bronchodilator IOS parameters between the former RSV positive and negative patients (data not shown). Likewise, there were no significant differences between former RSV A and RSV B cases. There were no significant differences in baseline, post-exercise, or post-bronchodilator IOS parameters between the former rhinovirus positive and negative patients (data not shown). The post-exercise Rrs at 20 Hz was lower in the former rhinovirus positive (mean −1.43, SD 1.73; $P = 0.04$) than in the former RSV positive cases (−0.72, 0.97).

There were no significant associations between tobacco smoke exposure during pregnancy or during infancy and baseline, post-exercise, or post-bronchodilator IOS parameters (data not shown).

When the 13 former bronchiolitis patients who had used and those 90 who had not used ICS regularly

TABLE 1— Characteristics of Study Population

No. of subjects	103
Male, n (%)	51 (49.5)
Female, n (%)	52 (50.5)
Age (y), mean (range)	6.3 (5.5–7.0)
Height (cm), mean (range)	120 (107–136)
Weight (kg), mean (range)	24 (16–39)
RSV-bronchiolitis, n (%)	62 (60.2)
Non-RSV-bronchiolitis, n (%)	41 (39.8)
Human rhinovirus-bronchiolitis, n (%)	16 (15.5)
Maternal smoking during pregnancy, n (%)	19 (20.6)
Parental smoking during infancy (<12 months age), n (%)	54 (52.4)
Regular use of ICS during preceding 12 months, n (%)	13 (12.6)

ICS, inhaled corticosteroid; RSV, respiratory syncytial virus.

TABLE 2— Baseline, Post-Exercise and Post-Bronchodilator Impulse Oscillometry Results as Relative (*z*-Scores) and Absolute (kPa/L/sec) Values in 103 Former Infantile Bronchiolitis Patients at the Mean Age of 6.3 years

	Baseline values			Post-exercise values			Post-bronchodilator values		
Parameter	Mean	95% CI	Range	Mean	95% CI	Range	Mean	95% CI	Range
(*z*-scores)									
Zrs 5 Hz	−0.01	−0.23, 0.22	−2.45, 2.68	0.27	0.03, 0.52	−2.64, 3.38	−1.75	−1.96, −1.56	−4.48, 1.23
Rrs 5 Hz	−0.09	−0.29, 0.12	−2.43, 2.14	0.16	−0.06, 0.39	−2.60, 3.08	−1.72	−1.91, −1.53	−4.43, 1.24
Rrs 20 Hz	−0.94	−1.16, −0.72	−3.95, 1.45	−0.91	−1.14, −0.68	−4.67, 1.75	−2.01	−2.23, −1.78	−4.78, 1.79
Xrs 5 Hz	−0.74	−0.98, −0.50	−6.26, 2.00	−1.08	−1.37, −0.80	−5.79, 1.48	0.33	0.20, 0.47	−1.71, 2.05
Fres	2.23	2.07, 2.40	−1.17, 4.08	2.38	2.21, 2.55	−0.64, 4.56	0.87	0.68, 1.07	−1.92, 2.76
dRrs/df	−1.02	−1.23, −0.80	−4.51, 1.09	−1.35	−1.62, −1.09	−5.65, 0.97	0.04	−0.10, 0.19	−3.12, 1.56
(kPa/L/sec)									
Zrs 5 Hz	0.81	0.78, 0.85	0.49, 1.31	0.85	0.82, 0.89	0.48, 1.34	0.63	0.60, 0.65	0.35, 0.95
Rrs 5 Hz	0.77	0.74, 0.80	0.45, 1.22	0.80	0.77, 0.83	0.45, 1.26	0.60	0.58, 0.62	0.33, 0.92
Rrs 20 Hz	0.58	0.56, 0.61	0.32, 0.91	0.59	0.56, 0.61	0.31, 0.89	0.50	0.48, 0.52	0.28, 0.85
Xrs 5 Hz	−0.26	−0.27, −0.24	−0.61, −0.10	−0.28	−0.30, −0.26	−0.55, −0.12	−0.19	−0.20, −0.18	−0.35, −0.10
Fres	21.9	21.2, 22.6	9.84, 30.9	22.6	21.8, 23.5	11.0, 33.6	16.7	16.1, 17.4	8.44, 24.7
dRs/df	−0.01	−0.01, −0.01	−0.03, 0.00	−0.01	−0.02, −0.01	−0.04, 0.00	−0.01	−0.01, −0.01	−0.02, 0.00

Zrs, total impedance; Rrs, resistance; Xrs, reactance; Fres, resonant frequency; dRrs/df, frequency dependency of resistance.

TABLE 3— Post-Exercise IOS Values (*z*-Scores) and Exercise Induced Changes (*z*-Scores) in the 13 Former Bronchiolitis Patients Who Have Used ICS Regularly During the Preceding 12 months, Compared to Those 90 Who Have Not Used

Parameter	Regular ICS use mean (SD)	*P* value*	No regular ICS use mean (SD)
Zrs 5 Hz	1.20 (1.39)	<0.01	0.14 (1.09)
Rrs 5 Hz	0.92 (1.22)	<0.01	0.05 (1.11)
Rrs 20 Hz	−0.91 (1.21)	0.58	−0.89 (1.00)
Xrs 5 Hz	−2.36 (1.90)	<0.01	−0.90 (1.28)
Fres	2.94 (0.79)	<0.01	2.30 (0.84)
dRrs/df	−2.52 (1.36)	<0.01	−1.18 (1.29)
ΔZrs 5 Hz	2.40 (3.73)	<0.01	0.40 (1.89)
ΔRrs 5 Hz	1.67 (2.79)	0.01	0.35 (1.59)
ΔXrs 5 Hz	−1.65 (2.19)	<0.01	−0.16 (0.95)
ΔFres	1.96 (2.85)	<0.01	0.26 (1.87)

Zrs, total impedance; Rrs, resistance; Xrs, reactance; Fres, resonant frequency; dRrs/df, frequency dependency of resistance, ICS, inhaled corticosteroid.

*Adjusted for atopy, maternal asthma, and RSV bronchiolitis.

during the preceding 12 months were compared, post-exercise values in all IOS parameters except in Rrs at 20 Hz were significantly higher (and Xrs and dRs/df lower) in the ICS users (Table 3). Instead, there were no significant differences in any baseline or post-bronchodilator IOS parameters between the ICS and non-ICS users.

In baseline measurements, either Rrs at 5 Hz was $\geq +1.65$ or Xrs at 5 Hz was ≤ -1.65 in 20.4% of the children (Table 4) By these criteria, Xrs at 5 Hz picked-up more cases than Rrs at 5 Hz. In the post-bronchodilator measurements, however, there was only one pathological result suggesting persistent lung function deficit.

Bronchial Reactivity

Bronchial reactivity was studied with exercise challenge and bronchodilatation tests, presented as Zrs, Rrs, Xrs at 5 Hz and Fres changes (Table 5).

There were no statistically significant differences between boys and girls, or former RSV positive and negative, former rhinovirus positive and negative, and former RSV positive and rhinovirus positive bronchiolitis patients (data not shown).

There were no statistically significant associations between bronchial reactivity and exposure to parental smoking during infancy (data not shown). However, the bronchodilator-induced change in Xrs at 5 Hz was smaller (mean −0.35, SD 0.87; $P = 0.04$) in children exposed to maternal smoking during pregnancy than in the non-exposed children (0.19, 1.04). Likewise, the 32 children exposed to maternal smoking during infancy had a lower bronchodilator-induced change in Xrs at 5 Hz (mean −0.26, SD 0.82; $P = 0.02$) than the 71 non-exposed children (0.26, 1.08).

Children who had used ICS regularly during 12 months before IOS measurement had larger changes in all four parameters used in the exercise challenge test (Table 3).

TABLE 4—Numbers of Study Subjects With Increased Airway Resistance (Rrs) and Decreased Airway Reactance (Xrs) at 5 Hz in Baseline and Post-Bronchodilator Values (*z*-Scores)

Parameters*	Baseline values, n (%)	Post-bronchodilator values, n (%)
Rrs 5 Hz $\geq+1.65$ (pathologic)	8 (7.8)	0 (0)
Rrs 5 Hz $<+1.65$ (normal)	95 (92.2)	103 (100)
Xrs 5 Hz ≤-1.65 (pathologic)	19 (18.4)	1 (1.0)
Xrs 5 Hz >-1.65 (normal)	84 (81.6)	103 (100)
Rrs 5 Hz or Xrs 5 Hz (pathologic)	21 (20.4)	1 (1.0)
Rrs 5 Hz and Xrs 5 Hz (pathologic)	6 (5.8)	0 (0)

Rrs, resistance; Xrs, reactance.
*In *z*-scores based on the Finnish reference material.[13]

However, there were no statistically significant differences in bronchodilator-induced changes between ICS users and non-users (data not shown).

Increased bronchial reactivity was diagnosed in 15.6% of the cases. Rrs 5 Hz change $\geq$35% in the exercise challenge test was found in 4.9% and in the bronchodilatation test in 10.7% (Table 5), and in both 0%.

DISCUSSION

There are four main results in the present prospective, 5- to 7-year post-bronchiolitis study focused on lung function by impulse oscillometry at preschool age. First, resistance at 5 Hz was $\geq$1.65 or reactance at 5 Hz was ≤-1.65 in *z*-scores compared to Finnish age-specific population-based references in 20% of the former infantile bronchiolitis patients in baseline values, but both were abnormal in only 6%. Post-bronchodilator values were abnormal in only one child. Thus, evidence for persistent lung function deficit was rare, less than 1%. Second, increased bronchial reactivity was diagnosed in 16% of the children. Resistance at 5 Hz increased $\geq$35% in the exercise challenge test in 5% and decreased in the bronchodilatation test in 11% of the children. Third, no significant association was found with RSV or rhinovirus etiology of bronchiolitis in baseline, post-exercise, or post-bronchodilator values, or in bronchial reactivity. Fourth, children exposed to maternal smoking during pregnancy or infancy had lower bronchodilator-induced changes in reactance.

TABLE 5—Exercise- and Bronchodilator-Induced Changes in IOS (*z*-scores) and Categorized Rrs5 Mean Crude Value Change in 103 Former Bronchiolitis Patients at the Mean Age of 6.3 years

Parameter	Exercise-induced change mean (95% CI)	Bronchodilator-induced change mean (95% CI)
ΔZrs 5 Hz	0.66 (0.21, 1.10)	0.10 (−0.08, 0.28)
ΔRrs 5 Hz	0.51 (0.16, 0.87)	0.12 (−0.05, 0.28)
ΔXrs 5 Hz	−0.35 (−0.60, −0.11)	0.09 (−0.11, 0.30)
ΔFres	0.498 (0.07, 0.88)	−0.46 (−0.71, −0.21)
ΔRrs 5 Hz $\geq$35%	5 (4.9%)	11 (10.7%)

Zrs, total impedance; Rrs, resistance; Xrs, reactance; Fres, resonant frequency.

Earlier studies have shown loss of lung function in 23–29% of cases and increased airway reactivity in 13–62% of cases 6–8 years after viral bronchiolitis in infancy.[4–6] The present results are in line with these previous findings obtained with spirometry. In the present study, the post-bronchodilator measurements were done 10 min after the exercise. In this time frame, there obviously is some exercise-induced bronchoconstriction left in the airways, and the post-bronchodilator values are expected to be worse than without preceding exercise. However, all the measured post-bronchodilator values were, on average, significantly better than the baseline values. A persistent reduction of lung function was documented in less than 1% of the cases. Thus, permanent lung function reduction demonstrated after infantile bronchiolitis in youth [7,21] and in young adulthood [22] seems to develop at later school age, though the process obviously has its origin in infancy.[23]

Increased bronchial reactivity was diagnosed at the age of 5–7 years in 16% of the former infantile bronchiolitis patients, though 27% of them had current or earlier asthma.[9] In addition, the average post-exercise values were not lower than baseline values. Bronchial hyperreactivity was assessed by 35% or greater responses in resistance at 5 Hz to exercise and bronchodilator administration. The exercise challenge test picked one-third and the bronchodilatation test two-thirds of the reactive cases. For both tests, five studies have suggested a change of 35–40% in resistance at 5 Hz as diagnostic for asthma. In a study from Finland,[20] 130 wheezy children and 79 non-atopic controls were evaluated at the age of 3–7 years. Wheezing children differed significantly from controls with the lower 95% CI limit for an Rrs 5 Hz increase being 32.5%. A Danish group [24] compared different lung function measurement techniques in 38 asthmatic children and 29 non-asthmatic controls at the age of 2–5 years, and showed that a 37% increase (three SDw, i.e., three weighted SDs) in resistance in cold air challenge separated asthmatic and non-asthmatic children. In a study from Finland, bronchodilator responses were studied in 108 healthy 2–7 years old children.[13] The 95% CI based limit for the change in resistance at 5 Hz was −36.9%. In a study from Belgium, bronchodilator responses were studied in 228 healthy 2–7 years old children.[25] The 95% CI based limit for the changes in resistance at 5 Hz was −41.4%. In a study from Australia, bronchodilator responses were studied in 78 healthy and

asthmatic 2–7 years old children.[26] The asthmatic and non-asthmatic children did not differ significantly. The 95% CI based limit for the change in resistance at 5 Hz was −42%.

The patients exposed to maternal smoking during pregnancy or infancy had lower bronchodilator-induced changes in reactance at 5 Hz, suggesting loss of elastic properties of lung tissue and poorer responses to bronchodilatation. There is increasing evidence suggesting that exposure to maternal smoking during pregnancy and infancy, more than exposure to paternal smoking during infancy, increases bronchial reactivity in childhood.[27,28] One-fifth of the children of the present study had been exposed to maternal smoking during pregnancy and one-third during infancy (31.4%).

In previous studies, bronchiolitis caused by RSV has been associated with both increased airway reactivity and reduced lung function.[6,29] Bronchiolitis caused by rhinoviruses has been associated with increased airway reactivity.[30,31] The present results were not able to confirm these virus-specific associations. On the other hand, the number of cases caused by other than RSV might have been too small to get enough statistical power in the analyses. However, there was a preliminary finding that the post-exercise resistance at 20 Hz, that is, in central airways,[18] was lower in the former rhinovirus positive patients. This may suggest a virus-depended influence on later lung function.

The main strengths of the study are the prospective design, homogeneity of the study group, careful data collection, and long follow-up time. At recruitment during hospitalization due to bronchiolitis, the patients were less than 6 months old, and were thereafter prospectively followed more than 5 years. Viral diagnosis was found in the great majority of cases. Thirteen patients were on regular ICSs for asthma, and the treatment was not interrupted. Post-exercise values in the IOS parameters describing airway resistance were higher and those describing reactance were lower in the ICS users. In addition, ICS users had larger exercise-induced changes. These findings mean that treatment with ICSs did not confound the analyses.

There are some shortcomings in the present study. We did not recruit controls, but instead, national population-based age-specific height-adjusted references were available. We think that the reference values, though obtained from healthy children without a diagnosed atopic disease, represent the Finnish population at this age rather well. Despite this, the lung function and rate of bronchial hyper-reactivity of an unselected age-matched control group without a history of early bronchiolitis is not known. The number of dropouts was rather high. In addition, IOS was not done in those attendants who were more than 7 years old. Technically, IOS succeeded well, and finally, an appropriate result was obtained from 81% of those who attended the clinical study. However, the exclusion of lung function measurements up to 5 min post-exercise may under-estimate bronchoconstriction at pre-school age,[32] which may partly explain our low figures. Bronchodilatation test was performed after the children had performed the exercise challenge test. Despite this, all the post-bronchodilator parameters were, on average, significantly better than the baseline ones. This means that the preceding exercise challenge did not increase the risk of false-positive findings in this modified bronchodilatation test, which allowed the performance of the study in 1 day. Some of the subgroup analyses, mainly concerning viral aetiology of bronchiolitis, were based on a small number of children. Therefore some of the results may be biased by type II error.

In conclusion, lung function deficit was revealed in 20% and increased airway reactivity in 16% by IOS in 5- to 7-year-old children who had been hospitalized for bronchiolitis at less than 6 months of age. No significant association was found with RSV or rhinovirus etiology of bronchiolitis in baseline, post-exercise, or post-bronchodilator values. Children exposed to maternal smoking during pregnancy or infancy had less bronchodilator-induced improvement of reactance, suggesting decreased lung compliance.

REFERENCES

1. Taussig LM, Wright AL, Holberg CJ, Halonen M, Morgan WJ, Martinez FD. Tucson children's respiratory study: 1980 to present. J Allergy Clin Immunol 2003;111:661–675; quiz 676.
2. Stein RT, Sherrill D, Morgan WJ, Holberg CJ, Halonen M, Taussig LM, Wright AL, Martinez FD. Respiratory syncytial virus in early life and risk of wheeze and allergy by age 13 years. Lancet 1999;354:541–545.
3. Piippo-Savolainen E, Korppi M. Wheezy babies–wheezy adults? Review on long-term outcome until adulthood after early childhood wheezing. Acta Paediatr 2008;97:5–11.
4. Korppi M, Kuikka L, Reijonen T, Remes K, Juntunen-Backman K, Launiala K. Bronchial asthma and hyperreactivity after early childhood bronchiolitis or pneumonia. An 8-year follow-up study. Arch Pediatr Adolesc Med 1994;148:1079–1084.
5. Kotaniemi-Syrjanen A, Reijonen TM, Korhonen K, Waris M, Vainionpaa R, Korppi M. Wheezing due to rhinovirus infection in infancy: bronchial hyperresponsiveness at school age. Pediatr Int 2008;50:506–510.
6. Fjaerli HO, Farstad T, Rod G, Ufert GK, Gulbrandsen P, Nakstad B. Acute bronchiolitis in infancy as risk factor for wheezing and reduced pulmonary function by seven years in Akershus county, Norway BMC Pediatr 2005;5:31.
7. Goksor E, Gustafsson PM, Alm B, Amark M, Wennergren G. Reduced airway function in early adulthood among subjects with wheezing disorder before two years of age. Pediatr Pulmonol 2008;43:396–403.
8. Piippo-Savolainen E, Remes S, Kannisto S, Korhonen K, Korppi M. Early predictors for adult asthma and lung function abnormalities in infants hospitalized for bronchiolitis: a prospective 18- to 20-year follow-up. Allergy Asthma Proc 2006;27:341–349.

9. Koponen P, Helminen M, Paassilta M, Luukkaala T, Korppi M. Preschool asthma after bronchiolitis in infancy. Eur Respir J 2012;39:76–80.
10. Dubois AB, Brody AW, Lewis DH, Burgess BF, Jr. Oscillation mechanics of lungs and chest in man. J Appl Physiol 1956;8: 587–594.
11. Marotta A, Klinnert MD, Price MR, Larsen GL, Liu AH. Impulse oscillometry provides an effective measure of lung dysfunction in 4-year-old children at risk for persistent asthma. J Allergy Clin Immunol 2003;112:317–322.
12. Oostveen E, MacLeod D, Lorino H, Farre R, Hantos Z, Desager K, Marchal F, ERS Task Force on Respiratory Impedance Measurements. The forced oscillation technique in clinical practice: methodology, recommendations and future developments. Eur Respir J 2003;22:1026–1041.
13. Malmberg LP, Pelkonen A, Poussa T, Pohianpalo A, Haahtela T, Turpeinen M. Determinants of respiratory system input impedance and bronchodilator response in healthy Finnish preschool children. Clin Physiol Funct Imaging 2002;22:64–71.
14. Stokes GM, Milner AD, Hodges IG, Groggins RC. Lung function abnormalities after acute bronchiolitis. J Pediatr 1981;98:871–874.
15. Henry RL, Milner AD, Stokes GM, Hodges IG, Groggins RC. Lung function after acute bronchiolitis. Arch Dis Child 1983;58:60–63.
16. Nuolivirta K, Koponen P, He Q, Halkosalo A, Korppi M, Vesikari T, Helminen M. Bordetella pertussis infection is common in nonvaccinated infants admitted for bronchiolitis. Pediatr Infect Dis J 2010;29:1013–1015.
17. Smith H, Reinhold P, Goldman M. Chapter 5. Forced oscillation technique and impulse oscillometry. Eur Respir Soc Monogr 2005;31:75–105.
18. Bates JH, Lutchen KR. The interface between measurement and modeling of peripheral lung mechanics. Respir Physiol Neurobiol 2005;148:153–164.
19. Beydon N, Davis SD, Lombardi E, Allen JL, Arets HG, Aurora P, Bisgaard H, Davis GM, Ducharme FM, Eigen H, et al. An Official American Thoracic Society/European Respiratory Society Statement: pulmonary function testing in preschool children. Am J Respir Crit Care Med 2007;175:1304–1345.
20. Malmberg LP, Makela MJ, Mattila PS, Hammaren-Malmi S, Pelkonen AS. Exercise-induced changes in respiratory impedance in young wheezy children and nonatopic controls. Pediatr Pulmonol 2008;43:538–544.
21. Sigurs N, Aljassim F, Kjellman B, Robinson PD, Sigurbergsson F, Bjarnason R, Gustafsson PM. Asthma and allergy patterns over 18 years after severe RSV bronchiolitis in the first year of life. Thorax 2010;65:1045–1052.
22. Stern DA, Morgan WJ, Halonen M, Wright AL, Martinez FD. Wheezing and bronchial hyper-responsiveness in early childhood as predictors of newly diagnosed asthma in early adulthood: a longitudinal birth-cohort study. Lancet 2008;372:1058–1064.
23. Saglani S, Molyneux C, Gong H, Rogers A, Malmstrom K, Pelkonen A, Makela M, Adelroth E, Bush A, Payne DN, et al. Ultrastructure of the reticular basement membrane in asthmatic adults, children and infants. Eur Respir J 2006;28:505–512.
24. Nielsen KG, Bisgaard H. Lung function response to cold air challenge in asthmatic and healthy children of 2–5 years of age. Am J Respir Crit Care Med 2000;161:1805–1809.
25. Hellinckx J, De Boeck K, Bande-Knops J, van der Poel M, Demedts M. Bronchodilator response in 3–6.5 years old healthy and stable asthmatic children. Eur Respir J 1998;12:438–443.
26. Thamrin C, Gangell CL, Udomittipong K, Kusel MM, Patterson H, Fukushima T, Schultz A, Hall GL, Stick SM, Sly PD. Assessment of bronchodilator responsiveness in preschool children using forced oscillations. Thorax 2007;62:814–819.
27. Cook DG, Strachan DP. Parental smoking, bronchial reactivity and peak flow variability in children. Thorax 1998;53:295–301.
28. Goksor E, Amark M, Alm B, Gustafsson PM, Wennergren G. The impact of pre- and post-natal smoke exposure on future asthma and bronchial hyper-responsiveness. Acta Paediatr 2007;96: 1030–1035.
29. Sigurs N, Gustafsson PM, Bjarnason R, Lundberg F, Schmidt S, Sigurbergsson F, Kjellman B. Severe respiratory syncytial virus bronchiolitis in infancy and asthma and allergy at age 13. Am J Respir Crit Care Med 2005;171:137–141.
30. Kotaniemi-Syrjanen A, Vainionpaa R, Reijonen TM, Waris M, Korhonen K, Korppi M. Rhinovirus-induced wheezing in infancy—the first sign of childhood asthma? J Allergy Clin Immunol 2003;111:66–71.
31. Hyvarinen MK, Kotaniemi-Syrjanen A, Reijonen TM, Korhonen K, Korppi MO. Lung function and bronchial hyper-responsiveness 11 years after hospitalization for bronchiolitis. Acta Paediatr 2007;96:1464–1469.
32. Vilozni D, Bentur L, Efrati O, Barak A, Szeinberg A, Shoseyov D, Yahav Y, Augarten A. Exercise challenge test in 3- to 6-year-old asthmatic children. Chest 2007;132:497–503.

