KIRSI NUOLIVIRTA

# Bronchiolitis in Early Infancy

## Predictive factors for post-bronchiolitis wheezing

ACADEMIC DISSERTATION
To be presented, with the permission of
the board of the School of Medicine of the University of Tampere,
for public discussion in the Small Auditorium of Building B,
School of Medicine of the University of Tampere,
Medisiinarinkatu 3, Tampere, on February 24th, 2012, at 12 o'clock.

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ACADEMIC DISSERTATION
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Tampere University Hospital, Department of Pediatrics
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taju@uta.fi
www.uta.fi/taju
http://granum.uta.fi

Cover design by
Mikko Reinikka

Acta Universitatis Tamperensis 1696
ISBN 978-951-44-8692-0 (print)
ISSN-L 1455-1616
ISSN 1455-1616

Acta Electronica Universitatis Tamperensis 1162
ISBN 978-951-44-8693-7 (pdf)
ISSN 1456-954X
http://acta.uta.fi

Tampereen Yliopistopaino Oy – Juvenes Print
Tampere 2012

To my Family

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# LIST OF ORIGINAL PUBLICATIONS

The present thesis is based on the following papers, which are referred to in the text by their Roman number.

I. Helminen M, Nuolivirta K, Virta M, Halkosalo A, Korppi M, Vesikari T, Hurme M.
*IL-10* gene polymorphism at –1082 A/G is associated with severe rhinovirus bronchiolitis in infants.
Pediatric Pulmonology 2008;43(4):391-395.

II. Nuolivirta K, Hurme M, Halkosalo A, Koponen P, Korppi M, Vesikari T, Helminen M.
Gene polymorphism of *IFNG* +874 T/A and *TLR4* +896 A/G and recurrent infections and wheezing in toddlers with history of bronchiolitis.
The Pediatric Infectious Disease Journal 2009;28(12):1121-1123.

III. Nuolivirta K, He Q, Gröndahl-Yli-Hannuksela K, Koponen P, Helminen M, Korppi M.
Mannose-binding lectin gene polymorphisms in infants with bronchiolitis and post-bronchiolitis wheezing.
Allergology International, accepted for publication

IV. 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.
The Pediatric Infectious Disease Journal 2010;29(11):1013-1015.

V. Nuolivirta K, Koponen P, Helminen M, Korppi M.
Weight gain in infancy and post-bronchiolitis wheezing.
Acta Paediatrica 2012;101(1):38-42.

# ABBREVIATIONS

| | |
|---|---|
| A | Alanine |
| AHR | Airway hyper-responsiveness |
| AOM | Acute otitis media |
| aOR | Adjusted odds ratio |
| BMI | Body mass index |
| C | Cysteine |
| CCR5 | Chemokine receptor 5 |
| CD | Cluster of differentiation |
| CI | Confidence interval |
| CSF | Colony-stimulating factor |
| DFA | Direct immunofluorescence assay |
| DNA | Deoxyribonucleic acid |
| ECLIA | Electro-chemiluminescence |
| FRC | Finnish Red Cross |
| G | Glycine |
| HBoV | Human bocavirus |
| HWE | Hardy-Weinberg equilibrium |
| hMPV | Human metapneumovirus |
| HRV | Human rhinovirus |
| I | Isoleucin |
| ICD-10 | International classification of diseases version 10 |
| IF | Immunofluorescence |
| *IFNG* | Interferon, gamma gene |
| IFN-γ | Interferon-gamma |
| Ig | Immunoglobulin |
| IL | Interleukin |
| IV | Influenza virus |
| LPS | Lipopolysaccharide |
| LRTI | Lower respiratory tract infection |
| MBL | Mannose- binding lectin |
| MHC | Major histocompatibility complex |
| M2 | Muscarine 2 (receptor) |
| NF-κB | Nuclear factor kappa light-chain-enhancer of activated B cells |
| NK | Natural killer (cell) |
| NO | Nitric oxide |
| NP | Nucleoprotein |
| NPA | Nasopharyngeal aspirate |
| PCR | Polymerase chain reaction |
| OR | Odds Ratio |
| PAMP | Pathogen-associated molecular pattern |
| PICU | Pediatric intensive care unit |
| PIV | Parainfluenza virus |
| PRR | Pattern recognition receptor |
| RNA | Ribonucleic acid |
| ROS | Reactive oxygen species |
| RSV | Respiratory syncytial virus |
| RT-PCR | Reverse transcriptase- polymerase chain reaction |

| | |
|---|---|
| SNP | Single nucleotide polymorphism |
| T | Threonin |
| TCR | T cell receptor |
| TGF | Transforming growth factor |
| Th | T helper cell |
| THL | Terveyden ja hyvinvoinnin laitos |
| TLR | Toll-like receptor |
| TNF | Tumor necrosis factor |
| Treg | Regulatory T cell |
| UV | Ultra violet |
| WFL | Weight for length |
| WHO | World Health Organization |

# ABSTRACT

**BACKGROUND:** Bronchiolitis is the leading cause of infant hospitalization in developed countries, especially in the age group of <6 months. Susceptibility to both severe bronchiolitis and post-bronchiolitis wheezing has been discovered to be multifactorial including factors related to the host, environment and the virus itself.

**AIMS:** The aim of this prospective study was to evaluate genetic, microbial and other early-life factors as risk factors for disease severity and subsequent wheezing in children hospitalized for bronchiolitis at <6 months of age and prospectively followed up until 1.5 years of age. The aim was to evaluate (1) if the polymorphisms of interleukin (*IL)-10* –1082 G/A, *IL-18* –137 G/C, toll-like receptor (*TLR) 4* +896 A/G, and interferon-gamma gene (*IFNG)* +874 T/A, and polymorphisms in the mannose-binding lectin (*MBL)2* gene are associated with the presence of bronchiolitis and the viral etiology of bronchiolitis; (2) if these polymorphisms are associated with recurrent wheezing and infections during the first 1.5 years of life; (3) how often viral infections, respiratory syncytial virus (RSV) infections in particular, are mixed with *Bordetella pertussis* infections in infants with bronchiolitis and how co-infection with *B. pertussis* influences the clinical picture and outcome of bronchiolitis; and (4) how birth weight, weight gain in infancy and overweight assessed by weight for length (WFL) at age 1-2 years influence the risk for wheezing after hospitalization for bronchiolitis.

**SUBJECTS AND METHODS:** This prospective study was conducted at the Department of Pediatrics, Tampere University Hospital during three infectious seasons from 2001 to 2004. Infants <6 months of age hospitalized for bronchiolitis were eligible for this study. One hundred and thirty-nine infants were eligible and 129 participated in the follow-up visit. The microbiological diagnosis was confirmed by polymerase chain reaction (PCR) for viruses and *B. pertussis*. Polymorphisms of the selected genes were studied by PCR from DNA extracted from blood samples. The control group for the gene polymorphism analysis, excluding the *MBL2* polymorphisms, consisted of 400 healthy Finnish blood donors. The demographic data and clinical characteristics were recorded during hospitalization. The clinical outcomes during the follow-up period were collected by diaries filled in by parents. The data were supplemented by interviewing the parents at the follow-up visit, and when the children were examined, at which point the weights and heights were measured and the WFLs were calculated. In the statistical analyses of the data, univariate and multivariate models were used as appropriate.

**RESULTS:** The viral etiology of bronchiolitis was most often associated with RSV (69.8%), followed by human rhinovirus (HRV) (6.5%) and influenza A virus (IVA) (4.3%). The infants hospitalized with non-RSV bronchiolitis were more likely to be homozygous for the A allele at –1082 of *IL-10* than were blood donors ($p<0.0001$). *MBL2* gene polymorphisms A/O or O/O were associated with multiple viral etiologies of bronchiolitis ($p=0.047$). The polymorphism at *IFNG* +874 T/A (homozygosity for A at +874) was associated with post-bronchiolitis wheezing, and the A allele was associated with less need for corticosteroid therapy (aOR=0.23). Conversely, the *MBL2* A/O or O/O genotypes were associated with the need for corticosteroid therapy during post-bronchiolitis wheezing ($p=0.016$). *TLR4* +896 A/G (G allele carriers, $p=0.012$ vs. non-carriers) and *IL-10* –1082 A/G (G allele carriers, $p=0.030$ vs. non-carriers) were associated with repeated otitis media assessed by tympanostomy tube insertions. *B. pertussis* diagnosed by PCR was common (8.6%) in unvaccinated or partially vaccinated infants. Two-thirds of the cases had mixed infections with RSV; although the clinical characteristics were rather similar, *B. pertussis* was associated with coughing spells. Both high birth weight >4000 g (aOR 3.21) and overweight WFL >110% (aOR 4.63) during the follow-up visit were associated with recurrent post-bronchiolitis wheezing.