Obesity and Bronchial Obstruction in Impulse Oscillometry at Age 5–7 Years in a Prospective Post-Bronchiolitis Cohort

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Summary. Background and Aims: Obesity has been linked with asthma symptoms, need for asthma treatment and reduced lung function but not with increased bronchial reactivity in children. The aim of this study was to evaluate the association between previous or current weight status and current lung function and bronchial reactivity to exercise at early school age. Methods: Ninety-nine children hospitalized for bronchiolitis at the age of less than 6 months were studied with impulse oscillometry (IOS) at the mean age of 6.3 years. Data on birth weight and weight gain in infancy before hospitalization were collected during hospitalization. Current weight and height data were transformed into age- and sex-specific height-related body mass index *z* scores (zBMI) using the Finnish national population-based weight and height data as reference. Results: Some significant though only low or modest correlations were found between current zBMI and baseline, post-exercise and post-bronchodilator IOS values in adjusted linear regression analysis. Seven obese children by zBMI had higher post-bronchodilator airway impedance (Zrs) and resistance (Rrs) at 5 Hz and lower post-bronchodilator frequency dependency of resistance (dRrs/df) than normal weight children. There were no significant differences in responses to exercise or to bronchodilators between currently obese or overweight children and normal weight children. Birth weight less than 3,000 g was associated with larger exercise-induced changes in Zrs and Rrs at 5 Hz, and in reactance (Xrs) at 5 Hz, than those with birth weight more than 3,000 g. Conclusions: Preliminary evidence was found that obesity may be associated with airway obstruction, but not with bronchial hyper-reactivity. **Pediatr Pulmonol.**

Key words: obesity; pulmonary function testing; asthma and early wheeze; impulse oscillometry.

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INTRODUCTION

A simultaneous increase in both childhood obesity and asthma prevalence suggests a link between obesity and pediatric asthma.[1–3] A specific obesity-related asthma phenotype has been identified in adults, with wheezing symptoms and reduced lung function without any prominent bronchial hyper-reactivity as typical features.[4,5] However, few studies support the existence of this phenotype in childhood.[6] Both preceding and current childhood obesity have been associated with asthma symptoms and need for asthma treatment in children with both atopic and nonatopic asthma.[7–9]

In adults, obesity causes changes in pulmonary function with reduced expiratory reserve volume (ERV) and small airways obstruction as typical features.[10] Recent studies on childhood obesity and lung function have suggested a presence of reduced lung function by flow-volume spirometry (FVS) also in school-aged children,[11–14] but the association of obesity with increased airway reactivity has been controversial.[15–17] In a recent study, obese

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children who became obese adults had poorer lung function than those normal weight children who became obese in adulthood.[18] No association with reduced lung function has been found in overweight preschool-aged children,[19] but this age group is poorly studied.

A reliable measurement of lung function is challenging before school age, since the methods like FVS need forced expiratory blows. Impulse oscillometry (IOS)[20] is a noninvasive method for lung function measurement during tidal breathing from the age of 2–3 years onward.[21–23] Population-based reference values have been published for Finnish 2–7 years old children.[24]

In our recent study on lung function by IOS at the mean age of 6.3 years in 107 children who were hospitalized for bronchiolitis at age less than 6 months, 20% had pathological resistance (Rrs) or reactance (Xrs) at 5 Hz, and 16% had bronchial hyper-reactivity documented with diagnostic Rrs at 5 Hz or Xrs at 5 Hz changes in the exercise challenge test (ECT) or bronchodilatation test.[25]

We hypothesized that high or low birth weight, rapid weight gain in infancy, and current overweight or obesity may be associated with reduced lung function at pre-school age after hospitalization for bronchiolitis in infancy. The aim of the present study was to evaluate the association between previous or current weight status and lung function with special focus on airway resistance and reactance, and airway reactivity to exercise and bronchodilator administration, at the mean age of 6.3 years in children who were hospitalized for bronchiolitis at less than 6 months of age.

MATERIALS AND METHODS

As published previously, 187 healthy, full-term infants were hospitalized for bronchiolitis at less than 6 months of age in Tampere University Children's Hospital, between December 1, 2001, and May 31, 2002, and between October 28, 2002, and May 31, 2004.[26] During hospitalization weight and height were measured from all of the 187 children. Birth weight data were obtained from the hospital's register, and weight gain before hospitalization was calculated as gram/week units.

In 2008–2009, 127 children attended the clinical control visit including pulmonary function testing.[27] IOS was measured in 107 children less than 7 years old. Four children were excluded; two refused to run on the exercise test, one measurement was done with a different IOS device, and in one case some other data were missing. Thirteen children (12.6%) had used inhaled corticosteroids (ICSs) regularly for asthma during the preceding 12 months. Data on birth weight, weight gain in infancy before hospitalization, and weight and height at the control visit were available from 100 of these 103 children. One boy was excluded from the analyses due to pathological under weight (zBMI<-2.35 corresponding BMI $<16\,kg/m^2$ in young adults). Thus, 99 children with complete weight and lung function data available form the subjects of the present study.

First, base-line IOS (Master Screen IOS, Jaeger, Hochberg, Germany) measurements were repeated until three acceptable curves were obtained. An experienced clinical physiologist (JT) pre-viewed the curves to be graphically appropriate and free from artifacts for the whole 30 sec time. Coherence threshold was set to >0.6 at 5 Hz and >0.9 at 10 Hz.[28]

Second, the children performed outdoors an 8-min free running ECT at ≥90% of expected heart rate [(205 − age)/2] for ≥ 2 min confirmed with a heart rate monitor (Polar Ltd, Kempele, Finland). The post-exercise IOS values were measured after running.

Thereafter, the children were given albuterol 300 μg (Ventoline®, GSK) inhalation through a spacer (Babyhaler®, GSK) and after 15 min the post-bronchodilator IOS values were measured. This allowed the children to perform the study protocol in 1 day.

Thus, baseline, post-exercise, and post-bronchodilator airway impedance (Zrs), resistance (Rrs) and reactance (Xrs) at 5 Hz, resonant frequency (Fres) and frequency dependency of resistance (dRs/df) were studied and registered.

The measured mean IOS values were transformed into height-adjusted standardized residuals (z scores) using a prediction equation from the Finnish population-based reference material.[24] The upper reference limit for z scores was set to +1.65 (95th percentile), and the lower reference limit, respectively, to −1.65 (5th percentile).

Bronchial reactivity was studied calculating exercise-induced changes and bronchodilator-induced changes in Zrs, Rrs, and Xrs at 5 Hz, and Fres z-score changes. Rrs at 5 Hz was categorized as pathological if exercise-induced change in mean was +35% or more,[29] or if bronchodilator-induced change in mean was −35% or more.[28,29]

ABBREVIATIONS

IOS	impulse oscillometry
FVS	flow-volume spirometry
zBMI	body mass index-for-age z-score
RSV	respiratory syncytial virus
ECT	exercise challenge test
SD	standard deviation
CI	confidence interval
ICS	inhaled corticosteroid
Rrs	resistance
Xrs	reactance
Fres	resonant frequency
dRs/df	frequency dependency of resistance
RV	residual volume
FRC	functional residual capacity
ERV	expiratory reserve volume
FEV_1	forced expiratory volume in one second
FVC	forced vital capacity
$FEF_{25–75\%}$	mean forced expiratory flow at 25–75% of FVC
$MEF_{50\%}$	maximal expiratory flow at 50% of FVC

Current weight and height data were transformed into age- and sex-specific height-related body mass index-for-age z scores (zBMI) using the Finnish national population-based weight and height data as reference.[30] The cut-off value of the zBMI for overweight was 1.16 for girls and 0.78 for boys (corresponding BMI of $>25\,kg/m^2$ in young adults) and for obesity 2.11 for girls and 1.70 for boys (corresponding BMI of $>30\,kg/m^2$ in young adults).

Birth weight limits less than 3,000 g and more than 4,000 g were used in analyses, corresponding the 5th and 95th percentile of birth weight in Finnish newborns.[31] Weight gain before hospitalization was categorized as low <25th percentile, medium 25th–75th percentile and high >75th percentiles.

Statistics

SPSS® Statistical Package Version 21 (IBM®) was used in the statistical analyses of the data. The results are expressed as means, standard deviations (SD), and 95% confidence intervals (95% CI) for continuous variables, and numbers and frequencies for categorized variables. Analysis of variance, adjusted for maternal asthma, atopic eczema in infancy, and maternal smoking during pregnancy and respiratory syncytial virus (RSV) etiology of bronchiolitis during hospitalization when appropriate was used in the analyses of continuous variables, and Chi square and Fisher's exact tests in the analyses of categorized variables. Multivariate linear regression was used to study the correlation between weight and IOS parameters, and the results are expressed as Pearson's R, Pearson's R^2, and adjusted p. R (R^2) more than 0.60 (>0.36) or less than −0.60 (>0.36) means a strong correlation, between 0.3 and 0.6 or between −0.3 and −0.6 (0.09–0.36) a modest correlation, and between 0 and 0.3 (<0.09) or 0 and −0.3 (<0.09) a low correlation.

Ethics

The Ethics Committee of the Tampere University Hospital District approved the study. Informed consent was obtained from parents both before enrolling the children in infancy and at the clinical control visit.

RESULTS

Background Factors

The mean age of the study subjects was 6.3 years (5.5–7.0), and 51.5% were girls. The mean zBMI at the control visit was 0.39 (−2.2 to 2.4), and 22 (22%) of the study subjects were overweight and 7 (7%) obese (Table 1).

Lung Function

The mean (SD) values of the basic, post-exercise, and post-bronchodilator (post-BD) IOS are presented in Table 2. There were no significant differences in any parameter between overweight and normal weight subjects (data not shown). In crude analyses, post-BD dRrs/df was marginally lower in the overweight than normal weight subjects (−0.18 vs. 0.14, $P = 0.05$), but statistical significance was lost in analyses adjusted for asthma in mothers, atopic eczema in children and RSV etiology of bronchiolitis in infancy.

TABLE 1— Characteristics of Study Population

No. of subjects	99
Male, n (%)	48 (48.5)
Female, n (%)	51 (51.5)
Age (years), mean (median; range)	6.3 (6.3; 5.5–7.0)
Height (cm), mean (range)	120 (107–136)
Weight (kg), mean (median; range)	24 (24; 16–39)
zBMI (z score), mean (range)	0.39 (−2.2 to 2.5)
Overweight by zBMI, n (%)	22 (22.2)
Obese by zBMI, n (%)	7 (7.1)
Birth weight (kg), mean (range)	3.5 (2.3–4.6)
Age at hospitalization (weeks), mean (range)	11 (1–24)
Weight at hospitalization (kg), mean (range)	5.5 (2.8–9.7)
Weight gain before hospitalization (g/week), mean (range)	190 (−150 to 580)

zBMI, body mass index-for-age z-score.

When obese children were compared with normal weight or overweight children, there were no significant differences in baseline or post-exercise IOS parameters (data not shown). Instead, post-BD Zrs at 5 Hz (−0.94 vs. −1.84, adjusted $P = 0.03$) and Rrs at 5 Hz (−0.87 vs. −1.80, adjusted $P = 0.02$) were significantly higher, and dRrs/df (−0.49 vs. 0.09, adjusted $P = 0.04$) was significantly lower in obese than in normal weight and/or overweight children (Table 3). The findings in overweight children were between those in normal weight and obese children, but closer to the findings in normal weight children.

There were no statistically significant differences between birth weight and basic, post-exercise, or post-BD IOS values (data not shown). Likewise weekly weight gain before hospitalization for bronchiolitis at less than 6 months of age did not show any significant associations with lung function parameters in crude (data not shown) or in adjusted (Table 4) analyses.

As seen in Table 2, linear regression analysis revealed statistically significant but only low or modest correlations between zBMI and basic Zrs at 5 Hz ($R = 0.32$), Rrs at 5 Hz ($R = 0.33$), Fres ($R = 0.24$), and dRs/df ($R = -0.29$). Likewise, there was a significant but low correlation between post-exercise Zrs at 5 Hz ($R = 0.27$), Rrs at 5 Hz ($R = 0.28$), Xrs at 5 Hz ($R = -0.21$), and dRs/df ($R = -0.29$). Further, there was a significant but low correlation between post-bronchodilator Rrs at 5 Hz ($R = 0.22$), Fres ($R = 0.23$), and dRs/df ($R = -0.27$) (Table 2). Birth weight <3,000 g had a low though statistically significant correlation with Zrs ($R = 0.25$,

TABLE 2— Linear Regression Analysis: IOS Values (*z* Scores) Versus zBMI

IOS parameter	Mean[a] (SD)	Pearson R	Pearson R^2	Crude *P*	Adjusted *P*[b]
Basic values					
Zrs5Hz	−0.03 (1.15)	0.32	0.10	0.01	0.02
Rrs5Hz	−0.10 (1.06)	0.33	0.11	0.01	0.01
Rrs20Hz	−0.95 (1.14)	0.27	0.08	0.55	0.58
Xrs5Hz	−0.70 (1.21)	−0.14	0.02	0.17	0.21
Fres	2.22 (0.83)	0.24	0.06	0.03	0.03
dRs/df	−1.00 (1.11)	−0.29	0.08	<0.01	<0.01
Post-exercise values					
Zrs5Hz	0.26 (1.27)	0.27	0.07	0.02	0.02
Rrs5Hz	0.16 (1.16)	0.28	0.08	0.02	0.02
Rrs20Hz	−0.91 (1.20)	0.29	0.08	0.63	0.70
Xrs5Hz	−1.05 (1.45)	−0.21	0.04	0.05	<0.05
Fres	2.37 (0.87)	0.20	0.04	0.08	0.08
dRs/df	−1.34 (1.37)	−0.29	0.08	0.01	<0.01
Post-BD values					
Zrs5Hz	−1.77 (1.00)	0.20	0.04	0.06	0.07
Rrs5Hz	−1.73 (0.99)	0.22	0.05	0.04	<0.05
Rrs20Hz	−2.02 (1.18)	0.20	0.04	0.45	0.53
Xrs5Hz	0.35 (0.65)	−0.13	0.02	0.23	0.26
Fres	0.85 (0.99)	0.23	0.05	0.04	0.04
dRs/df	0.05 (0.75)	−0.27	0.07	0.04	0.03

Zrs, total impedance; Rrs, resistance; Xrs, reactance; Fres, resonant frequency; dRrs/df, frequency dependency of resistance.
[a]Basic, post-exercise, and post-BD values (mean, 95%CI) have been published recently.[25] The minor differences are due to differences in the numbers of cases, 103 in previous and 99 in present analyses.
[b]Adjusted for atopic dermatitis, maternal asthma, and RSV-bronchiolitis.

adjusted $P < 0.01$), Rrs (R = 0.24, adjusted $P = 0.01$), and Xrs (R = −0.24, adjusted $P = 0.01$).

Bronchial Reactivity

Bronchial reactivity was assessed by ECT and bronchodilation test. There were no significant differences in changes of Zrs, Rrs, or Xrs at 5 Hz or Fres between the 29 overweight or obese study subjects compared with 70 normal weight subjects, nor between the 7 obese study subjects compared with normal weight subjects or compared with 92 normal weight and overweight subjects (data not shown).

In adjusted analyses, 17 children with birth weight <3,000 g had larger Zrs (1.95 vs. 0.41, $P < 0.01$), Rrs (1.50 vs. 0.33, $P = 0.01$), and Xrs (−1.03 vs. −0.23, $P = 0.01$) mean changes at 5 Hz in the ECT compared to 82 children with birth weight ≥3,000 g (Table 5).

The 13 children with birth weight more than 4,000 g did not differ significantly from those 86 with birth weight 4,000 g or less in either crude or adjusted analyses (data not shown). Likewise, weight gain in infancy had no

TABLE 3— Post-BD IOS Values (*z* Scores) as Categorized in Obese, Overweight, Normal Weight, and Non-Obese

Parameter	Obese (n = 7); mean (SD)	Overweight (obese not included) (n = 22); mean (SD)	Normal weight (n = 70); mean (SD)	Non-obese (normal weight or overweight) (n = 92); mean (SD)	Crude *P*-values (obese vs. non-obese)	Adjusted *P*-values[1] (obese vs. non-obese)
Zrs5Hz	−0.94 (1.12)	−1.74 (1.34)	−1.86 (0.84)	−1.84 (0.97)	0.02	0.03[2]
Rrs5Hz	−0.87 (1.05)	−1.69 (1.32)	−1.83 (0.82)	−1.80 (0.95)	0.02	0.02[3]
Rrs20Hz	−1.37 (0.87)	−2.14 (1.69)	−2.04 (1.00)	−2.07 (1.19)	0.14	0.18
Xrs5Hz	0.10 (0.70)	0.26 (0.69)	0.41 (0.63)	0.37 (0.64)	0.28	0.28
Fres	1.38 (0.86)	0.98 (1.05)	0.75 (0.98)	0.81 (0.99)	0.14	0.14
dRrs/df	−0.49 (0.55)	−0.09 (0.76)	0.14 (0.75)	0.09 (0.75)	0.05	0.04[4]

Zrs, total impedance; Rrs, resistance; Xrs, reactance; Fres, resonant frequency; dRrs/df, frequency dependency of resistance.
[1]Adjusted for atopy, maternal asthma, and RSV-bronchiolitis.
[2]$P = 0.01$.
[3]$P = 0.01$.
[4]$P = 0.03$ obese vs. normal weight.

TABLE 4— Basic, Post-Exercise, and Post-BD IOS Values (*z* Scores) Versus Weekly Weight Gain Before Hospitalization in Categorized <25th, 25th–75th, and >75th Percentiles

IOS parameter	Weight gain <25th percentile, mean (SD)	Weight gain 25th–75th percentile, mean (SD)	Weight gain >75th percentile, mean (SD)	Crude *P*-value	Adjusted *P*-value[a]
Basic values					
Zrs5Hz	−0.10 (0.92)	0.01 (1.28)	−0.01 (1.12)	0.94	0.99
Rrs5Hz	−0.14 (0.86)	−0.07 (1.17)	−0.11 (1.05)	0.96	0.99
Rrs20Hz	−0.94 (0.90)	−0.97 (1.20)	−0.94 (1.13)	0.99	0.99
Xrs5Hz	−0.57 (0.90)	−0.77 (1.38)	−0.71 (1.14)	0.79	0.95
Fres	2.14 (0.79)	2.25 (0.95)	2.23 (0.59)	0.85	0.88
dRs/df	−0.94 (1.19)	−1.06 (1.13)	−0.96 (1.01)	0.89	0.95
Post-exercise values					
Zrs5Hz	0.27 (1.08)	0.25 (1.41)	0.29 (1.19)	0.99	0.98
Rrs5Hz	0.21 (1.01)	0.13 (1.27)	0.16 (1.11)	0.96	0.99
Rrs20Hz	−0.64 (0.99)	−1.05 (1.34)	−0.92 (1.10)	0.37	0.48
Xrs5Hz	−0.85 (1.07)	−1.14 (1.67)	−1.05 (1.45)	0.73	0.58
Fres	2.28 (0.72)	2.41 (1.04)	2.39 (1.37)	0.83	0.63
dRs/df	−1.15 (1.28)	−1.41 (1.47)	−1.40 (1.28)	0.73	0.70
Post-BD values					
Zrs5Hz	−1.70 (0.70)	−1.70 (1.15)	−2.00 (0.97)	0.44	0.49
Rrs5Hz	−1.64 (0.69)	−1.64 (1.10)	−2.00 (1.00)	0.29	0.33
Rrs20Hz	−1.87 (0.85)	−2.00 (1.31)	−2.22 (1.21)	0.58	0.53
Xrs5Hz	0.44 (0.53)	0.33 (0.71)	0.33 (0.64)	0.77	0.84
Fres	0.66 (1.09)	0.93 (1.02)	0.89 (0.84)	0.54	0.60
dRs/df	0.07 (0.75)	−0.03 (0.82)	0.18 (0.61)	0.51	0.72

Zrs, total impedance; Rrs, resistance; Xrs, reactance; Fres, resonant frequency; dRrs/df, frequency dependency of resistance.
[a]Adjusted for atopic dermatitis, maternal asthma, smoking during pregnancy, RSV-bronchiolitis, and birth weight <3,000 g.

significant associations with changes in any IOS parameter in the ECT or in the bronchodilatation test in crude or adjusted linear regression analyses (data not shown).

DISCUSSION

There are three main results in the present prospective 5- to 7-year post-bronchiolitis follow-up study, focusing on the effect of overweight and obesity on lung function by IOS. First, preliminary evidence was found that obesity may be associated with lung function at preschool age in former bronchiolitis patients. Obese children had increased airway resistance and lower frequency dependency compared to normal weight and overweight children in post-BD measurements, suggesting irreversible structural changes in their airways. In addition, statistically significant though only low or modest correlations were found between obesity and baseline, post-exercise, and post-BD IOS parameters in adjusted linear regression analysis. Second, no significant association was found between bronchial reactivity and current weight status. Third, low birth weight less than 3,000 g was associated with larger changes in airway resistance and reactance in the ECT. Otherwise, birth weight or weight gain in infancy was not associated with lung function or bronchial reactivity measured with IOS at the mean age of 6.3 years in this post-bronchiolitis cohort.

Previous pulmonary function and bronchial hyperreactivity studies focusing on obesity have been mainly performed using FVS in school-aged children. Such study from Greece in 6- to 11-year-old children detected that

TABLE 5— Exercise-Induced Changes in IOS *z* Scores in 17 Children With Birth Weight <3,000 g Compared to 82 Children With Birth Weight ≥3,000 g

Parameter	Birth weight <3,000 g, mean (SD)	Birth weight ≥3,000 g, mean (SD)	Adjusted *P*-value[a]
ΔZrs5Hz	1.95 (2.96)	0.41 (2.09)	<0.01
ΔRrs5Hz	1.50 (2.15)	0.33 (1.73)	0.01
ΔXrs5Hz	−1.03 (1.89)	−0.22 (1.07)	0.01
ΔFres	1.30 (2.91)	0.35 (1.88)	0.10

Zrs, total impedance; Rrs, resistance; Xrs, reactance; Fres, resonant frequency.
[a]Adjusted for atopic dermatitis, maternal asthma, maternal smoking during pregnancy, RSV-bronchiolitis, and weight gain in infancy.

overweight and obesity were associated with reduced lung function parameters forced expiratory volume in one second (FEV_1), forced vital capacity (FVC), FEV_1/FVC ratio, and mean forced expiratory flow at 25–75% of FVC ($FEF_{25-75\%}$).[12] A Canadian retrospective cohort study of 327 children revealed that increasing BMI was associated with decreasing tidal volumes residual volume (RV), functional residual capacity (FRC), and ERV and absolute FEV_1/FVC-ratio by FVS in 8- to 15-year-old children.[14] A random population sample study of Australian children aged 9, 12, and 15 years found that FEV1 and FVC decreased with increasing proportion of body fat.[11] In a Finnish follow-up study, children hospitalized for wheezing associated infection at age <24 months had obesity-related reduced FEV_1/FVC and maximal expiratory flow at 50% of FVC ($MEF_{50\%}$) at age 7 years.[13] However, the only study performed with IOS at age of 6 years in an unselected population did not show any significant reduction in baseline lung function with increasing BMI.[19] Our results, however, give preliminary evidence that obesity-related lung function deficit may be present already at preschool age.

In an Israeli cohort study, wheezing, asthma, and other chest symptoms were more common in obese than in non-obese children, but obesity was not associated with bronchial hyper-reactivity in the bronchodilatation test.[16] In line, there was no significant association between high BMI and bronchial hyper-reactivity to inhaled histamin in Australian children aged 7–12 years, although cough and wheezing were more reported with increasing weight.[15] However, in a prospective study of 275 children aged 8 years, current high BMI was associated with increased reactivity in metacholine inhalation test, and the association was lost if earlier overweight had normalized before the age of 6–7 years.[17] Thus, our negative result on the link between obesity and bronchial reactivity at the mean age of 6.3 years in former bronchiolitis patients is in line with previous observations in populations of school-aged children.

In this study, the 17 children with birth weight less than 3,000 g presented with an increased bronchial reactivity to exercise at preschool age. The children born as preterm were excluded before entering the study, and the finding was robust to adjustments with maternal smoking during pregnancy and weight gain in infancy. A recent literature review[32] stated that studies on the association between birth weight and childhood asthma have given inconsistent results, but some evidence exists for the link between reduced birth weight, rapid post-natal weight gain and later asthma. In accordance, rapid weight gain in the fist three months of life was associated with recurrent wheezing and lower baseline FEV_1 and $FEF_{25-75\%}$ in 5-year old Dutch children, but the finding was not dependent on birth weight.[33] In the present study, instead, weight gain before hospitalization for bronchiolitis at age under 6 months had no significant associations with later lung function or bronchial reactivity. Thus, the found association between low birth weight and increased bronchial reactivity cannot be explained by prematurity or by more weight gain during the first months of life. Since low birth weight is associated with the risk of severe bronchiolitis,[34,35] our finding may reflect the fact that all study subjects were hospitalized for bronchiolitis in infancy.

The strengths of this post-bronchiolitis study are the prospective design with a follow-up time more than 5 years, homogeneity of the study group and careful data collection in infancy and at the mean age of 6.3 years. For example, weight and height were measured and registered both during hospitalization in infancy and at the current control visit in 93% of cases. The association between obesity and lung function at pre-school age is poorly studied, though increasing evidence from measurements with spirometry suggests that obesity is associated with reduced lung function at school age.[11–14] Furthermore, studies on bronchial reactivity versus earlier or current weight status are totally lacking at preschool age.

The lack of controls is a clear shortcoming of the study. On the other hand, new national population-based height-adjusted weight references separately for boys and girls were available[30] increasing the accuracy of the observations. IOS was not measured in the attendants who were over 7 years of age, which decreased the number of study subjects but added the homogeneity of the group. Data on maternal weight, educational level or postnatal feeding habits were not collected, but maternal asthma and smoking during pregnancy were included in the analyses as confounding factors. Similarly, viral etiology of bronchiolitis was included in the multivariate models, though no association was found between viral etiology and later pulmonary function. The subgroup of obese children (7%) was small with a risk of the type II error. Our post-bronchiolitis study group was without any doubt selected but represents a risk group for pulmonary illnesses and lung function disorders which, therefore, especially should avoid excessive weight gain.

In conclusion, our IOS study provided preliminary evidence that current obesity at age of 5–7 years in children hospitalized for bronchiolitis at age of 0–6 months may be associated with bronchial obstruction, including irreversible obstruction after bronchodilator administration. No significant association was found between current overweight or obesity and airway reactivity to exercise or in the bronchodilatation test. Birth weight less than 3,000 g, but not rapid weight gain in early infancy, was associated with increased airway reactivity to exercise.

REFERENCES

1. Shore SA, Johnston RA. Obesity and asthma. Pharmacol Ther 2006;110:83–102.

2. Ford ES. The epidemiology of obesity and asthma. J Allergy Clin Immunol 2005;115:897–909; quiz910.
3. Beuther DA, Sutherland ER. Overweight, obesity, and incident asthma: a meta-analysis of prospective epidemiologic studies. Am J Respir Crit Care Med 2007;175:661–666.
4. Schachter LM, Salome CM, Peat JK, Woolcock AJ. Obesity is a risk for asthma and wheeze but not airway hyperresponsiveness. Thorax 2001;56:4–8.
5. Moore WC, Meyers DA, Wenzel SE, Teague WG, Li H, Li X, D'Agostino R Jr, Castro M, Curran-Everett D, Fitzpatrick AM, et al. Identification of asthma phenotypes using cluster analysis in the severe asthma research program. Am J Respir Crit Care Med 2010;181:315–323.
6. Papoutsakis C, Priftis KN, Drakouli M, Prifti S, Konstantaki E, Chondronikola M, Antonogeorgos G, Matziou V. Childhood overweight/obesity and asthma: is there a link? A systematic review of recent epidemiologic evidence. J Acad Nutr Diet 2013;113:77–105.
7. Holguin F, Bleecker ER, Busse WW, Calhoun WJ, Castro M, Erzurum SC, Fitzpatrick AM, Gaston B, Israel E, Jarjour NN, et al. Obesity and asthma: an association modified by age of asthma onset. J Allergy Clin Immunol 2011;127:1486–1493.e2.
8. Visness CM, London SJ, Daniels JL, Kaufman JS, Yeatts KB, Siega-Riz AM, Calatroni A, Zeldin DC. Association of childhood obesity with atopic and nonatopic asthma: results from the national health and nutrition examination survey 1999–2006. J Asthma 2010;47:822–829.
9. Rasmussen F, Hancox RJ. Mechanisms of obesity in asthma. Curr Opin Allergy Clin Immunol 2014;14:35–43.
10. Littleton SW. Impact of obesity on respiratory function. Respirology 2012;17:43–49.
11. Lazarus R, Colditz G, Berkey CS, Speizer FE. Effects of body fat on ventilatory function in children and adolescents: cross-sectional findings from a random population sample of school children. Pediatr Pulmonol 1997;24:187–194.
12. Spathopoulos D, Paraskakis E, Trypsianis G, Tsalkidis A, Arvanitidou V, Emporiadou M, Bouros D, Chatzimichael A. The effect of obesity on pulmonary lung function of school aged children in Greece. Pediatr Pulmonol 2009;44:273–280.
13. Sidoroff V, Hyvarinen M, Piippo-Savolainen E, Korppi M. Lung function and overweight in school aged children after early childhood wheezing. Pediatr Pulmonol 2011;46:435–441.
14. Davidson WJ, Mackenzie-Rife KA, Witmans MB, Montgomery MD, Ball GD, Egbogah S, Eves ND. Obesity negatively impacts lung function in children and adolescents. Pediatr Pulmonol 2013; doi: 10.1002/ppul.22915 [Epub ahead of print].
15. Schachter LM, Peat JK, Salome CM. Asthma and atopy in overweight children. Thorax 2003;58:1031–1035.
16. Bibi H, Shoseyov D, Feigenbaum D, Genis M, Friger M, Peled R, Sharff S. The relationship between asthma and obesity in children: is it real or a case of over diagnosis? J Asthma 2004;41:403–410.
17. Scholtens S, Wijga AH, Seidell JC, Brunekreef B, de Jongste JC, Gehring U, Postma DS, Kerkhof M, Smit HA. Overweight and changes in weight status during childhood in relation to asthma symptoms at 8 years of age. J Allergy Clin Immunol 2009;123:1312–1318.e2.
18. Curry BA, Blizzard CL, Schmidt MD, Walters EH, Dwyer T, Venn AJ. Longitudinal associations of adiposity with adult lung function in the childhood determinants of adult health (CDAH) study. Obesity (Silver Spring) 2011;19:2069–2075.
19. Kalhoff H, Breidenbach R, Smith HJ, Marek W. Impulse oscillometry in preschool children and association with body mass index. Respirology 2011;16:174–179.
20. Dubois AB, Brody AW, Lewis DH, Burgess BF Jr. Oscillation mechanics of lungs and chest in man. J Appl Physiol 1956;8:587–594.
21. Marotta A, Klinnert MD, Price MR, Larsen GL, Liu AH. Impulse oscillometry provides an effective measure of lung dysfunction in 4-year-old children at risk for persistent asthma. J Allergy Clin Immunol 2003;112:317–322.
22. Oostveen E, MacLeod D, Lorino H, Farre R, Hantos Z, Desager K, Marchal F, ERS Task Force on Respiratory Impedance Measurements. The forced oscillation technique in clinical practice: methodology, recommendations and future developments. Eur Respir J 2003;22:1026–1041.
23. Smith H, Reinhold P, Goldman M. Chapter 5. Forced oscillation technique and impulse oscillometry. Eur Respir Soc Monograph 2005;31:75–105.
24. Malmberg LP, Pelkonen A, Poussa T, Pohianpalo A, Haahtela T, Turpeinen M. Determinants of respiratory system input impedance and bronchodilator response in healthy Finnish preschool children. Clin Physiol Funct Imaging 2002;22:64–71.
25. Lauhkonen E, Koponen P, Nuolivirta K, Paassilta M, Toikka J, Korppi M. Lung function by impulse oscillometry at age 5–7 years after bronchiolitis at age 0–6 months. Pediatr Pulmonol 2014; doi: 10.1002/ppul.23039 [Epub ahead of print].
26. Nuolivirta K, Koponen P, He Q, Halkosalo A, Korppi M, Vesikari T, Helminen M. *Bordetella pertussis* infection is common in nonvaccinated infants admitted for bronchiolitis. Pediatr Infect Dis J 2010;29:1013–1015.
27. Koponen P, Helminen M, Paassilta M, Luukkaala T, Korppi M. Preschool asthma after bronchiolitis in infancy. Eur Respir J 2012;39:76–80.
28. Beydon N, Davis SD, Lombardi E, Allen JL, Arets HG, Aurora P, Bisgaard H, Davis GM, Ducharme FM, Eigen H, et al. An Official American Thoracic Society/European Respiratory Society statement: pulmonary function testing in preschool children. Am J Respir Crit Care Med 2007;175:1304–1345.
29. Malmberg LP, Makela MJ, Mattila PS, Hammaren-Malmi S, Pelkonen AS. Exercise-induced changes in respiratory impedance in young wheezy children and nonatopic controls. Pediatr Pulmonol 2008;43:538–544.
30. Saari A, Sankilampi U, Hannila ML, Kiviniemi V, Kesseli K, Dunkel L. New Finnish growth references for children and adolescents aged 0 to 20 years: length/height-for-age, weight-for-length/height, and body mass index-for-age. Ann Med 2011;43: 235–248.
31. Pihkala J, Hakala T, Voutilainen P, Raivio K. Characteristics of recent fetal growth curves in Finland (in Finnish). Duodecim 1989;105:1540–1546.
32. Turner S. Perinatal programming of childhood asthma: early fetal size, growth trajectory during infancy, and childhood asthma outcomes. Clin Dev Immunol 2012;2012:962923.
33. van der Gugten AC, Koopman M, Evelein AM, Verheij TJ, Uiterwaal CS, van der Ent CK. Rapid early weight gain is associated with wheeze and reduced lung function in childhood. Eur Respir J 2012;39:403–410.
34. Cilla G, Sarasua A, Montes M, Arostegui N, Vicente D, Perez-Yarza E, Perez-Trallero E. Risk factors for hospitalization due to respiratory syncytial virus infection among infants in the Basque country, Spain. Epidemiol Infect 2006;134:506–513.
35. Lanari M, Silvestri M, Rossi GA. Respiratory syncytial virus risk factors in late preterm infants. J Matern Fetal Neonatal Med 2009;22:102–107.

PLOS ONE

RESEARCH ARTICLE

IL-10 Gene Polymorphisms Are Associated with Post-Bronchiolitis Lung Function Abnormalities at Six Years of Age

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Abstract

Aim

Interleukin-10 (IL-10) has been associated with wheezing and asthma in children and the genetic variation of the IL-10 cytokine production may be linked to post-bronchiolitis lung function. We used impulse oscillometry (IOS) to evaluate the associations of *IL10* polymorphisms with lung function at a median age of 6.3 years in children hospitalised for bronchiolitis before six months of age.

Methods

We performed baseline and post-exercise IOS on 103 former bronchiolitis patients. Data on single nucleotide polymorphisms (SNP) of *IL10* rs1800896 (–1082G/A), rs1800871 (–819C/T), rs1800872 (–592C/A) were available for 99 children and of *IL10* rs1800890 (–3575T/A) for 98 children.

Results

IL10 rs1800896, rs1800871 and rs1800872 combined genotype AA+CT+CA and carriage of haplotype ATA, respectively, were associated with higher resistance and lower reactance in baseline IOS in adjusted analyses. At *IL10* rs1800890, the A/A-genotype and carriers of A-allele were associated with lower reactance in baseline IOS. There were no significant associations between the studied SNPs and airway hyper-reactivity to exercise.

Conclusion

Low-IL-10-producing polymorphisms in the IL-10 encoding gene were associated with obstructive lung function parameters, suggesting an important role for IL-10 in development of lung function deficit in early bronchiolitis patients.

Introduction

It is estimated that 2–3% of each age cohort are hospitalised for viral bronchiolitis during the first year of life. [1] There is increasing evidence that both virus-specific factors [2,3] and genetic variations of innate immunity contribute to later outcomes, including post-bronchiolitis wheezing [4,5] and the development of asthma. [6]

Interleukin-10 (IL-10) is a pivotal regulatory cytokine produced by blood monocytes and alveolar macrophages in the lungs [7] and reduced IL-10 levels have been reported in the alveolar fluid of asthmatics. [8] The *IL-10* gene is highly polymorphic and single nucleotide polymorphisms (SNP) in the proximal promoter region rs1800896, rs1800871 and rs1800872 form distinct haplotypes associated with IL-10 production. [9,10] Furthermore, these polymorphisms have been associated with paediatric asthma. [6,11] In the distal promoter region, carriage of allele A in the *IL10* rs1800890 has been associated with low IL-10 production in experimental studies. [12]

Earlier studies on this cohort of 166 infants hospitalised for bronchiolitis at less than six months of age reported that SNPs in the *IL-10* gene were associated with severe rhinovirus bronchiolitis and post-bronchiolitis asthma. [2,13]

The use of impulse oscillometry (IOS) [14] to measure lung function during tidal breathing is a promising technique for children under the age of seven. [15,16] We have previously published lung function results by IOS at age of five to seven years in 103 children hospitalised for bronchiolitis at less than six months of age. [17] Resistance or reactance at 5 Hz were pathological in 20% of the children compared to Finnish population-based, height-adjusted reference values.

We hypothesised that polymorphisms in the *IL-10* gene could be associated with post-bronchiolitis lung function disorders. This study evaluated the association of polymorphisms in the proximal promoter region *IL10* rs1800896 (–1082G/A), rs1800871 (–819C/T) and rs1800872 (–592C/A) or in the distal promoter region *IL10* rs1800890 (–3575T/A) with IOS results at a median age of 6.3 years in children hospitalised for bronchiolitis before six months of age.

Patients and Methods

Design

We prospectively followed up 166 children hospitalised for bronchiolitis before six months of age until they were five to seven years of age [18]. Of these, 127 attended a clinical follow-up visit at a median age of 6.3 years [18] and 103 of the children under the age of seven underwent impulse oscillometry (IOS), including baseline and post-exercise measurements. [17] As previously published [19], weight and height were measured and body mass index (BMI) was calculated and expressed as Z-scores from the population means. One child was excluded from the analyses because of a pathologically low BMI Z-score, corresponding to less than 16 kg/m^2 in a young adult. Early-life risk factors like maternal asthma, atopic eczema in infancy, maternal smoking during pregnancy and respiratory syncytial virus (RSV) aetiology of bronchiolitis had been collected during the hospitalisation for infant bronchiolitis. [5]

Data on SNPs of *IL10* rs1800896, rs1800871 and rs1800872 were available from 99 of the 103 children included in the IOS study, and of *IL10* rs1800890 were available from 98. The median age of the children was 6.3 years, with a standard deviation of 0.46 years, and 51 (50%) were boys. RSV was identified in 60.2% of cases during bronchiolitis.

Impulse oscillometry

Impulse oscillometry technique is a modification of the forced oscillations method [14] described in detail elsewhere. [20] The lung function examination is based on the measurement of total airway impedance (Zrs) in response to 5–35Hz oscillations conducted to the bronchial tree through a mouthpiece during quiet breathing. The airways resistance (Rrs) describes the resistive forces to the airflow and reactance (Xrs) reflects the elastic properties of the airway and the surrounding lung tissue, both components being derived from the measured Zrs. Other measured parameters describe the changes in bronchial tone and recoil: the rate of change in the Rrs as a function of the oscillation frequency (dRrs/df) and the point where the resistive and the elastic forces equal each other marks the resonant frequency (Fres). Thus, the main parameters observed are the Rrs and Xrs, where low <15Hz frequencies represent the measurement of more peripheral and >15 Hz more central airways. In a typical peripheral obstructive pattern, the Rrs at 5Hz (Rrs5Hz) rises above normal and the dRrs/df becomes more negative, and due to dynamic loss of lung compliance, the reactance at 5Hz (Xrs5Hz) decreases below normal resulting in increased Fres. IOS is applicable in exercise-challenge testing and bronchodilator testing, where change in resistance at 5Hz more than 35% is considered pathological. [21,22]

Lung function measurement

We used the Masterscreen IOS (Jaeger, Hochberg, Germany) to obtain baseline measurements, under the supervision of an experienced clinical physiologist (JOT), and these were pre-analysed to be graphically appropriate, free from artefacts and coherent in a set criteria (>0.6 at 5Hz and >0.9 at 10Hz) [15,16]. The post-exercise IOS measurements were taken after the children performed an outdoor, eight-minute free-running exercise challenge test (ECT) at more than 90% of the expected maximal heart rate. The mean IOS values were then transformed to height-adjusted Z-scores. [21]

As published previously, [17] either Rrs5Hz or Xrs5Hz was pathological in the baseline IOS measurements in 21 of the study subjects (20.4%) when compared to Finnish population-based, height-adjusted references. [21] Rrs5Hz was pathological in eight (7.8%) cases and Xrs5Hz was pathological in 19 (18.4%) cases. Only one case showed an irreversible lung function reduction that was not responsive to bronchodilators.