**CONCLUSION:** The results of this study confirm that the most common viral finding in young infants with bronchiolitis is RSV, and that *B. pertussis* co-infections comprise 8–10% of cases. Preliminary evidence was found that birth weight and overweight at 1–2 years are risk factors for recurrent wheezing after hospitalization for bronchiolitis. The main result of the study was that gene polymorphisms may have some association with the severity of bronchiolitis, post-bronchiolitis wheezing and recurrent infections after bronchiolitis. Greater understanding of the interaction between genes and viruses increases the potential for identifying at-risk infants, in whom preventing infections or tailoring anti-inflammatory treatments could alter the presentation of recurrent wheezing, recurrent infections and even later asthma.

# TIIVISTELMÄ

**TAUSTAA**: Bronkioliitti eli ilmatiehyttulehdus on yleinen sairaalahoidon syy vauvoilla etenkin alle 6 kuukauden iässä. Alttius sairastua vaikeaan bronkioliittiin ja sen jälkeiseen toistuvaan hengenahdistukseen on todettu olevan monen tekijän yhteisvaikutus, mihin kuuluvat sairastuneeseen vauvaan, ympäristöön ja mahdollisesti virukseen itseensä liittyvät tekijät.

**TUTKIMUKSEN TARKOITUS**: Tämän prospektiivisen tutkimuksen tarkoitus oli selvittää perinnöllisiä, mikrobiologisia ja muita varhaisvaiheen tekijöitä, jotka voivat vaikuttaa taudin vaikeuteen ja toistuvien hengenahdistuksien esiintymisiin alle puolivuotiailla vauvoilla sairaalahoitoa vaatineen bronkioliitin jälkeen. Seuranta-aika oli vuosi sairastetun bronkioliitin jälkeen. Tarkoitus oli selvittää (1) onko *IL-10* –1082 G/A, *IL-18* –137 G/C, *TLR4* +896 A/G, *IFNG* +874 T/A ja *MBL2* geenien monimuotoisuudella yhteyttä bronkioliitin taudinkuvaan ja aiheuttajaviruksiin; (2) onko näiden geenien monimuotoisuudella yhteyttä toistuvien hengitystietulehdusten ja hengenahdistusten esiintymiseen vuosi sairastetun bronkioliitin jälkeen.(3) Miten usein virusten, etenkin respiratory syncytial viruksen (RSV), aiheuttamat hengitystietulehdukset vauvoilla ovat sekainfektiota hinkuyskää aiheuttavan *Bordetella pertussis* bakteerin kanssa ja onko sekainfektiolla vaikutus taudin kliiniseen kuvaan tai toistuviin hengenahdistuksiin bronkioliitin jälkeen; sekä (4) miten syntymäpaino, painonnousu imeväisiässä ja ylipaino arvioituna pituuspaino prosentteina (WFL) 1–2 vuoden iässä vaikuttavat toistuvien hengenahdistusten esiintymiseen bronkioliitin jälkeen.

**AINEISTO JA MENETELMÄT:** Tutkimus tehtiin Tampereen Yliopistollisen sairaalan Lastentautien klinikassa kolmen infektiokauden aikana vuosina 2001–2004. Alle 6 kuukauden ikäiset imeväiset, joilla oli sairaalahoitoa vaatinut bronkioliitti, olivat soveltuvia tutkimukseen. 139 imeväistä osallistui tutkimukseen ja näistä 129 saapui jälkitarkastuskäynnille. Mikrobiologinen diagnoosi virusten ja *B. pertussis* bakteerin osalta varmistettiin PCR- menetelmällä. Tutkittavien geenien monimuotoisuutta selvitettiin verinäytteestä eristetyn DNA:n PCR tutkimuksella. Kontrolliryhmä geenien monimuotoisuuden selvittelyssä koostui 400 vapaaehtoisen suomalaisen verenluovuttajan näytteiden tuloksista lukuun ottamatta *MBL2* geenin monimuotoisuuden määritystä. Imeväisten taustatiedot ja kliinisen taudinkuvan mittarit kirjattiin ylös sairaalahoitojakson aikana. Tiedot sairastetuista infektiosta ja hengenahdistuksista kerättiin vanhempien pitämistä päiväkirjoista. Seurantakäynnin yhteydessä tiedot täydennettiin vanhempien

haastattelulla. Lapset tutkittiin, mitattiin ja punnittiin, sekä pituus-paino prosentit(WFL) laskettiin. Aineiston tilastolliset testit tehtiin yksi- tai monimuuttujamallilla.