Genetics

We carried out genotyping of the *IL10* rs1800896 (–1082G/A) SNP using the ABI PRISM 7000 Sequence Detection System for both polymerase chain reaction (PCR) and allelic discrimination. [2] The *IL10* rs1800890 (–3575A/T) genotypes were determined by pyrosequencing as described previously. [23] The detection of the IL10 polymorphism sites, rs1800871 (-819A/G) and rs1800872 (-592T/G), were performed using PCR and sequencing of the amplified region. The SNPs at positions -592 and -819 were detected simultaneously in one PCR reaction. The sequence of PCR primers was: forward 5'-TAGGTCTCTGGGCCTTAGTT-3' and reverse 5'-AAGGCCAATTTAATCCAAGGTT-3'. The correct PCR product size (440 bp) was verified with agarose gel electrophoresis. The forward primer was used also for the sequencing reaction. Sequencing reactions were performed at the Institute for Molecular Medicine Finland in Helsinki. All PCR reactions were performed in the following conditions: initial denaturation at 95°C, denaturation at 95°C for two minutes, annealing at 60°C for 30 seconds and extension at 72°C for 40 seconds. After 40 cycles, final denaturation was carried out at 72°C for seven minutes. Our test provided the reverse transcription sequence, instead of the forward transcription sequence reported in most previous publications. Therefore, to enable us to compare the results

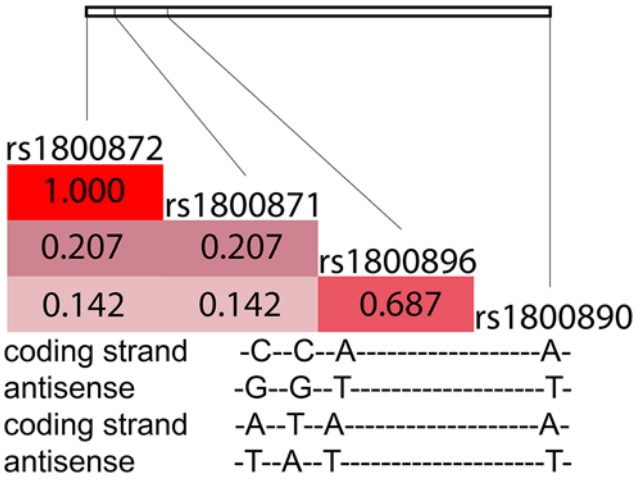

Fig 1. R^2 -values for the studied *IL-10* polymorphisms in Finnish population based data. *The formation of ACC(A)/ATA(A) genotype from the parental DNA strands described as an example. Colour = R^2-correlation, all pairs D´ = 1. Modified from* [28].

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with other studies, we have expressed the alleles G/A (*IL10* rs1800871) and G/T (*IL10* rs1800872) as C/T (*IL10* rs1800871) and C/A (*IL10* rs1800872), respectively.

The measured SNPs were tested to be in HWE ($p>0.05$) and haplotype frequencies of rs1800896, rs1800871 and rs1800872 were calculated with Haploview 4.2. [24] We compared the SNP minor allele frequencies to population-based public data. [25] The *IL10* rs1800896, rs1800871 and rs1800872 fall in distinct haplotypes in the Caucasian population. [9,26] Individual haplotypes of rs1800896, rs1800871 and rs1800872 were counted from unphased genotype data using the Clark´s algorithm [27] where all homozygote and single-site heterozygotes were identified and haplotypes resolved by direct counting. The SNPs rs1800871 and rs1800872 were co-segregating. This way 87% of the haplotypes were unambiguous, and the rest 13% could be resolved using the haplotype frequencies. All the studied four SNPs are highly linked, (Fig 1) but we were not able to resolve all four allele haplotypes, thus the rs1800890 was left out of the haplotype analyses.

Statistics

SPSS version 21 (IBM, NY, USA) was used in the statistical analyses of the data. The results are expressed as means and standard deviations (SD) for continuous variables and as numbers and frequencies for categorised variables. Analysis of co-variance (ANCOVA) was used in the analyses of continuous data, when appropriate, adjusted for current age, BMI Z-score, maternal smoking during pregnancy, respiratory syncytial virus (RSV) and rhinovirus aetiology of bronchiolitis.

Firstly, we compared genotypes separately and then we compared carriers of major alleles, for example dominant XX+Xx versus non-carriers xx, and thereafter carriers of minor alleles, for example recessive xx+Xx versus non-carriers XX.

Ethics

The Ethics Committee of the Tampere University Hospital District approved the study and a written informed consent was obtained from the parents before the children were enrolled as infants and at the clinical control visit. The genetic studies were carried out anonymously.

Table 1. Minor allele frequencies of the studied *IL-10* polymorphisms and comparison to population genetics.

Single nucleotide polymorphism	Minor allele frequency	FIN	EUR
rs1800896 (G>A)	0.45	0.40	0.45
rs1800871(C>T) /. . .72 (C>A)	0.18	0.24	0.24
rs1800890 (A>T)	0.39	0.31	0.37

FIN = Finnish, EUR = European as in [25]

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Results

The minor allele frequency of rs1800896 was comparable to Finnish and European populations. (Table 1) In rs1800871 and rs1800872 the minor allele frequencies were slightly lower and in rs1800890 somewhat higher compared to Finnish and European populations. (Table 1)

Single nucleotide polymorphisms

The frequencies and IOS results of the *IL10* rs1800896, rs1800871, rs1800872 and rs1800890 genotypes and allele carriage were first analysed separately.

We found that 26 of the 99 children tested had the G/G genotype of *IL10* rs1800896, 48 had the A/G genotype and 25 had the A/A genotype. There were no significant associations between the genotypes (Table 2) or allele carriage (Table 2) of *IL10* rs1800896 and the IOS results.

Our results showed that 68/99 had the C/C genotype of *IL10* rs1800871/ *IL10* rs1800872, 27 had the C/T or C/A genotype and four had the T/T or A/A genotype. Baseline Xrs5Hz was lowest in children with the T/T (*IL10* rs1800871) or A/A (*IL10* rs1800871) genotypes and highest in those with the C/C genotype. (Table 3) The carriage of major allele C was associated with higher baseline Xrs5Hz (adjusted p = 0.03) and post-exercise Xrs5Hz and Fres (adjusted p = 0.02 and p = 0.05, respectively). The carriage of minor allele T (*IL10* rs1800871) or A (*IL10* rs1800872) was associated with higher Rrs5Hz and lower Xrs5Hz in baseline IOS than non-T or non-A carriers, respectively. (Table 3) There were no significant associations between *IL10* rs1800871/ *IL10* rs1800872 minor alleles T or A carriage, and the post-exercise IOS results.

Table 2. Impulse oscillometry presented as Z-scores in relation to *IL-10* rs1800896 polymorphism in 99 children under the age of seven after hospitalisation for bronchiolitis at less than six months of age.

IOS parameter	G/G n = 26	A/G n = 48	A/A n = 25	p[a]	G-allele n = 74	p[a]*	A-allele n = 73	p[a]*
Baseline	**Mean (SD)**	**Mean (SD)**	**Mean (SD)**		**Mean (SD)**		**Mean (SD)**	
Rrs5Hz	-0.16 (0.96)	-0.02 (1.11)	-0.05 (1.07)	0.86	-0.07 (1.05)	0.59	-0.03 (1.09)	0.82
Xrs5Hz	-0.76 (1.25)	-0.72 (1.08)	-0.71 (1.46)	0.93	-0.73 (1.13)	0.79	0.71 (1.21)	0.74
Fres	2.32 (0.64)	2.27 (0.87)	2.06 (0.94)	0.67	2.29 (0.79)	0.39	2.20 (0.89)	0.57
dRs/df	-0.86 (1.00)	-1.16 (1.14)	-0.91 (1.19)	0.65	-1.06 (1.10)	0.54	-1.08 (1.16)	0.67
Post-exercise values								
Rrs5Hz	0.05 (1.12)	0.34 (1.12)	0.04 (1.25)	0.90	0.24 (1.12)	0.97	0.24 (1.16)	0.68
Xrs5Hz	-1.08 (1.37)	-1.09 (1.37)	-1.01 (1.74)	0.93	-1.09 (1.36)	0.97	-1.07 (1.49)	0.71
Fres	2.48 (0.75)	2.44 (0.87)	2.19 (1.01)	0.69	2.45 (0.83)	0.55	2.35 (0.92)	0.42
dRs/df	-1.20 (1.08)	-1.54 (1.40)	-1.17 (1.61)	0.62	-1.42 (1.30)	0.45	-1.41 (1.47)	0.77

Zrs = Total Impedance; Rrs = Resistance; Xrs = Reactance; Fres = Resonant Frequency; dRrs/df = Frequency Dependency of Resistance

[a]adjusted for age, maternal smoking during pregnancy, RSV and rhinovirus aetiology of bronchiolitis and BMI Z-score

*allele carriers vs. non-carriers

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Table 3. Impulse oscillometry presented as Z-scores in relation to *IL-10* rs1800871/rs1800872 polymorphisms in 99 children under the age of seven after hospitalisation for bronchiolitis at less than six months of age.

IOS parameter	C/C n = 68	C/T or C/A n = 27	T/T or A/A n = 4	p[a]	C-allele n = 95	p[a]*	T- /A-allele n = 31	p[a]*
Baseline	**Mean (SD)**	**Mean (SD)**	**Mean (SD)**		**Mean (SD)**		**Mean (SD)**	
Rrs5Hz	-0.17 (1.00)	0.15 (1.16)	0.27 (1.16)	0.08	-0.08 (1.05)	0.64	0.17 (1.14)	**0.02**
Xrs5Hz	-0.60 (0.98)	-0.85 (1.58)	-2.07 (1.47)	**0.04**	-0.67 (1.18)	**0.03**	-1.01 (1.60)	**0.04**
Fres	2.17 (0.84)	2.32 (0.85)	2.73 (0.63)	0.33	2.21 (0.84)	0.26	2.37 (0.82)	0.20
dRs/df	-0.95 (1.00)	-1.12 (1.38)	-1.48 (1.13)	0.33	-1.00 (1.12)	0.37	-1.17 (1.34)	0.16
Post-exercise values								
Rrs5Hz	0.09 (1.14)	0.29 (1.13)	1.11 (1.34)	0.11	0.15 (1.13)	0.11	0.40 (1.17)	0.07
Xrs5Hz	-1.02 (1.36)	-0.93 (1.60)	-2.70 (1.50)	0.07	-1.00 (1.43)	**0.02**	-1.16 (1.67)	0.39
Fres	2.33 (0.91)	2.41 (0.74)	3.20 (1.06)	0.13	2.35 (0.86)	**0.05**	2.51 (0.81)	0.22
dRs/df	-1.29 (1.36)	-1.38 (1.46)	-2.34 (0.94)	0.25	-1.32 (1.38)	0.13	-1.50 (1.43)	0.25

Zrs = Total Impedance; Rrs = Resistance; Xrs = Reactance; Fres = Resonant Frequency; dRrs/df = Frequency Dependency of Resistance

[a]adjusted for age, maternal smoking during pregnancy, RSV and rhinovirus aetiology of bronchiolitis and BMI Z-score

*allele carriers vs. non-carriers

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With regard to *IL10* rs1800890, 35/98 had the A/A genotype, 50 (51%) the A/T genotype and 13 (13%) had the T/T genotype. Baseline Xrs5Hz was highest in children with the T/T genotype and lowest in those with the A/A genotype. (Table 4) The carriage of the major allele A was associated with lower baseline Xrs5Hz (adjusted p = 0.03) when compared to the non-carriers. (Table 4) The carriage of the minor allele T was associated with lower baseline Rrs5Hz (adjusted p = 0.04, respectively) and higher Xrs5Hz (adjusted p = 0.04) and lower post-exercise Rrs5Hz (adjusted p = 0.03). (Table 4)

Combined genotypes and haplotypes

The three bi-allelic polymorphisms rs1800896, rs1800871 and rs1800872 in the proximal promoter region of *IL-10* gene were analysed as combined genotypes and according to haplotype carriage.

Table 4. Impulse oscillometry presented as Z-scores in relation to *IL-10* rs1800890 polymorphism in 98 children under the age of seven after hospitalisation for bronchiolitis at less than six months of age.

IOS parameter	A/A n = 35	A/T n = 50	T/T n = 13	p[a]	A-allele n = 85	p[a]*	T-allele n = 74	p[a]*
Baseline	**Mean (SD)**	**Mean (SD)**	**Mean (SD)**		**Mean (SD)**		**Mean (SD)**	
Rrs5Hz	0.10 (1.03)	-0.17 (1.10)	-0.22 (0.77)	0.13	-0.06 (1.08)	0.58	-0.18 (1.04)	**0.04**
Xrs5Hz	-1.02 (1.25)	-0.64 (1.11)	-0.06 (1.08)	**0.03**	-0.79 (1.18)	**0.03**	-0.52 (1.12)	**0.04**
Fres	2.38 (0.85)	2.16 (0.84)	2.02 (0.66)	0.20	2.25 (0.85)	0.36	2.13 (0.81)	0.08
dRs/df	-1.23 (1.09)	-0.93 (1.14)	-0.64 (0.93)	0.20	-1.06 (1.12)	0.24	-0.87 (1.10)	0.10
Post-exercise values								
Rrs5Hz	0.37 (1.12)	0.09 (1.20)	-0.07 (0.85)	0.10	0.20 (1.17)	0.37	0.05 (1.13)	**0.03**
Xrs5Hz	-1.27 (1.53)	-1.06 (1.44)	-0.33 (0.90)	0.11	-1.14 (1.47)	0.06	-0.91 (1.37)	0.15
Fres	2.49 (0.78)	2.36 (0.92)	2.04 (0.79)	0.12	2.41 (0.86)	0.12	2.30 (0.90)	0.09
dRs/df	-1.63 (1.49)	-1.26 (1.30)	-0.83 (1.12)	0.16	-1.41 (1.39)	0.19	-1.17 (1.27)	0.08

Zrs = Total Impedance; Rrs = Resistance; Xrs = Reactance; Fres = Resonant Frequency; dRrs/df = Frequency Dependency of Resistance

[a] adjusted for age, maternal smoking during pregnancy, RSV and rhinovirus aetiology of bronchiolitis and BMI Z-score

*allele carriers vs. non-carriers

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Table 5. Combined *IL-10* rs1800896, rs1800871 and rs1800872 genotypes and haplotype frequencies of 99 children hospitalised for bronchiolitis in infancy.

Genotype rs1800896+rs1800871+rs1800872 (haplotype)	n (%)
GG+CC+CC (GCC/GCC)	16 (16.2)
GG+CT+CA (GCC/GTA or GTA/GCC)	7 (7.1)
GG+TT+AA (GTA/GTA)	3 (3.0)
GA+CC+CC (GCC/ACC or ACC/GCC)	35 (35.4)
GA+CT+CA (GCC/ATA or ATA/GCC)*	13 (13.1)
GA+TT+AA (GTA/ATA or ATA/GTA)	0 (0.0)
AA+CC+CC (ACC/ACC)	17 (17.2)
AA+CT+CA (ACC/ATA or ATA/ACC)	7 (7.1)
AA+TT+AA (ATA/ATA)	1 (1.0)
Haplotype frequency	f
GCC	0.44
GTA	0.07
ACC	0.39
ATA	0.11

**GTA/ACC or ACC/GTA not present as estimated from the study population frequencies.*

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Table 5 summarises the frequencies of the combined genotypes and haplotype frequencies of the *IL10* rs1800896, rs1800871 and rs1800872 genes. The haplotype GCC (major alleles) was present in 71.7%, ACC was present in 59.6%, ATA (minor alleles) was present in 21.2% and GTA was present in 10.1%.

The genotype AA+CT+CA was significantly associated with higher baseline Rrs5Hz and lower baseline Xrs5Hz than the non-AA+CT+CA genotypes. (Table 6) The genotype GG+TT+AA was significantly associated with lower baseline and post-exercise Xrs5Hz. (Table 6)

The carriage of haplotype ATA, consisting of three minor alleles, was associated with higher baseline Rrs5Hz. (Table 7)

Table 6. Impulse oscillometry presented as Z-scores in relation to combined *IL-10* rs1800896, rs1800871 and rs1800872 genotypes in 99 children under the age of seven after hospitalisation for bronchiolitis in infancy.

IOS parameter	**GG+CC+CC n = 16**	**GG+CT+CA n = 7**	**GG+TT+AA n = 3**	**GA+CC+CC n = 35**	**GA+CT+CA n = 13**	**AA+CC+CC n = 17**	**AA+CT+CA n = 7**
Baseline	**mean (SD)**	**mean (SD)**	**mean (SD)**	**mean (SD)**	**mean (SD)**	**mean (SD)**	**mean (SD)**
Rrs5Hz	-0.20 (0.57)	-0.30 (1.47)	0.40 (1.38)	-0.06 (1.11)	0.09 (1.14)	-0.36 (1.09)	**0.72 (0.68)[a]**
Xrs5Hz	-0.70 (1.15)	-0.14 (0.75)	**-2.52 (1.42)[a]**	-0.66 (0.95)	-0.86 (1.39)	-0.36 (0.88)	**-1.55 (2.30)[a]**
Fres	2.23 (0.58)	2.29 (0.73)	2.89 (0.66)	2.27 (0.88)	2.29 (0.86)	1.91 (0.93)	2.39 (1.03)
dRs/df	-0.87 (0.91)	-0.56 (1.06)	-1.55 (1.37)	-1.11 (1.12)	-1.31 (1.40)	-0.72 (1.00)	-1.32 (1.64)
Post-exercise values							
Rrs5Hz	-0.19 (0.83)	0.11 (1.33)	1.20 (1.63)	0.36 (1.15)	0.29 (1.09)	-0.19 (1.29)	0.49 (1.14)
Xrs5Hz	-0.82 (1.15)	-0.78 (1.26)	**-3.15 (1.48)[a]**	-1.03 (1.29)	-1.26 (1.59)	-1.21 (1.71)	-0.47 (1.97)
Fres	2.31 (0.63)	2.48 (0.58)	3.40 (1.20)	2.41 (0.96)	2.51 (0.63)	2.19 (1.04)	2.13 (1.06)
dRs/df	-1.04 (0.90)	-0.99 (1.26)	-2.54 (1.04)	-1.50 (1.44)	-1.64 (1.34)	-1.09 (1.55)	-1.28 (1.96)

Zrs = Total Impedance; Rrs = Resistance; Xrs = Reactance; Fres = Resonant Frequency; dRrs/df = Frequency Dependency of Resistance

[a]adjusted p<0.05 vs. non-AA+CT+CA (n = 92) or non-GG+TT+AA (n = 96), as adjusted for age, maternal smoking during pregnancy, RSV- and rhinovirus etiology of bronchiolitis and BMI Z-score

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Table 7. Impulse oscillometry presented as Z-scores in relation to *IL-10* rs1800896, rs1800871 and rs1800872 haplotype carriage in 99 pre-school-aged children after hospitalization for bronchiolitis in infancy.

IOS parameter	GCC n = 71	GTA n = 10	ACC n = 59	ATA n = 21	p[a]
Baseline	**Mean (SD)**	**Mean (SD)**	**Mean (SD)**	**Mean (SD)**	
Rrs5Hz	-0.09 (1.04)	-0.09 (1.40)	-0.05 (1.09)	0.29 (1.01)	**0.03**
Xrs5Hz	-0.66 (1.07)	-0.86 (1.46)	-0.68 (1.19)	-1.08 (1.69)	0.07
Fres	2.27 (0.79)	2.47 (0.73)	2.18 (0.91)	2.32 (0.88)	0.44
dRs/df	-1.04 (1.09)	-0.86 (1.18)	-1.02 (1.11)	-1.32 (1.41)	0.10
Post-exercise values					
Rrs5Hz	0.20 (1.09)	0.43 (1.43)	0.22 (1.20)	0.38 (1.06)	0.24
Xrs5Hz	-1.00 (1.30)	-1.49 (1.69)	-1.02 (1.49)	-1.00 (1.69)	0.98
Fres	2.41 (0.79)	2.76 (0.86)	2.31 (0.98)	2.39 (0.78)	0.81
dRs/df	-1.37 (1.29)	-1.46 (1.34)	-1.36 (1.52)	-1.53 (1.50)	0.39

Zrs = Total Impedance; Rrs = Resistance; Xrs = Reactance; Fres = Resonant Frequency; dRrs/df = Frequency Dependency of Resistance

[a]ATA vs. non-ATA (n = 78) adjusted for age, maternal smoking during pregnancy, RSV- and rhinovirus etiology of bronchiolitis and BMI z-score

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Discussion

The main results of this post-bronchiolitis lung function study at a median age of 6.3 years were that *IL10* polymorphisms rs1800896, rs1800871, rs1800872 and rs1800890 were associated with lung function measured with IOS. Low IL-10 producing polymorphisms were associated with obstructive IOS baseline results. There were no significant associations between *IL10* polymorphisms and airway hyper-reactivity in response to exercise. Although some knowledge has accumulated on the association between cytokines or cytokine genetics, such as *IL-10* polymorphisms, and wheezing or asthma in preschool-aged children, no lung function studies have so far been published.

IL-10 is a key regulator of human immune responses and possesses many immunosuppressive functions that protect the host from abnormally strong inflammation and subsequent tissue damage playing a protective role in allergic disease. [7] The other side of the coin is that the suppression of immune responses may lead to more severe infections that last longer. For example, *IL-10* gene polymorphisms have been associated with severe RSV and rhinovirus bronchiolitis and with post-infectious wheezing in childhood. [2,4] In experimental studies, IL-10-deficient mice developed allergic responses but airway reactivity did not increase in response to allergen challenge. [29]

The studied *IL-10* gene polymorphisms are functional as they regulate IL-10 production. In rs1800890, the presence of allele A has been associated with low IL-10 production [12] and, similarly in rs1800896, rs1800871 and rs1800872, the haplotype ATA has been associated with low IL-10 levels. [10] An earlier study stated that the presence of allele A at *IL10* rs1800896 (G/A) was associated with lower IL-10 production regardless of the alleles at rs1800871 or rs1800872. [9] However, another study suggested that only the ACC haplotype was associated with high IL-10 levels. [26]

In this study, the presence of *IL10* rs1800896, rs1800871 and rs1800872 haplotype ATA was associated with increased resistance in the baseline IOS measurements. Our findings are in line with a study of 518 asthmatic children at a mean age of 8.1 years, which reported that haplotype ATA was associated with low forced expiratory volume in one second (FEV1) and that haplotype GCC was associated with high FEV1. [11] In addition, the AA+CT+CA genotype was associated with abnormal baseline IOS results. This genotype includes two *IL10* rs1800896

minor A alleles and the ATA haplotype, which both have a known association with low IL-10 production. [10]

Previously in this cohort, the *IL10* rs1800896 A/A genotype was associated with non-RSV aetiology of bronchiolitis [2], and under the age of seven, the G/G genotype was protective for post-bronchiolitis asthma. Correspondingly, the presence of allele A increased the risk of asthma [13]. In the current study, SNP at rs1800896 did not show any significant association with lung function measurements, but the minor allele A was present in the combined genotype and haplotype associated with reduced IOS results.

Data on the *IL-10* rs1800890 polymorphisms were now available in this study, though not possible to be included in the haplotype analyses. The presence of the major allele A was associated with post-bronchiolitis lung function disorders under the age of seven. These findings suggest that *IL10* polymorphisms play a role in the pathogenesis of post-bronchiolitis lung dysfunction, likely via differences in IL-10 production during bronchiolitis.

The SNP distribution in *IL10* rs1800896 was not different from the Finnish blood donors in an earlier study [2] and the SNP distributions of *IL10* rs1800871, rs1800872 and rs1800890 were comparable with those of Finnish and European populations. (see Table 1) Although GTA haplotypes are uncommon in Caucasian populations [9,26] their frequency was 0.07 in our study. In previous studies this haplotype has been identified in Dutch population with a frequency of 0.01 [26] and in Asian (Hong Kong) population with a frequency of 0.04. [30] Thus this haplotype is overrepresented in our study population, which could be explained with different, maybe more eastern ancestral background of the Finnish population compared to other European populations.

The main weakness of the present study was the somewhat small number of patients for a genetic study. Due to this, the power of the study was not enough for stratified analyses and it carried a risk of type-two statistical errors. However, the design and data of the study allowed adjusted analyses, which increase the reliability of the revealed associations. The findings were robust to adjustments for the most important confounders like maternal smoking, atopic eczema in infants and viral aetiology of infant bronchiolitis. On the other hand, multiple analyses of different IOS parameters from the same data mean a risk of type-one statistical error. Any control groups with sufficient IOS and genetic data were not available, which is a clear limitation of our study. However, national population-based age-specific and height-adjusted reference values were available for the IOS measurements [21]. We generated IOS measurements of good-quality for more than 96% of the study subjects, and genetic data were available for 92% of them.

The results of this study provide additional evidence that lung function decline measured by IOS in former bronchiolitis patients under the age of seven may be associated with low-producing genotypes and haplotypes of the IL-10 encoding gene polymorphisms at rs1800896, rs1800871, rs1800872 and rs1800890. This preliminary evidence of cytokine-associated lung function disorder after viral bronchiolitis might reflect the impact of the infection itself during the first months of life or result from a predisposition to a subsequent disease processes in individuals with low IL-10 production.

Author Contributions

Conceived and designed the experiments: PK KN MH JOT QH MK. Performed the experiments: PK JT KG JV KN JOT. Analyzed the data: EL PK MK. Contributed reagents/materials/analysis tools: JT KG JV JOT QH. Wrote the paper: EL PK JT KG JV KN JOT MH QH MK.

References

1. Smyth RL, Openshaw PJ. Bronchiolitis. Lancet. 2006; 368: 312–322. PMID: 16860701
2. Helminen M, Nuolivirta K, Virta M, Halkosalo A, Korppi M, Vesikari T, et al. IL-10 gene polymorphism at -1082 A/G is associated with severe rhinovirus bronchiolitis in infants. Pediatr Pulmonol. 2008; 43: 391–395. doi: 10.1002/ppul.20793 PMID: 18286551
3. Garcia C, Soriano-Fallas A, Lozano J, Leos N, Gomez AM, Ramilo O, et al. Decreased innate immune cytokine responses correlate with disease severity in children with respiratory syncytial virus and human rhinovirus bronchiolitis. Pediatr Infect Dis J. 2012; 31: 86–89. doi: 10.1097/INF.0b013e31822dc8c1 PMID: 21829141
4. Bont L, Heijnen CJ, Kavelaars A, van Aalderen WM, Brus F, Draaisma JT, et al. Monocyte IL-10 production during respiratory syncytial virus bronchiolitis is associated with recurrent wheezing in a one-year follow-up study. Am J Respir Crit Care Med. 2000; 161: 1518–1523. PMID: 10806148
5. Nuolivirta K, Hurme M, Halkosalo A, Koponen P, Korppi M, Vesikari T, et al. Gene polymorphism of IFNG +874 T/A and TLR4 +896 A/G and recurrent infections and wheezing in toddlers with history of bronchiolitis. Pediatr Infect Dis J. 2009; 28: 1121–1123. doi: 10.1097/INF.0b013e3181af37ee PMID: 19773677
6. Hyun MH, Lee CH, Kang MH, Park BK, Lee YH. Interleukin-10 promoter gene polymorphisms and susceptibility to asthma: a meta-analysis. PLoS One. 2013; 8: e53758. doi: 10.1371/journal.pone.0053758 PMID: 23335974
7. Akdis M, Burgler S, Crameri R, Eiwegger T, Fujita H, Gomez E, et al. Interleukins, from 1 to 37, and interferon-gamma: receptors, functions, and roles in diseases. J Allergy Clin Immunol. 2011; 127: 701–21.e1–70. doi: 10.1016/j.jaci.2010.11.050 PMID: 21377040
8. Borish L, Aarons A, Rumbyrt J, Cvietusa P, Negri J, Wenzel S. Interleukin-10 regulation in normal subjects and patients with asthma. J Allergy Clin Immunol. 1996; 97: 1288–1296. PMID: 8648025
9. Turner DM, Williams DM, Sankaran D, Lazarus M, Sinnott PJ, Hutchinson IV. An investigation of polymorphism in the interleukin-10 gene promoter. Eur J Immunogenet. 1997; 24: 1–8. PMID: 9043871
10. Crawley E, Kay R, Sillibourne J, Patel P, Hutchinson I, Woo P. Polymorphic haplotypes of the interleukin-10 5' flanking region determine variable interleukin-10 transcription and are associated with particular phenotypes of juvenile rheumatoid arthritis. Arthritis Rheum. 1999; 42: 1101–1108. PMID: 10366102
11. Lyon H, Lange C, Lake S, Silverman EK, Randolph AG, Kwiatkowski D, et al. IL10 gene polymorphisms are associated with asthma phenotypes in children. Genet Epidemiol. 2004; 26: 155–165. PMID: 14748015
12. Gibson AW, Edberg JC, Wu J, Westendorp RG, Huizinga TW, Kimberly RP. Novel single nucleotide polymorphisms in the distal IL-10 promoter affect IL-10 production and enhance the risk of systemic lupus erythematosus. J Immunol. 2001; 166: 3915–3922. PMID: 11238636
13. Koponen P, Nuolivirta K, Virta M, Helminen M, Hurme M, Korppi M. Polymorphism of the rs1800896 IL10 promoter gene protects children from post-bronchiolitis asthma. Pediatr Pulmonol. 2014; 49: 800–806. doi: 10.1002/ppul.22909 PMID: 24167151
14. Dubois AB, Brody AW, Lewis DH, Burgess BF Jr. Oscillation mechanics of lungs and chest in man. J Appl Physiol. 1956; 8: 587–594. PMID: 13331841
15. Oostveen E, MacLeod D, Lorino H, Farre R, Hantos Z, Desager K, et al. The forced oscillation technique in clinical practice: methodology, recommendations and future developments. Eur Respir J. 2003; 22: 1026–1041. PMID: 14680096
16. Beydon N, Davis SD, Lombardi E, Allen JL, Arets HG, Aurora P, et al. An official American Thoracic Society/European Respiratory Society statement: pulmonary function testing in preschool children. Am J Respir Crit Care Med. 2007; 175: 1304–1345. PMID: 17545458
17. Lauhkonen E, Koponen P, Nuolivirta K, Paassilta M, Toikka J, Korppi M. Lung function by impulse oscillometry at age 5–7 years after bronchiolitis at age 0–6 months. Pediatr Pulmonol. 2015; 50: 389–395. doi: 10.1002/ppul.23039 PMID: 24668616
18. Koponen P, Helminen M, Paassilta M, Luukkaala T, Korppi M. Preschool asthma after bronchiolitis in infancy. Eur Respir J. 2012; 39: 76–80. doi: 10.1183/09031936.00040211 PMID: 21700604
19. Lauhkonen E, Koponen P, Nuolivirta K, Paassilta M, Toikka J, Saari A, et al. Obesity and bronchial obstruction in impulse oscillometry at age 5–7 years in a prospective post-bronchiolitis cohort. Pediatr Pulmonol. 2015; 50: 908–914. doi: 10.1002/ppul.23085 PMID: 25044353
20. Smith H, Reinhold P, Goldman M. Chapter 5. Forced oscillation technique and impulse oscillometry. European Respiratory Society Monograph. 2005; 31: 75–105.

21. Malmberg LP, Pelkonen A, Poussa T, Pohianpalo A, Haahtela T, Turpeinen M. Determinants of respiratory system input impedance and bronchodilator response in healthy Finnish preschool children. Clin Physiol Funct Imaging. 2002; 22: 64–71. PMID: 12003103

22. Malmberg LP, Makela MJ, Mattila PS, Hammaren-Malmi S, Pelkonen AS. Exercise-induced changes in respiratory impedance in young wheezy children and nonatopic controls. Pediatr Pulmonol. 2008; 43: 538–544. doi: 10.1002/ppul.20805 PMID: 18433041

23. Vuononvirta J, Toivonen L, Grondahl-Yli-Hannuksela K, Barkoff AM, Lindholm L, Mertsola J, et al. Nasopharyngeal bacterial colonization and gene polymorphisms of mannose-binding lectin and toll-like receptors 2 and 4 in infants. PLoS One. 2011; 6: e26198. doi: 10.1371/journal.pone.0026198 PMID: 22022564

24. Barrett JC, Fry B, Maller J, Daly MJ. Haploview: analysis and visualization of LD and haplotype maps. Bioinformatics. 2005; 21: 263–265. PMID: 15297300

25. 1000 Genomes Project Consortium, Abecasis GR, Auton A, Brooks LD, DePristo MA, Durbin RM, et al. An integrated map of genetic variation from 1,092 human genomes. Nature. 2012; 491: 56–65. doi: 10.1038/nature11632 PMID: 23128226

26. Eskdale J, Keijsers V, Huizinga T, Gallagher G. Microsatellite alleles and single nucleotide polymorphisms (SNP) combine to form four major haplotype families at the human interleukin-10 (IL-10) locus. Genes Immun. 1999; 1: 151–155. PMID: 11196662

27. Clark AG. Inference of haplotypes from PCR-amplified samples of diploid populations. Mol Biol Evol. 1990; 7: 111–122. PMID: 2108305

28. Cunningham F, Amode MR, Barrell D, Beal K, Billis K, Brent S, et al. Ensembl 2015. Nucleic Acids Res. 2015; 43: D662–9. doi: 10.1093/nar/gku1010 PMID: 25352552

29. Makela MJ, Kanehiro A, Borish L, Dakhama A, Loader J, Joetham A, et al. IL-10 is necessary for the expression of airway hyperresponsiveness but not pulmonary inflammation after allergic sensitization. Proc Natl Acad Sci U S A. 2000; 97: 6007–6012. PMID: 10811896

30. Mok CC, Lanchbury JS, Chan DW, Lau CS. Interleukin-10 promoter polymorphisms in Southern Chinese patients with systemic lupus erythematosus. Arthritis Rheum. 1998; 41: 1090–1095. PMID: 9627019

PLOS ONE

RESEARCH ARTICLE

Gene Polymorphism of Toll-Like Receptors and Lung Function at Five to Seven Years of Age after Infant Bronchiolitis

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Abstract

Aim

Toll-like receptors (TLR) play a crucial role in innate immunity, protecting the host from pathogens such as viruses. Genetic variations in TLRs have been associated with the severity of viral bronchiolitis in infancy and with the later occurrence of post-bronchiolitis asthma. The aim of the present study was to evaluate if there are any exploratory associations between *TLR* gene polymorphisms and lung function at 5 to 7 years of age in former bronchiolitis patients.

Methods

We performed impulse oscillometry (IOS) at the median age of 6.3 years for 103 children who had been hospitalized for bronchiolitis at less than six months of age. The main parameters evaluated were airway resistance and reactance at 5Hz in baseline and post-exercise measurements. Data on single nucleotide polymorphisms (SNP) of *TLR1* rs5743618, *TLR2* rs5743708, *TLR6* rs5743810 and *TLR10* rs4129009 (TLR2 subfamily) and *TLR3* rs3775291, *TLR4* rs4986790, *TLR7* rs179008, *TLR8* rs2407992 and *TLR 9* rs187084 were available for analyses.

Results

The *TLR4* rs4986790 wild genotype A/A was associated with a greater Rrs5 response (0.72 vs. -0.42, p = 0.03) to exercise. In *TLR6* rs5743810, the minor allele T was associated with greater Rrs5 response (0.80 vs. -0.03, p = 0.04) to exercise. In *TLR7* rs179008, the major allele A was associated with baseline decline in dRrs/df (-1.03 vs 0.61, p = 0.01) and increased Fres (2.28 vs. 0.89, p = 0.01) in girls.

Conclusion

Among the nine studied TLRs, only *TLR7* rs179008 showed some exploratory associations with post-bronchiolitis lung function deficiency, and polymorphisms of *TLR4* rs4986790, and *TLR6* rs5743810 in particular, with airway reactivity. These findings call for further confirmatory studies.

Introduction

In a recent Finnish study, on average 37 per 1000 infants under the age of six months were annually admitted to the emergency room due to viral bronchiolitis and 70% of them were hospitalized. [1] At this age, respiratory syncytial virus (RSV) is the predominant cause of bronchiolitis, and most of the affected infants have no previous medical history. [2,3] Toll-like receptors (TLR), which recognize pathogens and initiate responses of both innate and adaptive immunity, are the gatekeepers of the immune system. [4] TLRs are important in airway mucosal responses to acute viral infections [5] and in later emergence and regulation of asthmatic inflammation. [6]

In the respiratory epithelial cells, TLRs 1, 2, 4, 6 and 10 are expressed on the cell surfaces. TLRs 1, 2, 6 and 10 are co-receptors that form a functional unit called the TLR2 subfamily encoded by genes in the chromosome 4, and play an important role in recognition of bacterial structures. [7] TLRs 3, 7, 8 and 9 are endosomal receptors that recognize viral RNA and DNA structures. [5] Bacterial lipopolysaccharide is the main ligand for TLR 4, but also the F-protein of the RSV is recognized by TLR 4, being highly expressed in airway epithelium during RSV infection promoting inflammation. [5,8] The genetic alterations in TLRs were associated with the severity of bronchiolitis in infancy [8] and the later development of asthma. [9] Attenuated TLR signaling and altered antigen presenting cell function can lead to more severe infection with a longer duration due to less viral clearance. [10] On the other hand, attenuated TLR signaling could lead to repeated viral infections, which may further increase the risk of later atopy. [10,11] Thus, genetic differences in the TLR function might be associated with direct lung injury during bronchiolitis, or contribute to the development of later lung function reduction.

Impulse oscillometry (IOS), [12] which measures lung function during tidal breathing, is a promising technique that can be used for preschool-aged children who are unable to perform the maximal expiratory blow needed in spirometry. [13] The method is also appropriate for the evaluation of airway reactivity to exercise and responses to bronchodilators. [14,15] Finnish age-specific height-adjusted reference values for IOS parameters were published recently. [16]

This study focused on children who had been hospitalized for bronchiolitis at less than 6 months of age. The aim of this exploratory study was to see whether we could find any associations between the single nucleotide polymorphisms (SNP) at *TLR1* rs5743618, *TLR2* rs5743708, *TLR3* rs3775291, *TLR4* rs4986790, *TLR6* rs5743810, *TLR7* rs179008, *TLR8* rs2407992, *TLR9* rs187084 or *TLR10* rs4129009 and lung function or airway reactivity measured by IOS at 5 to 7 years of age.

Materials and Methods

Design

As previously published, we prospectively followed-up a cohort of 166 children hospitalized for bronchiolitis at less than 6 months of age until they were 5 to 7 years of age. [17] Of these,

127 attended a clinical follow-up visit at a median age of 6.3 years, [17] and 107 children aged less than 7 years performed impulse oscillometry (IOS). Four children were excluded from the IOS analysis for technical reasons. Body mass index (BMI) was calculated and expressed as z-scores from the population means (zBMI) and one child was excluded from the analyses due to pathologically low BMI (<16 kg/m^2) at follow up. This meant that IOS data on 102 children were available for the analysis and data on SNPs of TLR 1, 2, 3, 4, 6, 7 and 8 were available for 98 and of TLR 9 and 10 for 97 of them.

Respiratory syncytial virus (RSV) etiology of bronchiolitis was registered while the infant was hospitalized for bronchiolitis. [18] Consumption of inhaled corticosteroids (ICS) (present in 12 cases) and presence of atopic eczema (present in 31 cases) currently and during the preceding 12 months were asked and registered at the controls visit at 5 to 7 years of age.

Impulse oscillometry

The IOS method [19] measures total airway impedance (Zrs) resulting from phase and pressure changes of the airflow when oscillation at 1–35 Hz frequency is conducted to the bronchial tree during quiet breathing. The lower (<15 Hz) frequencies vibrate the peripheral and higher frequencies the more central airways. The main clinical parameters airway resistance (Rrs) and airway reactance (Xrs) are derived from the Zrs mathematically. Rrs describes the resistive forces to the airflow and Xrs the elastic forces of the tissues surrounding the moving air column. Additional parameters describing the change in bronchial tone and recoil as a function of the oscillation frequency are the frequency dependency of resistance (dRs/df), and the resonant frequency (Fres), a point where the resistive and elastic forces equal each other. As an example, in small airway obstruction, as in asthma, resistance at 5Hz increases above normal and the frequency dependency of resistance (dRs/df) becomes more negative, and the Xrs decreases and as a result the Fres rises. [15] Rrs and Xrs can change somewhat independently, as they are vector components of the measured Zrs in a mathematical sense. [19] Thus different lung pathology can be described with these IOS parameters, as for example reactance at 5Hz decreases in both peripheral obstruction and in restrictive conditions, as in hyperinflation or fibrosis. [15]

Lung function measurement and analysis

First, the baseline IOS (Masterscreen IOS, Jaeger, Hochberg, Germany) measurements were obtained and pre-analyzed by an experienced clinical physiologist (JOT) to ensure they were graphically appropriate, free from artefacts and coherent in set criteria (>0.6 at 5Hz and >0.9 at 10Hz) [14,15].

Then, the children performed an outdoor free running exercise challenge test (ECT) for 8 minutes at >90% of expected heart rate (205-age/2) using a heart rate monitor (Polar, Kempele, Finland) and post-exercise IOS measurements were taken in a manner identical to the baseline measurements.

Finnish population-based, height-adjusted, age-specific reference values were published recently [16] and we used these to calculate all baseline and post-exercise IOS results as height-adjusted z-scores in resistance at 5 Hz (Rrs5), reactance at 5 Hz (Xrs5), frequency dependency of resistance (dRs/df) and resonant frequency (Fres). For the main parameters Rrs5 and Xrs5 a z-score cut-off 1.65SD is considered pathological in the baseline measurement. [16] Bronchial hyper-reactivity was evaluated as changes (Δ) in z-scores of Rrs5 and Xrs5.

As previously published [20], either Rrs5 or Xrs5 was pathological in the baseline IOS in 21 study subjects (20.4%), compared to the reference values. [16] Baseline Rrs5 was pathological in 8 (7.8%) cases and Xrs5 in 19 (18.4%) cases. Bronchial reactivity was considered pathological if the post-exercise Rrs5 change was 35% or more [14,21] and such hyper-reactivity was seen in

5 (4.9%) cases. Irreversible pathological changes of resistance or reactance at 5Hz were only seen in one child.

Genetic studies

The genotyping of SNPs *TLR1* rs5743618, *TLR2* rs5743708 and *TLR 6* rs5743810 has previously been described in detail. [22,23] Polymorphisms of *TLR3* rs3775291 (1234 C/T), *TLR4* rs4986790 (1194 A/G), *TLR7* rs179008 (171 A/T), *TLR8* rs2407992 (2040 C/G), *TLR9* rs187084 (1486 T/C) and *TLR10* rs4129009 (2322 A/G) were selected due to their evident functional properties. (Table 1) The genotyping of *TLR3* rs3775291 (1234 C/T) was performed by pyrosequencing (PSQ™96MA Pyrosequencer, Biotage, Uppsala, Sweden), using a PSQ™96 Pyro Gold Q96 reagent kit according to the manufacturer's protocol. [24] The genotyping of *TLR4* rs4986790 (299 A/G) was performed by pyrosequencing with the ABIPRISM 7000 Sequence Detection System (Applied Biosystems, CA), [25] supplemented later with pyrosequencing (PSQ™96MA Pyrosequencer, Biotage, Uppsala, Sweden), using a PSQ™96 Pyro Gold Q96 reagent kit. [26,27] The genotyping of *TLR8* rs2407992 (2040 C/G) was performed in the same manner as described for *TLR3* rs3775291 (1234 C/T). [24] For *TLR7* rs179008 (171 A/T), the PCR products were first purified using the QIA quick PCR Purification Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. PCR products with low deoxyribonucleic acid (DNA) content were eluted to 30μl of elution buffer. After purification, the PCR products were pipetted to 96-well plate (5μl) together with *TLR7* rs179008 (171 A/T) forward primer (1.6μl) and the 96-well plate was sent to the Institute for Molecular Medicine laboratory in Helsinki, Finland, for sequencing. *TLR9* rs187084 (1486 T/C) genotyping was performed according to Etem et al [28] by using BspTI restriction enzyme (ThermoFischer Scientific, Waltham, USA) for digestion of PCR product. High resolution melting analysis (HMRA) (Roche Diagnostics Light Cycler 480, Basel, Switzerland), was used for genotyping of *TLR10* rs4129009 (2322 A/G). HMRA PCR reactions were run at 95°C for 10 min followed by 45 cycles amplification at 95°C for 10 s, at 59°C for 10 s and at 72°C for 15 s. After PCR process final melting cycle conditions were as outlined by Roche: first heating to 95°C and hold for 1 min, cooling to pre-hold temperature (40°C) to make sure that all PCR products have re-associated and encourages heteroduplex formation. Melting interval for collecting fluorescence from 60°C -95° at ramp rate 0.02°C per second. In each run, known *TLR10* rs4129009 standards (wild type, heterozygote and homozygote) were used.

The PCR and sequencing primers used for the *TLR7*, *TLR8*, *TLR9* and *TLR10* genes are described in Table 1. The primers were purchased from Sigma-Aldrich, Finland.

Statistics

SPSS Statistical Package Version 21 (IBM, NY, USA) was used in the statistical analyses of the data. The IOS outcome parameters were graphically estimated to be normally distributed. The results are expressed as means, standard deviations (SD) and 95% confidence intervals (95% CI) for continuous variables and as numbers and frequencies for categorized variables. Chi-square and Fisher's exact tests were used in the analyses of categorized data. Analysis of co-variance (ANCOVA) was used in the analyses of continuous data and, when appropriate, adjusted for age, sex, RSV vs. non-RSV etiology of bronchiolitis, current BMI z-score, current atopic eczema and the use of ICS medication during the last 12 months. The p-value <0.05 was considered statistically significant.

Since the *TLR7* rs179008 and *TLR8* rs2407992 genes are located in the X chromosome, the analyses were carried out separately for 48 boys and 50 girls. When the data from the boys and

Table 1. Primer sequences, allele and amino acid changes in toll-like receptor (TLR) genes *TLR7* rs179008 (171A/T), *TLR8* rs2407992 (2040C/G), *TLR9* rs187084 (1486T/C) and *TLR10* rs4129009 (2322A/G).*

TLR7 11Gln>Leu (171A/T) (rs179008)
for 5´-AGATGTCTGGTATGTGGTT-3´
rev 5´-TGATTCTTGGTATGTTTTAGA-3´
TLR8 651Leu>Leu (2040C/G) (rs2407992)
for 5´-TGGAAAGCAAGTCCCTGGTA-3´
rev 5´-Biotin-AGTGAGACTCGCTGGCAAAT-3´
seq 5´-ATCCCTTAATAGGCT-3´
TLR9 (silent mutation) (1486T/C) (rs187084)
for 5´-ACTATGGAGCCTGCCTGCCATGATACC-3´
rev 5´-ATCCAGCCTTCTTACAAACCTCCCACC-3´
restriction enzyme BspTI
TLR10 775Ile>Val (2322A/G) (rs4129009)
for 5´-CTTACTGGAACCCATTCCATTCTATTGC-3´
rev 5´-TCAATGTACATCCCAACAGTGTATGTGG-3´
restriction enzyme VspI
TLR10 775Ile>Val (2322A/G) (rs4129009)
for 5´-AGTTCATACATTTCTCTGGTGGCT-3´
rev 5´-GTGGGCTTTTCTGGGCAAAC-3´

**TLR7 SNP was detected by common sequencing, TLR8 SNP by pyrosequencing, TLR9 by digestion and TLR10 by high resolution analysis*

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girls were analyzed together, the male allele carries were combined with female homozygotes or, alternately, with female heterozygotes.

Further genotype combination analyses were carried out for the TLR2 subfamily (*TLR1* rs5743618, *TLR2* rs5743708, *TLR6* rs5743810 and *TLR10* rs4129009).

Ethics

The study was approved by the Ethics Committee of the Tampere University Hospital District, approval number R13025. Informed written consent was obtained from the parents before we enrolled the infants hospitalized for bronchiolitis in infancy and at the control visit at the age of 5 to 7 years. Genetic studies were carried out anonymously and were limited to the evaluation of asthma risk.

Results

The genotypes, presence of major and minor alleles as homozygotes and herezygotes, and minor allele frequencies (MAF) of TLRs 1, 2, 3, 4, 6, 7, 8, 9 and 10 in 98 children hospitalised for bronchiolitis in infancy and MAFs in the Finnish population are presented in Table 2. The MAFs for all nine TLR SNPs in our patients were surprisingly similar to those in the non-selected Finnish population.

TLR 4

In *TLR4* rs4986790, the wild A/A genotype was significantly associated with a greater response to exercise in Rrs5 (0.72 *vs*. -0.42 in those with A/G genotype, p = 0.03). (Table 3) Since there were no cases with the homozygous variant G/G genotype, separate analyses based on the presence or absence of the A or T allele in the child were not possible.

Table 2. Toll-like receptor 1, 2, 3, 4, 6, 7, 8, 9 and 10 genotype and minor allele frequencies in 98 children hospitalised for bronchiolitis and the Finnish population.

SNP (Major>Minor)	Major/Major	Major/Minor	Minor/Minor	MAF	FIN
	(Wild*)	(Variant*)	(Variant*)		
TLR1 rs5743618 (G>T)	0.78	0.18	0.04	0.13	0.17
TLR2 rs5743708 (C>T)	0.94	0.06	0	0.03	0.03
TLR3 rs3775291 (C>T)	0.49	0.41	0.10	0.31	0.33
TLR4 rs4986790 (A>G)	0.85	0.15	0	0.08	0.12
TLR6 rs5743810 (C>T)	0.31	0.45	0.24	0.47	0.42
TLR7 rs179008 (A>T)	0.62 (girls)	0.32 (girls)	0.06 (girls)	0.27 (all)	0.31
	0.81 (boys)	0	0.19 (boys)		
TLR8 rs2407992 (G>C)	0.38 (girls)	0.42 (girls)	0.20 (girls)	0.42 (all)	0.36
	0.56 (boys)	0	0.44 (boys)		
**TLR9 rs187084 (T>C)	0.30	0.44	0.26	0.48	0.45
**TLR10 rs4129009 (A>G)	0.85	0.14	0.01	0.08	0.08

Wild genotype means that minor allele is not presented and variant genotype that minor allele presents either as heterozygous or homozygous, *MAF = minor allele frequency*, *FIN = Finnish MAFs as in* [29]

***n = 97 for TLR9 and TLR10*.

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TLR2 subfamily

In *TLR1* rs5743618 and *TLR2* rs5743708, there were no significant associations between genotypes or presence or absence of major or minor alleles and IOS parameters at baseline or presence or absence of bronchial hyper-reactivity. (Data not shown)

In *TLR6* rs5743810, there were no significant associations between genotypes and IOS parameters at baseline or presence of hyper-reactivity. (Data not shown) However, the presence of the minor T allele was significantly associated with greater responses to exercise in Rrs5 (increase in resistance), when compared with those without T allele. In line, there was a trend that responses in Xrs5 (decrease in reactance) were greater if the minor T allele was present (Table 4)

Table 3. *TLR4* rs4986790 genotypes and baseline IOS (z-scores) and exercise-induced changes (z-scores) in the 98 former bronchiolitis patients.

Parameter	A/A (wild)	p-value*	A/G (variant)
	n = 83		n = 15
	Mean (SD)		Mean (SD)
Rrs5	-0.13 (1.07)	0.15	0.35 (0.91)
Xrs5	-0.74 (1.28)	0.82	-0.66 (0.90)
Fres	2.20 (0.79)	0.43	2.46 (1.05)
dRrs/df	-1.03 (1.15)	0.93	-1.03 (0.96)
ΔRrs5	0.72 (1.81)	**0.03**	-0.42 (1.84)
ΔXrs5	-0.39 (1.35)	0.40	-0.10 (0.90)

Rrs5 = Resistance at 5 Hz; Xrs5 = Reactance at 5 Hz; Fres = resonant frequency; dRs/df = Frequency dependency of resistance

**A/A vs. A/G, adjusted for age, sex, RSV etiology of bronchiolitis, current BMI z-score, current atopic eczema and use of inhaled corticosteroids during the preceding 12 months. p<0.05 marked in bold*.

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Table 4. Presence of *TLR6* rs5743810 genotypes (wild vs. variant) and baseline IOS (z-scores) and exercise-induced changes (z-scores) in the 98 former bronchiolitis patients.

Parameter	C/T or T/T (variant)	p-value*	C/C (wild)
	n = 68		n = 30
	Mean (SD)		Mean (SD)
Rrs5	-0.08 (1.05)	0.62	0.00 (1.07)
Xrs5	-0.71 (1.26)	0.78	-0.77 (1.17)
Fres	2.28 (0.69)	0.55	2.15 (1.10)
dRrs/df	-1.06 (1.05)	0.95	-0.99 (1.26)
ΔRrs5	0.80 (1.90)	**0.04**	-0.03 (1.61)
ΔXrs5	-0.52 (1.37)	0.08	0.05 (0.98)

Rrs5 = Resistance at 5 Hz; Xrs5 = Reactance at 5 Hz; Fres = resonant frequency; dRrs/df = frequency dependency of resistance.

**T-allele present vs. T-allele not present, adjusted for age, sex, RSV etiology of bronchiolitis, BMI z-score, current atopic eczema and use of inhaled corticosteroids during the preceding 12 months. p<0.05 marked in bold.*

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The joint analyses of the *TLR1* rs5743618, *TLR2* rs5743708, *TLR6* rs5743810 and *TLR10* rs4129009 genes were carried out for 97 cases with complete data available, and the analyses were adjusted for age, sex, RSV etiology of bronchiolitis, current BMI z-score, current atopic eczema and the use of ICSs during the last 12 months. Of the 16 possible combinations of wild or variant genotypes, 8 were present in the study population. (Table 5) We found that 20 children had the combination of four wild genotypes as homozygous, and this combination was associated with a smaller response to exercise in Rrs5 (-0.17 *vs*. 0.73, p = 0.049) compared to those 77 with a combination of one or more variant genotypes. In addition, there was a trend of improving Xrs5 (0.12 vs. -0.48, p = 0.07), but the association was not statistically significant. The combination that included the wild *TLR1* rs5743618, *TLR2* rs5743708 and *TLR10* rs4129009 genotypes, together with the variant *TLR6* rs5743810 genotype, was present in 50 children and was associated with a greater response to exercise in Rrs5 (0.91 vs. 0.16, p = 0.043) compared to those 47 children with other genotype combinations. Any other combination of genotypes, including one, two, three or four variant genotypes, did not show significant associations with IOS results. (Table 5)

TLR 3, *7*, *8* and *9*

In *TLR3* rs3775291, there were no significant associations between genotypes or alleles and IOS measurements of baseline lung function or bronchial hyper-reactivity. (Data not shown) In *TLR7* rs179008, the test result showed heterozygosity A/T in one boy, and the case was deleted from the analyses. There were no significant associations between the presence of A or T alleles and any IOS parameter in boys (Data not shown).

The girls with the *TLR7* rs179008 variant heterozygous A/T genotype had the highest Fres and lowest dRrs/df in baseline IOS measurements, but there were no significant findings in hyper-reactivity parameters. (Table 6)

Similarly, girls carrying the A allele had significantly higher baseline Fres and lower dRrs/df compared to the non-carriers of allele A, and there was a trend towards lower Xrs at 5Hz but the association was not statistically significant. No signifcant differences were seen in hyper-reactivity parameters. (Table 7)

Table 5. The joint analyses of the *TLR1* rs5743618, *TLR2* rs5743708, *TLR6* rs5743810 and *TLR10* rs4129009 genotype combinations and baseline (z-scores) and hyper-reactivity IOS measurements (Δz-scores) in 97 children hospitalized for bronchiolitis.

TLR1/TLR2/TLR6/TLR10 genotype combination	n	Rrs5 Mean SD	Xrs5 Mean SD	Fres Mean SD	dRrs/df Mean SD	ΔRrs5 Mean SD	ΔXrs5 Mean SD
wild/wild/wild/wild	20	0.14	-0.81	2.31	-1.22	**-0.17***	0.12
		(1.19)	(1.19)	(1.05)	(1.40)	**(1.70)**	(1.16)
variant/wild/wild/wild	3	-0.21	-0.64	2.12	-0.94	0.20	-0.21
		(0.77)	(0.39)	(0.12)	(0.31)	(1.07)	(0.68)
wild/wild/variant/wild	50	-0.26	-0.71	2.23	-1.01	**0.91****	-0.53
		(1.08)	(1.38)	(0.69)	(1.09)	**(1.87)**	(1.30)
variant/wild/variant/wild	3	0.86	-1.38	3.02	1.93	1.47	-1.44
		(1.07)	(0.75)	(0.93)	(0.80)	(2.83)	(3.44)
variant/wild/wild/variant	7	-0.31	-0.71	1.69	-0.35	0.25	-0.06
		(0.74)	(1.43)	(1.42)	(0.86)	(1.67)	(0.44)
wild/variant/variant/wild	5	0.64	-0.64	2.29	-0.89	-0.37	-0.17
		(0.41)	(0.91)	(0.50)	(1.18)	(0.98)	(0.87)
variant/wild/variant/variant	8	0.14	-0.52	2.28	-1.20	0.65	-0.48
		(0.93)	(0.94)	(0.72)	(0.95)	(2.42)	(1.36)
variant/variant/variant/wild	1	0.89	-0.85	2.59	-1.09	0.81	-0.78

Rrs5 = Resistance at 5 Hz; Xrs5 = Reactance at 5 Hz; Fres = Resonant frequency, dRs/df = Frequency dependency of resistance

**p = 0.049*

***p = 0.043 vs. all other genotype combinations, as adjusted for age, sex, RSV etiology of bronchiolitis, current BMI z-score, current atopic eczema and use of inhaled corticosteroids during the preceding 12 months.*

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We also carried out an analysis that included the boys and girls in the same model. There were no significant associations with the IOS results when we compared 1) the 70 children in the male A and female A/A group with the 28 children in the male T and female TT group and 2) the 86 children in the male A, female A/A and female A/T group and the 12 children in the the male T and female TT group. (Data not shown)

Table 6. *TLR7* rs179008 genotypes and baseline IOS (z-scores) and exercise-induced changes (z-scores) in the 50 former bronchiolitis cases who were girls.

Parameter	A/A (wild) n = 31 Mean (SD)	A/T (variant) n = 16 Mean (SD)	T/T (variant) n = 3 Mean (SD)	p-value*
Rrs5	-0.26 (0.96)	0.28 (1.08)	-0.12 (1.10)	0.30
Xrs5	-0.57 (1.14)	-0.90 (0.78)	0.49 (0.56)	0.17
Fres	2.15 (0.84)	2.53 (0.59)	0.89 (1.38)	**0.02**
dRrs/df	-0.85 (0.82)	-1.36 (1.34)	0.61 (0.49)	**0.01**
ΔRrs5	0.67 (1.59)	0.39 (2.40)	0.23 (2.22)	0.58
ΔXrs5	-0.32 (1.13)	0.00 (1.19)	0.14 (0.57)	0.42

Rrs5 = Resistance at 5 Hz; Xrs = Reactance at 5 Hz; Fres = resonant frequency; dRrs/df = frequency dependency of resistance.

**between the three groups p, adjusted for age, RSV etiology of bronchiolitis, current BMI z-score, current atopic eczema and use of inhaled corticosteroids during the preceding 12 months. p<0.05 marked in bold*

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Table 7. Presence of *TLR7* rs179008 major allele A and baseline IOS results (z-scores) and exercise-induced changes (z-scores) in the 50 former bronchiolitis cases who were girls.

Parameter	A-allele (major) present	p-value*	A-allele not present
	n = 47		n = 3
	Mean (SD)		Mean (SD)
Rrs5	-0.08 (1.03)	0.90	-0.12 (1.10)
Xrs5	-0.68 (1.03)	0.08	0.49 (0.56)
Fres	2.28 (0.78)	**0.01**	0.89 (1.38)
dRrs/df	-1.03 (1.04)	**0.01**	0.61 (0.49)
ΔRrs5	0.57 (1.88)	0.61	0.57 (1.88)
ΔXrs5	-0.21 (1.15)	0.68	0.14 (0.57)

Rrs5 = Resistance at 5 Hz; Xrs = Reactance at 5 Hz; Fres = resonant frequency; dRrs/df = frequency dependency of resistance

**A-allele present vs. A-allele not present, adjusted for age, RSV etiology of bronchiolitis, current BMI z-score, current atopic eczema and use of inhaled corticosteroids during the preceding 12 months. p<0.05 marked in bold.*

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In *TLR8* rs2407992, there were no significant associations between the presence of the G or C alleles and any baseline IOS parameters or hyper-reactivity parameters in boys or in girls (Data not shown).

The analyses were also carried out by including the boys and girls in the same model. There were no significant associations with the IOS results when we compared 1) the 46 children in the male G and female G/G group and the 52 children in the male C, female C/C and female G/C group and 2) the 67 children in the male G, female G/G and female G/C group and 31 children in the male C and female C/C group. (Data not shown)

In *TLR9* rs187084, there were no significant associations between genotypes or presence of alleles and IOS results in baseline lung function or bronchial hyper-reactivity measurements. (Data not shown)

Discussion

This was an exploratory case-control study on lung function measured with IOS at a median age of 6.3 years in 103 children, who were prospectively followed-up since hospitalization for bronchiolitis at less than 6 months of age. The aim of the study was to explore if there are any associations between the TLRs encoding genes and lung function. There are two key findings. Firstly, *TLR4* and *TLR6* polymorphisms were associated with responses to exercise, but not with baseline lung function by IOS. Secondly, polymorphism in the X-chromosomally inherited *TLR7* was associated with abnormal baseline lung function by IOS in girls. This study reveals some novel but preliminary associations of TLR genetics and lung dysfunction in a high-risk post-bronchiolitis cohort.

TLRs are conserved pattern-recognizing proteins that function in the first line of innate immunity, either promoting or enhancing inflammatory processes and influencing the orientation of immunity into Th1 or Th2 directions. [30] In newborn infants, the Th1/Th2 balance is Th2-oriented and in atopy, the normal shift to the Th1-oriented balance does not happen, which leads to Th2 dominance and immunoglobulin E (IgE) production. [6] For instance, polymorphism in *TLR4* rs4986790 (Asp299Gly) was first seen to be associated with decreased airway reactivity to inhaled gram-negative bacterial lipopolysaccharide. [31] In later studies,

exposures to environmental lipopolysaccharides in childhood were associated with less atopy, atopic asthma and rhinitis at school age. [10] Our finding that the *TLR4* rs4986790 wild type genotype was associated with some non-beneficial change in response to exercise suggests that polymorphism A to G may play a protective role controlling the emergence of hyper-reactivity.

A recent meta-analysis of TLR6 genetics provided inconsistent results about the association of *TLR6* polymorphisms with childhood asthma. [9] Polymorphism in *TLR6* rs5743810 was associated with atopic asthma in some studies, but most of the associations were non-significant or even protective from asthma. [9] Previous research in this cohort found an association between *TLR6* rs5743810 minor allele T and atopic eczema at 5 to 7 years of age. [22] Our present results suggest an association between *TLR6* rs5743810 C to T polymorphism and bronchial hyper-reactivity. Interestingly, the finding was robust to adjustment with atopic eczema, which suggests that the association between TLR6 and bronchial hyper-reactivity is not dependent on atopy.

TLR6 forms functional complexes with TLR1,TLR2, and TLR10, and therefore, we further analysed different combinations of the *TLR1*, *TLR2*, *TLR6* and *TLR10* genotypes. These supplementary analyses showed that the *TLR6* variant genotype was associated with bronchial hyper-reactivity jointly with the wild *TLR1*, *TLR2* and *TLR10* genotypes. In addition, the combination of the four wild genotypes *TLR1*, *TLR2*, *TLR6* and *TLR10* was associated with less airway reactivity. This suggests that wild *TLR2* subfamily genotypes may even be protective for later airway hyper-reactivity, and, in particular, *TLR6* rs5743810 variant genotypes may be associated with increased airway reactivity after infant bronchiolitis. The result is in line with our previous clinical observations in the same cohort. Only two children (8%) with wild genotypes in the *TLR1*, *TLR2* and *TLR6* genes had asthma during the first six years of life, compared to 30% in those with variant genotypes. [22]

The endoplasmic retinaculum TLRs 3, 7, 8 and 9 have not been as widely studied as other TLRs, although they play an important role in detecting respiratory viruses, such as single strand RNA structures in RSV and rhinoviruses. [5] There is some evidence that polymorphisms of *TLR7* and *TLR8* genes may have an association with asthma in adolescents and adults. [32] That association was not gender-specific, although some *TLR7* and *TLR8* genes, like *TLR7* rs179008 and *TLR8* rs2407992, which were applied in the present study, are located in the X chromosome. In the present lung function study, there were no significant associations between *TLR7* or *TLR8* polymorphisms and lung function or airway reactivity by IOS in boys, but some associations were found in girls. In *TLR7* rs179008, the variant A/T genotype was associated with highest baseline Fres and lowest dRs/df, reflecting mild peripheral obstruction or dynamic restriction. The same changes were seen with presence of the *TLR7* rs179008 major allele A. This finding might reflect the lability of the small airways at preschool age, and although there were no significant differences in the main parameters, the reactance at 5Hz was lowest in those having the allele A, which suggests decreased lung elastic properties. These are preliminary findings of gender-specific structural differences in the airways or gender-specific differences in the inflammatory processes regulated by the *TLR7* gene.

The main shortcoming of this study was the small number of cases for a genetic study. In addition, the cohort size was insufficient for further stratified analyses of defined risk groups. Therefore, there is a risk of type-two statistical errors. On the other hand, analyzing many different IOS parameters as continuous variables between different genotype-specific and allele-specific subgroups increased the risk of type-one statistical errors. The 12 (12.2%) children who were using ICSs at the time of the study were included in the analyses, which might explain our somewhat lower figures for post-exercise hyper-reactivity. On the other hand, the analyses were adjusted for the use of ICSs, and when we compared the results of adjusted and non-adjusted analyses, the conclusions were similar.

We explored the possible associations of polymorphisms of 9 different TLRs, including genotypes and haplotypes in separate analyses, and used 4 lung function outcomes and 2 airway reactivity outcomes. This means 72 individual tests for lung function and 36 for airway reactivity outcomes. We considered our study as an exploratory study to find preliminary evidence for associations, if present, which needs to be confirmed or rejected in coming confirmatory studies. Therefore, we did not regard any multiplicity adjustments necessary. [33,34]

The strengths of our study were the long, prospective, 5 to 7 years follow-up after hospitalization for viral bronchiolitis in infancy. Though the present design was a case-control study, the long prospective follow-up upgraded the reliability of the data on confounding and disease modifying factors. In addition, the material is unique since the follow-up started before six months of age. At preschool age, we were able to determine lung function by IOS in more than 95% of the participants. All study subjects were of Finnish origin, which is a benefit in genetic studies. The IOS results were compared to new, up-to-date, national reference values that were population-based, height-adjusted and age-specific. [16]

Conclusions

In conclusion, the *TLR1*, *TLR2*, *TLR3*, *TLR4*, *TLR6*, *TLR8*, *TLR9* or *TLR10* polymorphisms that we studied did not show significant associations with lung function, as measured by IOS at five to seven years of age after infant bronchiolitis. However, we found in this exploratory study that *TLR4*, and *TLR6* in particular, might be associated with airway reactivity, and that in girls *TLR7* might be associated with decreased lung function. To confirm these preliminary results the corresponding hypotheses need to be tested in further confirmatory studies

Supporting Information

S1 Dataset. 98 children at age 5–7 years after bronchiolitis.
(XLSX)

Author Contributions

Conceived and designed the experiments: PK KN JOT MH QH MK. Performed the experiments: PK JV JT KN JOT. Analyzed the data: EL PK MK. Contributed reagents/materials/analysis tools: JOT MH QH MK. Wrote the paper: EL PK JV JT KN MH QH MK.

References

1. Pruikkonen H, Uhari M, Dunder T, Pokka T, Renko M. Infants under 6 months with bronchiolitis are most likely to need major medical interventions in the 5 days after onset. Acta Paediatr. 2014; 103: 1089–1093. doi: 10.1111/apa.12704 PMID: 24862359
2. Hall CB, Weinberg GA, Iwane MK, Blumkin AK, Edwards KM, Staat MA, et al. The burden of respiratory syncytial virus infection in young children. N Engl J Med. 2009; 360: 588–598. doi: 10.1056/NEJMoa0804877 PMID: 19196675
3. Stockman LJ, Curns AT, Anderson LJ, Fischer-Langley G. Respiratory syncytial virus-associated hospitalizations among infants and young children in the United States, 1997–2006. Pediatr Infect Dis J. 2012; 31: 5–9. doi: 10.1097/INF.0b013e31822e68e6 PMID: 21817948
4. Akira S, Uematsu S, Takeuchi O. Pathogen recognition and innate immunity. Cell. 2006; 124: 783–801. PMID: 16497588
5. Parker D, Prince A. Innate immunity in the respiratory epithelium. Am J Respir Cell Mol Biol. 2011; 45: 189–201. doi: 10.1165/rcmb.2011-0011RT PMID: 21330463
6. Hansel TT, Johnston SL, Openshaw PJ. Microbes and mucosal immune responses in asthma. Lancet. 2013; 381: 861–873. PMID: 23428115
7. Tapping RI, Omueti KO, Johnson CM. Genetic polymorphisms within the human Toll-like receptor 2 subfamily. Biochem Soc Trans. 2007; 35: 1445–1448. PMID: 18031242

8. Ramet M, Korppi M, Hallman M. Pattern recognition receptors and genetic risk for rsv infection: value for clinical decision-making? Pediatr Pulmonol. 2011; 46: 101–110. doi: 10.1002/ppul.21348 PMID: 20963841
9. Klaassen EM, Thonissen BE, van Eys G, Dompeling E, Jobsis Q. A systematic review of CD14 and toll-like receptors in relation to asthma in Caucasian children. Allergy Asthma Clin Immunol. 2013; 9: 10-1492-9-10.
10. Braun-Fahrlander C, Riedler J, Herz U, Eder W, Waser M, Grize L, et al. Environmental exposure to endotoxin and its relation to asthma in school-age children. N Engl J Med. 2002; 347: 869–877. PMID: 12239255
11. Prefontaine D, Banville-Langelier AA, Fiset PO, Guay J, An J, Mazer M, et al. Children with atopic histories exhibit impaired lipopolysaccharide-induced Toll-like receptor-4 signalling in peripheral monocytes. Clin Exp Allergy. 2010; 40: 1648–1657. doi: 10.1111/j.1365-2222.2010.03570.x PMID: 20636402
12. Dubois AB, Brody AW, Lewis DH, Burgess BF Jr. Oscillation mechanics of lungs and chest in man. J Appl Physiol. 1956; 8: 587–594. PMID: 13331841
13. Marotta A, Klinnert MD, Price MR, Larsen GL, Liu AH. Impulse oscillometry provides an effective measure of lung dysfunction in 4-year-old children at risk for persistent asthma. J Allergy Clin Immunol. 2003; 112: 317–322. PMID: 12897737
14. Beydon N, Davis SD, Lombardi E, Allen JL, Arets HG, Aurora P, et al. An official American Thoracic Society/European Respiratory Society statement: pulmonary function testing in preschool children. Am J Respir Crit Care Med. 2007; 175: 1304–1345. PMID: 17545458
15. Oostveen E, MacLeod D, Lorino H, Farre R, Hantos Z, Desager K, et al. The forced oscillation technique in clinical practice: methodology, recommendations and future developments. Eur Respir J. 2003; 22: 1026–1041. PMID: 14680096
16. Malmberg LP, Pelkonen A, Poussa T, Pohianpalo A, Haahtela T, Turpeinen M. Determinants of respiratory system input impedance and bronchodilator response in healthy Finnish preschool children. Clin Physiol Funct Imaging. 2002; 22: 64–71. PMID: 12003103
17. Koponen P, Helminen M, Paassilta M, Luukkaala T, Korppi M. Preschool asthma after bronchiolitis in infancy. Eur Respir J. 2012; 39: 76–80. doi: 10.1183/09031936.00040211 PMID: 21700604
18. Nuolivirta K, Hurme M, Halkosalo A, Koponen P, Korppi M, Vesikari T, et al. Gene polymorphism of IFNG +874 T/A and TLR4 +896 A/G and recurrent infections and wheezing in toddlers with history of bronchiolitis. Pediatr Infect Dis J. 2009; 28: 1121–1123. doi: 10.1097/INF.0b013e3181af37ee PMID: 19773677
19. Smith H, Reinhold P, Goldman M. Chapter 5. Forced oscillation technique and impulse oscillometry. European Respiratory Society Monograph. 2005; 31: 75–105.
20. Lauhkonen E, Koponen P, Nuolivirta K, Paassilta M, Toikka J, Korppi M. Lung function by impulse oscillometry at age 5–7 years after bronchiolitis at age 0–6 months. Pediatr Pulmonol. 2015; 50: 389–395. doi: 10.1002/ppul.23039 PMID: 24668616
21. Malmberg LP, Makela MJ, Mattila PS, Hammaren-Malmi S, Pelkonen AS. Exercise-induced changes in respiratory impedance in young wheezy children and nonatopic controls. Pediatr Pulmonol. 2008; 43: 538–544. doi: 10.1002/ppul.20805 PMID: 18433041
22. Koponen P, Vuononvirta J, Nuolivirta K, Helminen M, He Q, Korppi M. The association of genetic variants in toll-like receptor 2 subfamily with allergy and asthma after hospitalization for bronchiolitis in infancy. Pediatr Infect Dis J. 2014; 33: 463–466. doi: 10.1097/INF.0000000000000253 PMID: 24445834
23. Ahmad-Nejad P. Pyrosequencing of toll-like receptor polymorphisms of functional relevance. Methods Mol Biol. 2009; 496: 73–87. doi: 10.1007/978-1-59745-553-4_7 PMID: 18839106
24. Nuolivirta K, He Q, Vuononvirta J, Koponen P, Helminen M, Korppi M. Toll-like receptor 3 L412F polymorphisms in infants with bronchiolitis and postbronchiolitis wheezing. Pediatr Infect Dis J. 2012; 31: 920–923. doi: 10.1097/INF.0b013e31825aff25 PMID: 22549436
25. Helminen M, Nuolivirta K, Virta M, Halkosalo A, Korppi M, Vesikari T, et al. IL-10 gene polymorphism at -1082 A/G is associated with severe rhinovirus bronchiolitis in infants. Pediatr Pulmonol. 2008; 43: 391–395. doi: 10.1002/ppul.20793 PMID: 18286551
26. Vuononvirta J, Toivonen L, Grondahl-Yli-Hannuksela K, Barkoff AM, Lindholm L, Mertsola J, et al. Nasopharyngeal bacterial colonization and gene polymorphisms of mannose-binding lectin and toll-like receptors 2 and 4 in infants. PLoS One. 2011; 6: e26198. doi: 10.1371/journal.pone.0026198 PMID: 22022564
27. Grondahl-Yli-Hannuksela K, Vuononvirta J, Barkoff AM, Viander M, Van Der Meeren O, Mertsola J, et al. Gene polymorphism in toll-like receptor 4: effect on antibody production and persistence after

acellular pertussis vaccination during adolescence. J Infect Dis. 2012; 205: 1214–1219. doi: 10.1093/infdis/jis182 PMID: 22383676

28. Etem EO, Elyas H, Ozgocmen S, Yildirim A, Godekmerdan A. The investigation of toll-like receptor 3, 9 and 10 gene polymorphisms in Turkish rheumatoid arthritis patients. Rheumatol Int. 2011; 31: 1369–1374. doi: 10.1007/s00296-010-1472-8 PMID: 20422193

29. 1000 Genomes Project Consortium, Abecasis GR, Auton A, Brooks LD, DePristo MA, Durbin RM, et al. An integrated map of genetic variation from 1,092 human genomes. Nature. 2012; 491: 56–65. doi: 10.1038/nature11632 PMID: 23128226

30. Cook DN, Pisetsky DS, Schwartz DA. Toll-like receptors in the pathogenesis of human disease. Nat Immunol. 2004; 5: 975–979. PMID: 15454920

31. Arbour NC, Lorenz E, Schutte BC, Zabner J, Kline JN, Jones M, et al. TLR4 mutations are associated with endotoxin hyporesponsiveness in humans. Nat Genet. 2000; 25: 187–191. PMID: 10835634

32. Moller-Larsen S, Nyegaard M, Haagerup A, Vestbo J, Kruse TA, Borglum AD. Association analysis identifies TLR7 and TLR8 as novel risk genes in asthma and related disorders. Thorax. 2008; 63: 1064–1069. doi: 10.1136/thx.2007.094128 PMID: 18682521

33. Bender R, Lange S. Adjusting for multiple testing—when and how? J Clin Epidemiol. 2001; 54: 343–349. PMID: 11297884

34. Streiner DL. Best (but oft-forgotten) practices: the multiple problems of multiplicity-whether and how to correct for many statistical tests. Am J Clin Nutr. 2015; 102: 721–728. doi: 10.3945/ajcn.115.113548 PMID: 26245806

Following Up Infant Bronchiolitis Patients Provided New Evidence For and Against the United Airway Disease Hypothesis

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Tables 1-4

ABSTRACT

Aim The united airway disease (UAD) hypothesis suggests that allergic rhinitis and asthma develop together. We evaluated the evidence for and against the UAD hypothesis at five to seven years of age after hospitalisation for bronchiolitis at less than six months.

Methods This study used prospective follow-up data for 102 children hospitalised for bronchiolitis under the age of six months. We included the presence of previous and current asthma, prolonged rhinitis and skin prick tests (SPT) to common inhaled allergens and lung function by impulse oscillometry (IOS) at five to seven years of age. Bronchial hyper-reactivity (BHR) was assessed using the exercise challenge test and bronchodilation test.

Results Current asthma, but not previous transient asthma, was associated with prolonged rhinitis and a positive SPT. BHR reflecting reactive airways, but not lung function, was associated with the combination of current asthma, prolonged rhinitis and a positive SPT called together as respiratory allergy.

Conclusion This post-bronchiolitis follow-up study suggested an association between respiratory allergy and reactive airways at five to seven years of age, which supported the UAD hypothesis. However, previous transient asthma and a reduction in lung function reduction did not support the hypothesis.

Keywords: Allergic rhinitis; Asthma; Bronchiolitis; Pulmonary function; United airway disease.

Key Notes

- The united airway disease (UAD) hypothesis suggests that allergic rhinitis and asthma develop together and we evaluated the evidence for the hypothesis after infant bronchiolitis at less than six months.
- Our findings suggested an association between respiratory allergy and reactive airways at five to seven years of age, which supported the UAD hypothesis.
- However, previous transient asthma and a reduction in lung function did not support the hypothesis.

INTRODUCTION

The united airway disease (UAD) hypothesis suggests that upper and lower airways share common pathogenetic mechanisms in allergic rhinitis and asthma, but the clinical manifestations differ.(1-3) It also maintains that allergic rhinitis and asthma often develop one after another or even at the same time. This hypothesis is reflected in the *Allergic rhinitis and its impact on asthma* (ARIA) document, which recommends that the presence of allergic rhinitis must be considered, and treated if present, in children with asthma and vice-versa.(4,5)

Atopy is genetically determined, but clinical manifestations and their occurrence depend on age.(6) Sensitisation to food allergens develops during infancy and the most common clinical manifestation is atopic dermatitis, while sensitisation to inhaled allergens starts to occur during the second or third year of life, leading to allergic rhinitis. In Finland, and similar countries such as Sweden and Germany, the incidence of allergic rhinitis in non-selected populations is 5-10% at six to seven years and 23-31% at 13 to 14 years. (7) In line with this, the incidence of allergic sensitisation to inhaled allergens, assessed by a positive skin prick test (SPT), is 10-20% at five to six years and 20-40% at 11 years.(8,9)

As published previously in this post-bronchiolitis cohort, 27.9% of the children had ever had a diagnosis of asthma and 12.7% had a current asthma at five to seven years of age.(10) Lung function by impulse oscillometry (IOS) showed small airways obstruction in the baseline measurements, being abnormal in 20% compared to Finnish population data, and that 16% had bronchial hyper-reactivity (BHR). (11)

This paper presents a secondary analysis of the data on the 102 children and reports on the information available on asthma, prolonged rhinitis, SPT results and baseline, post-exercise and post-bronchodilator IOS results. Previous papers on this cohort have not included data on rhinitis or SPT results. We aimed to evaluate evidence for and against the UAD hypothesis at five to seven years of age in children who had been hospitalised for bronchiolitis when they were less than six months of age. We analysed whether there are significant associations between previous or current asthma, current prolonged rhinitis, current SPT positivity, current reduced lung function assessed by IOS, and current BHR, reflecting reactive airways, based on IOS responses to exercise and, or, bronchodilation.

PATIENTS AND METHODS

We prospectively followed up 166 healthy, term children who had been hospitalised for bronchiolitis before six months of age until they were five to seven years of age. Of these, 127 attended the follow-up visit, which consisted of a clinical examination by a paediatrician, data collection using a structured questionnaire and an interview, skin prick tests (SPT) to common inhaled allergens and lung function measurements, including testing for BHR.(10)

Of these 127 children, complete IOS measurements at baseline and after the exercise challenge test (ECT) and bronchodilation (BD) test were available in 103 children, as previously published.(11) One child with no history of prolonged rhinitis or asthma refused to undergo the SPT. This meant that complete data was available from 102 children on current or previous asthma, current lung function measured using IOS, current BHR in the ECT and BD tests, current SPT results and the presence or absence of prolonged rhinitis during the last 12 months. These 102 children formed our final study population.

The IOS outcome variables resistance (Rrs5) and reactance at 5Hz (Xrs5) were transformed to height-adjusted standard deviation (SD) Z-scores in order to describe the difference from the population predicted value.(12-14) In the baseline IOS, the limit of pathological Rrs5 was +1.65 SD and the limit of pathological Xrs5 was -1.65 SD representing the upper and lower 95% confidence intervals of the Z-score scale, respectively. (12) BHR was considered to be present if the child presented with a rise of at least 35% in Rrs5 in the ECT (n=5) or with a decrease of at least 35% in Rrs5 in the BD test (n=11), compared to baseline values.(12-15)

We categorised 13 children as having current asthma because they had been on continuous or intermittent inhaled corticosteroid (ICS) medication during the last 12 months. Another two were added to the list because BHR was diagnosed at the follow-up visit and there were asthma-presumptive symptoms during the last 12 months, such as wheezing or prolonged cough for more than four weeks or a night cough when no infection was present. The symptoms and the consumption of medicines were covered in the questionnaire and checked during the interview. Children were classified as having previous transient asthma if they had previously used ICS medication, but not during the past 12 months.

The diagnosis of current prolonged rhinitis was based on the answers given in the questionnaire and interview. The questionnaire included questions on rhinitis symptoms and their occurrence due to the season and the presence or absence of infection. Prolonged rhinitis was considered to be present if allergic rhinitis had been diagnosed by a doctor or the children had suffered from a runny, sneezing or stuffy nose during the last 12 months when they didn't have an infection, in connection with seasonal pollen exposure or contact with animals.

Skin prick tests (SPT) were performed using standardised extracts (ALK, Copenhagen, Denmark) for the outdoor allergens of birch, timothy grass and mugwort pollen and spores of the mould *Alternaria alternata*, for cat and dog dander and for the house dust mites *Dermatophagoides pteronyssimus* and *D. farinae*. We used 10 mg/ml of histamine hydrochloride as a positive control and glycerol as a negative control. One drop of each allergen extract was applied to healthy forearm skin and punctuated with a sharp lancet and an experienced nurse measured the reaction after 15 minutes. The test was positive if the skin wheal reached a diameter of 3mm. Antihistamine medication had to be discontinued for five or more days before the SPTs.

Statistics

SPSS version 21 (IBM Corp, New York, USA) was used in the statistical analyses. Pearson's chi-square test or Fischer´s exact test were used, as appropriate, to analyse categorised variables. ANOVA was used to analyse continuous variables in order to compare differences in subgroups. The Bonferroni correction was used for paired comparisons, by multiplying the p value by two, when only two of the three groups were included

Ethics

The Ethics Committee of the Tampere University Hospital district approved the study and written, informed consent was obtained from the parents during the hospitalisation for bronchiolitis and at the control visit at five to seven years of age.

RESULTS

Rhinitis and asthma

Current asthma was present in 15 (14.7%) of the 102 children and prolonged rhinitis was present in 27 (26.5%) children. We also found that eight of the 15 children (53.3%) with current asthma had prolonged rhinitis, compared with 21.8% of the 87 children without current asthma (p=0.016), and that eight of the 27 children (29.6%) with prolonged rhinitis had current asthma, compared with 9.3% of the 75 children without. On the other hand, 46.7% of the children with current asthma did not have any rhinitis and 70.4% of the children with prolonged rhinitis did not have current asthma.

Current asthma (53.3%) and prolonged rhinitis (62.9%) were significantly associated with a positive SPT to inhaled allergens (Table 1) and the most common responses were to birch and timothy pollen, followed by dog and cat dander. In addition, five children were allergic to dust mites, mugwort pollen and mould spores. However, we found that only 53.1% of those who tested positive to inhaled allergens had prolonged rhinitis and only 25.0% had current asthma (Table 1).

There were 12 children who presented with previous transient childhood asthma without current asthma at five to seven years of age. Only two (16.7%) of them had prolonged rhinitis and neither were SPT positive. In

comparison 22.7% of the 75 children with no previous or current asthma had rhinitis (p=0.972) and 32.0% had a positive SPT (p=0.0016).

Lung function, reactive airways and asthma

The proportions of children with either increased Rrs5 or decreased Xrs5 in their baseline IOS were not significantly different in children with current asthma, previous transient asthma or no asthma (Table 2). There was a significant association between current asthma and BHR. (Table 2)

We found that eight children had pathologic Rrs5 in their baseline IOS, but none of them had a pathologic 35% Rrs5 increase in their ECT. However, four (50.0%) of them had a pathological 35% decrease of Rrs5 in their BD test compared with 7.4% of the 94 children with a normal baseline Rrs5 (p=0.004). Similarly, 19 children had pathologic Xrs5 in their baseline IOS and none had a pathological 35% increase in Rrs5 in their ECT. However, four (21.4%) of them had a pathologic 35% decrease of Rrs5 in their BD test compared to 16.5% of the 84 children with a normal baseline Xrs5 (p=0.120).

Lung function, reactive airways and rhinitis

We analysed the IOS and BHR results in relation to the presence of prolonged rhinitis and, or, SPT positivity (Table 3) and this showed that pathologic results in the baseline IOS were not associated with prolonged rhinitis or with SPT positivity. Instead, BHR was associated with SPT positivity and with the simultaneous presence of prolonged rhinitis and SPT positivity (Table 3).

Respiratory allergy

Respiratory allergy was defined by combining current asthma, prolonged rhinitis and a positive SPT to provide four different definitions: 1) all three elements 2) current asthma plus either prolonged rhinitis or a positive SPT, 3) current asthma on its own or rhinitis combined with a positive SPT and 4) at least one of the three elements (Table 4). Baseline IOS was not associated with respiratory allergy by any of the definitions. Reactive airways, that is BHR, was associated with the first and most strict definition, and with two more lenient definitions. (Table 4).

DISCUSSION

Three main results emerged from this study that provided evidence for or against the UAD hypothesis, based on infants who were hospitalised for bronchiolitis under the age of six months and then followed up at five to seven years of age. Firstly, current asthma was associated with prolonged rhinitis and skin test positivity, but previous asthma was not. Secondly, baseline lung function, measured by IOS, showed no significant association with rhinitis, a positive SPT or respiratory allergy, which combined prolonged rhinitis, a positive SPT and current asthma. Thirdly, BHR, which was established by the ECT or BD test, was associated with rhinitis, a positive SPT and respiratory allergy.

In this cohort, asthma at five to seven years of age was strongly associated with prolonged rhinitis and, or, SPT positivity to inhaled allergens, in line with the UAD hypothesis.(1-3) On the other hand, nearly half of the children with current asthma did not have prolonged rhinitis or a positive SPT, which does not support the UAD hypothesis. Although five to seven years old is an appropriate age at which to study allergy and allergic sensitisation to inhaled allergens, new allergies also develop after that age and new UAD cases can be diagnosed at a later age. Another study showed that a positive SPT was present in 15% of children at the age of five but that this prevalence had increased to 39% at 11 years of age. In addition, new sensitivities emerged in 23% of the children over those six years.(9)

There was also a strong association between BHR, prolonged rhinitis and SPT positivity for inhaled allergens, in line with the UAD hypothesis.(1-3) This observation means that the development of allergies to inhaled allergens after infant bronchiolitis happens jointly with continuing or emerging airway reactivity, in line with the UAD hypothesis.(1-3) On the other hand, one-third of the BHR cases were not associated with rhinitis or SPT positivity, at least by the age of five to seven. The nine children who had BHR, but

not asthma, formed an interesting group: seven (78%) of them had either prolonged rhinitis or were sensitised to inhaled allergens and it is very probable that they would have become asthmatic at a later age.(16,17)

Reactive airways, assessed in this study by BHR, has been closely associated with rhinitis in childhood, with a BHR prevalence of 30-50% in children with allergic rhinitis and 25% in non-allergic rhinitis.(18,19) In a study of children aged three to five years, the severity of rhinitis was strongly associated with wheezing.(20) In another study allergic rhinitis until age of five years predicted a subsequent wheezing onset. (21) A review concluded that many allergic rhinitis patients had subclinical BHR and that allergic rhinitis often preceded the development of asthma.(17) In line with these findings, our current study revealed that prolonged rhinitis, a positive SPT and respiratory allergy, which combined current asthma, rhinitis and a positive SPT, were significantly associated with reactive airways in former bronchiolitis patients.

We did not find any significant associations between baseline lung function and prolonged rhinitis, SPT positivity or respiratory allergy. This means that baseline lung function disorder does not belong to the UAD entity, in contrast to BHR, or possibly that this connection emerges later than at five to seven years of age. However, this is unlikely as the Tucson and Denmark birth cohorts both showed that lung function disorders developed by the age of six to seven years. (22,23)

Flow-volume spirometry is better established than IOS for examining lung function in children, including tests for BHR (13,14) but may be unreliable at the age of five to seven years because of the need for long forced expiratory blows. (13,24,25) IOS has been shown applicable in children from two to three years of age onwards, (26-28)and the method may be even superior in detecting reversible obstruction compared to FVS in children. (29) A Finnish study showed that IOS in 158 wheezing children at the age of 2-7 years accurately predicted flow-volume spirometry results at 12-18 years of age. (30) Increased baseline Rrs5 in IOS in that study was also related to a six-fold risk of pathologic post-BD forced expiratory volume in 1 second in later flow-volume spirometry. (30)

In our study, the 12 children with previous asthma who wheezed and used ICS but stopped wheezing by five to seven years of age, provided strong but indirect evidence for the UAD hypothesis. Only 16.7% reported prolonged rhinitis, none were sensitised to inhaled allergens and none were shown to have BHR by the ECT or BD tests. The most likely explanation is that they stopped wheezing because their respiratory allergy and subsequent airway reactivity did not develop. However, 33.3% of them presented with decreased lung function, in particular low baseline reactance, reflecting small airways obstruction and/or diminished lung elastic properties. This was in line with the theory that an early infection with RSV, which was the main causative agent of bronchiolitis in our cohort, was more strongly associated with a restrictive than obstructive pattern of later lung function. (31)

The small number of patients was a clear weakness of the study, as it increased the chance of type two errors, and the lack of a control group was also a weakness which lessens the generalizability of our results. In addition, our study population was highly selective and only included bronchiolitis patients who were hospitalised before six months of age. Thus careful consideration has to be undertaken when interpreting the results, and it is clear that conclusions cannot be transferred straight forward to the non-selected population. We used the term bronchial hyper-reactivity (BHR) as combined to reflect reactive airways, though to be exact, exercise induced bronchoconstriction and bronchodilator responsiveness represent different physiological phenomena. However they both reflect a risk of later asthma.

The strengths of our study were the prospective design, the long follow-up period and careful data collection during and after bronchiolitis in early childhood and at the age of five to seven years. The lung function results were based on highly professional analysis of the IOS findings and were compared to population-based, age-specific, height-adjusted reference values. (12) In

addition, the definition of allergy was not just based on symptoms, but also on objective evidence of sensitisation to inhaled allergens in the SPTs. The history of prolonged rhinitis was based on parental reports, but it was obtained from structured questionnaires and confirmed by clinical interviews.

CONCLUSION

This study found a significant association between allergic sensitisation, prolonged rhinitis, BHR reflecting reactive airways, and asthma in five to seven-year-old children who were hospitalised for bronchiolitis when they were less than six months of age. This means that we were able to document new evidence for the UAD hypothesis, based on former bronchiolitis patients. However, there was no significant association between any of the above parameters and a decline in baseline lung function, as measured by IOS. This suggests that lung function disorder, in contrast to BHR, may not be a part of the UAD entity, or will be established later than at preschool age in a post-bronchiolitis cohort.

References

1. Rimmer J, Ruhno JW. 6: Rhinitis and asthma: United airway disease. *Med J Aust* 2006; 185: 565-71.

2. Garcia-Marcos L, Rubi Ruiz T, Garcia-Hernandez G, Suarez-Varela MM, Valverde-Molina J, Sanchez-Solis M. Asthma and rhinoconjunctivitis comorbidity: United airway disease or inherited target organs? *Pediatr Allergy Immunol* 2010; 21: e142-8.

3. Ciprandi G, Caimmi D, Miraglia Del Giudice M, La Rosa M, Salpietro C, Marseglia GL. Recent developments in united airways disease. *Allergy Asthma Immunol Res* 2012; 4: 171-7.

4. Bousquet J, Van Cauwenberge P, Khaltaev N, Aria Workshop Group, World Health Organization. Allergic rhinitis and its impact on asthma. *J Allergy Clin Immunol* 2001; 108: S147-334.

5. Bousquet J, Schunemann HJ, Samolinski B, Demoly P, Baena-Cagnani CE, Bachert C, et al. Allergic rhinitis and its impact on asthma (ARIA): Achievements in 10 years and future needs. *J Allergy Clin Immunol* 2012; 130: 1049-62.

6. Shaker M. New insights into the allergic march. *Curr Opin Pediatr* 2014; 26: 516-20.

7. Strachan D, Sibbald B, Weiland S, Ait-Khaled N, Anabwani G, Anderson HR, et al. Worldwide variations in prevalence of symptoms of allergic rhinoconjunctivitis in children: The international study of asthma and allergies in childhood (ISAAC). *Pediatr Allergy Immunol* 1997; 8: 161-76.

8. Kulig M, Bergmann R, Klettke U, Wahn V, Tacke U, Wahn U. Natural course of sensitization to food and inhalant allergens during the first 6 years of life. *J Allergy Clin Immunol* 1999; 103: 1173-9.

9. Pesonen M, Kallio MJ, Siimes MA, Ranki A. Allergen skin prick testing in early childhood: Reproducibility and prediction of allergic symptoms into early adulthood. *J Pediatr* 2015; 166: 401,6.e1.

10. Koponen P, Helminen M, Paassilta M, Luukkaala T, Korppi M. Preschool asthma after bronchiolitis in infancy. *Eur Respir J* 2012; 39: 76-80.

11. Lauhkonen E, Koponen P, Nuolivirta K, Paassilta M, Toikka J, Korppi M. Lung function by impulse oscillometry at age 5-7 years after bronchiolitis at age 0-6 months. *Pediatr Pulmonol* 2015; 50: 389-95.

12. Malmberg LP, Pelkonen A, Poussa T, Pohianpalo A, Haahtela T, Turpeinen M. Determinants of respiratory system input impedance and bronchodilator response in healthy finnish preschool children. *Clin Physiol Funct Imaging* 2002; 22: 64-71.

13. Beydon N, Davis SD, Lombardi E, Allen JL, Arets HG, Aurora P, et al. An official american thoracic society/european respiratory society statement: Pulmonary function testing in preschool children. *Am J Respir Crit Care Med* 2007; 175: 1304-45.

14. Oostveen E, MacLeod D, Lorino H, Farre R, Hantos Z, Desager K, et al. The forced oscillation technique in clinical practice: Methodology, recommendations and future developments. *Eur Respir J* 2003; 22: 1026-41.

15. Malmberg LP, Makela MJ, Mattila PS, Hammaren-Malmi S, Pelkonen AS. Exercise-induced changes in respiratory impedance in young wheezy children and nonatopic controls. *Pediatr Pulmonol* 2008; 43: 538-44.

16. Castro-Rodriguez JA, Holberg CJ, Wright AL, Martinez FD. A clinical index to define risk of asthma in young children with recurrent wheezing. *Am J Respir Crit Care Med* 2000; 162: 1403-6.

17. Cruz AA, Popov T, Pawankar R, Annesi-Maesano I, Fokkens W, Kemp J, et al. Common characteristics of upper and lower airways in rhinitis and asthma: ARIA update, in

collaboration with GA(2)LEN. *Allergy* 2007; 62 Suppl 84: 1-41.

18. Choi SH, Yoo Y, Yu J, Rhee CS, Min YG, Koh YY. Bronchial hyperresponsiveness in young children with allergic rhinitis and its risk factors. *Allergy* 2007; 62: 1051-6.

19. Kim SW, Han DH, Lee SJ, Lee CH, Rhee CS. Bronchial hyperresponsiveness in pediatric rhinitis patients: The difference between allergic and nonallergic rhinitis. *Am J Rhinol Allergy* 2013; 27: e63-8.

20. Pereira AM, Morais-Almeida M, Santos N, Nunes C, Bousquet J, Fonseca JA. Severity of rhinitis and wheezing is strongly associated in preschoolers: A population-based study. *Pediatr Allergy Immunol* 2015.

21. Rochat MK, Illi S, Ege MJ, Lau S, Keil T, Wahn U, et al. Allergic rhinitis as a predictor for wheezing onset in school-aged children. *J Allergy Clin Immunol* 2010; 126: 1170,5.e2.

22. Martinez FD, Wright AL, Taussig LM, Holberg CJ, Halonen M, Morgan WJ. Asthma and wheezing in the first six years of life. the group health medical associates. *N Engl J Med* 1995; 332: 133-8.

23. Bisgaard H, Jensen SM, Bonnelykke K. Interaction between asthma and lung function growth in early life. *Am J Respir Crit Care Med* 2012; 185: 1183-9.

24. Arets HG, Brackel HJ, van der Ent CK. Forced expiratory manoeuvres in children: Do they meet ATS and ERS criteria for spirometry? *Eur Respir J* 2001; 18: 655-60.

25. Aurora P, Stocks J, Oliver C, Saunders C, Castle R, Chaziparasidis G, et al. Quality control for spirometry in preschool children with and without lung disease. 2004; 169: 1152-9.

26. Delacourt C, Lorino H, Herve-Guillot M, Reinert P, Harf A, Housset B. Use of the forced oscillation technique to assess airway obstruction and reversibility in children. *Am J Respir Crit Care Med* 2000; 161: 730-6.

27. Ducharme FM, Davis GM. Measurement of respiratory resistance in the emergency department: Feasibility in young children with acute asthma. *Chest* 1997; 111: 1519-25.

28. Marotta A, Klinnert MD, Price MR, Larsen GL, Liu AH. Impulse oscillometry provides an effective measure of lung dysfunction in 4-year-old children at risk for persistent asthma. *J Allergy Clin Immunol* 2003; 112: 317-22.

29. Komarow HD, Skinner J, Young M, Gaskins D, Nelson C, Gergen PJ, et al. A study of the use of impulse oscillometry in the evaluation of children with asthma: Analysis of lung parameters, order effect, and utility compared with spirometry. *Pediatr Pulmonol* 2012; 47: 18-26.

30. Knihtila H, Kotaniemi-Syrjanen A, Makela MJ, Bondestam J, Pelkonen AS, Malmberg LP. Preschool oscillometry and lung function at adolescence in asthmatic children. *Pediatr Pulmonol* 2015.

31. Hyvarinen MK, Kotaniemi-Syrjanen A, Reijonen TM, Korhonen K, Korppi MO. Lung function and bronchial hyper-responsiveness 11 years after hospitalization for bronchiolitis. *Acta Paediatr* 2007; 96: 1464-9.

Table 1. Skin prick test results in relation to current asthma and prolonged rhinitis in 102 former bronchiolitis patients at five to seven years of age.

Skin prick test results	Current asthma n=15	p-value versus no current asthma	Prolonged rhinitis n=27	p-value versus no rhinitis
At least one inhaled allergen positive (n=32)	8 (53.3%)	**p=0.049**	17 (62.9%)	**p=0.002**
Cat dander (n=12)	6	**p=0.002**	8	**p=0.002**
Dog dander (n=13)	7	**p<0.001**	8	**p=0.005**
Dust mites (n=4)*	0	p=0.542	1	p=0.715
Birch (n=20)	6	**p=0.042**	11	**p=0.002**
Timothy (n=19)	7	**p=0.007**	9	**p=0.026**
Mugwort (n=4)	2	p=0.102	2	p=0.285
A. alternata (n=3)**	2	p=0.056	2	p=0.170

**Dermatophagoides farinae* in no cases, *D. pteronyssimus* in four cases. ***A.alternata=Alternaria alternata*. Current asthma = continuous ICS or intermittent medication during the last 12 months (n=13) or current symptoms and BHR (n=2). p-value<0.05 marked in bold.

Table 2. Association of baseline impulse oscillometry and bronchial hyper-reactivity (BHR) with the presence of current asthma, previous transient asthma and no asthma.

Asthma	Resistance or reactance at 5Hz pathologic	p-value versus no current asthma	BHR present	p-value versus BHR absent
Current asthma	4/15 (26.7%)	0.604*	5/13** (38.5%)	**0.042***
Previous transient asthma***	4/12 (33.3%)	0.358*	1/12 (8.3%)	1.00*
No asthma	13/75 (17.3%)	---	8/75 (10.9%)	---

Resistance at 5Hz was pathological (>+1.65 SD) in eight cases and reactance was pathological (<–1.65 SD) in 19 cases.

Presence of BHR was defined as a >35% increase in resistance at 5Hz in the ECT test (n=5) or >35% decrease in the BD test (n=11).

* p-values were multiplied by two (Bonferroni correction).

** The asthma diagnosis was partially based on BHR documented by IOS in two cases and these cases are not included in the analyses between BHR and asthma.

*** Children with current asthma not included.

p-value<0.05 marked in bold.

Table 3. Baseline impulse oscillometry and bronchial hyper-reactivity in relation to prolonged rhinitis and, or, skin prick test (SPT) positivity to inhaled allergens

Prolonged rhinitis and/or SPT positivity	Resistance or reactance at 5Hz pathologic (n=21)	p-value versus resistance and reactance at 5Hz normal	Bronchial hyper-reactivity present (n=16)	p-value versus bronchial hyper-reactivity absent
Prolonged rhinitis (n=27)	5 (23.8%)	p=0.498	7 (43.8%)	p=0.084
Positive SPT (n=32)	6 (28.6%)	p=0.756	9 (56.3%)	**p=0.020**
Prolonged rhinitis and positive SPT (n=15)	3 (14.3%)	p=0.629	5 (31.3%)	p=0.057
Prolonged rhinitis or positive SPT (n=44)	8 (38.1%)	p=0.601	11 (68.8%)	**p=0.024**

Resistance at 5Hz was pathological (>+1.65 SD) in eight cases and reactance was pathological (<–1.65 SD) in 19 cases.

Bronchial hyper-reactivity was defined as >35% increase in resistance at 5Hz in the ECT (n=5) or >35% decrease in the BD test (n=11).

p-value<0.05 marked in bold

Table 4. Baseline impulse oscillometry and bronchial hyper-reactivity in the groups constructed on the basis of respiratory allergy and current asthma.

Respiratory allergy	Resistance or reactance at 5Hz pathologic (n=20)*	Resistance and reactance at 5Hz normal (n=80)*	Bronchial hyper-reactivity present** (n=14)*	Bronchial hyper-reactivity absent** (n=86)*
Current asthma and (prolonged rhinitis and positive SPT) (n=6)	1 (5.0%) p=0.833	5 (6.3%)	3 (21.4%) **p=0.034**	3 (3.5%)
Current asthma and (prolonged rhinitis or positive SPT) (n=8)	2 (10.0%) p=0.712	6 (7.5%)	3 (21.4%) p=0.081	5 (5.8%)
Current asthma or (prolonged rhinitis and positive SPT) (n=21)	5 (25.0%) p=0.623	16 (20.0%)	6 (42.9%) **p=0.041**	15 (17.4%)
Current asthma or (prolonged rhinitis or positive SPT) (n=48)	9 (45.0%) p=0.764	39 (48.8%)	12 (85.7%) **p=0.002**	36 (41.9%)

* The current asthma diagnosis was partially based on BHR documented by IOS in two cases and these cases are not included in the analyses (n=100).

** See methods section for details of diagnosis.

p-value<0.05 marked in bold