**TULOKSET:** Bronkioliitin aiheuttaja oli useimmiten RSV (69.8 %), mutta muutkin virukset kuten rhinovirus (HRV) (6.5 %) ja influenssa A virus (IVA) (4.3 %) olivat aiheuttamassa bronkioliittia. Imeväiset, joilla oli muun kuin RSV:n aiheuttama sairaalahoitoa vaatinut bronkioliitti, olivat todennäköisimmin homotsygootteja *IL-10* alleeli A –1082 suhteen kuin verenluovuttajien kontrolliryhmä ($p<0.0001$). *MBL2* geenin monimuotoisuus A/O tai O/O liittyi useammin monen viruksen aiheuttamaan bronkioliittiin ($p=0.047$). Monimuotoisuus geenissä *IFNG* +874 T/A (homotsygoottisuus alleelissa A +874) voitiin yhdistää toistuvaan hengenahdistukseen bronkioliitin jälkeen niin, että alleeli A oli suojaava tekijä suhteessa kortisonihoitoon (aOR=0.23). *MBL2* A/O tai O/O voitiin yhdistää useammin kortisonihoidon tarpeeseen toistuvien hengenahdistusten yhteydessä ($p=0.016$). Monimuotoisuus geeneissä *TLR4* +896 A/G (alleeli G kantajat) ja *IL-10*–1082 A/G (alleeli G kantajat) lisäsi ilmeisimmin toistuviin korvatulehduksiin sairastumista, sillä tympanostomiaputket oli asetettu useammin ($p=0.012$ ja $p=0.030$). *B. pertussis* bakteerin aiheuttama hengitystietulehdus todennettuna PCR- menetelmällä oli yleinen (8.6 %) rokottamattomissa tai osittain rokotetuissa imeväisissä. Kaksi kolmasosaa tapauksista oli sekainfektiota RSV:n kanssa. Pelkästään viruksen aiheuttamia tai sekainfektiota *B. pertussis* bakteerin kanssa ei voitu yskän perusteella erottaa, mutta sekainfektioihin liittyi enemmän yskänpuuskia. Korkea syntymäpaino (>4000 g) (aOR 3.21) ja ylipaino (WFL >110 %) (aOR 4.63) seurantakäynnin aikana olivat yhteydessä toistuviin hengenahdistuksiin bronkioliitin jälkeen.

**JOHTOPÄÄTÖKSET:** Tutkimuksen tulokset vahvistavat, että RSV on yleisin bronkioliitin aiheuttaja ja *B. pertussis* sekainfektio on yleinen (8–10 %) imeväisillä. Tuloksissa oli viitteitä siitä, että suuri syntymäpaino ja ylipaino 1–2 vuoden iässä ovat riskitekijöitä toistuville hengenahdistuksille sairastetun bronkioliitin jälkeen. Varsinainen tulos oli se, että geenien monimuotoisuudella on yhteyttä bronkioliitin taudinkuvaan, toistuviin hengenahdistuksiin ja hengitystietulehduksiin bronkioliitin jälkeen. Tietämys geenien ja virusten yhteistyöstä voisi tarjota hyvän mahdollisuuden tunnistaa riskialttiit imeväiset, joilla infektioiden estäminen tai tulehdusten yksilöllinen tulehdusreaktiota rauhoittava hoito voisi estää toistuvien hengitystietulehdusten, hengenahdistusten ja jopa myöhemmän astman synnyn.

# 1. INTRODUCTION

Bronchiolitis is a distressing, potentially life-threatening respiratory illness that affects young babies. Viral bronchiolitis is the most common lower respiratory tract infection (LRTI) in children less than 12 months of age and is the most frequent cause of hospitalization in infants under 6 months of age (Panitch. 2001, AAP. *et al.* 2006, Smyth & Openshaw. 2006). The hospitalization rate for bronchiolitis is 1–3% of previously healthy infants (Shay *et al.* 1999, Simoes & Carbonell-Estrany. 2003, Stockman *et al.* 2012). Among the various respiratory viruses causing LRTI, respiratory syncytial virus (RSV) is the single most important virus (Jartti *et al.* 2004, Manoha *et al.* 2007, Mansbach *et al.* 2007, Marguet *et al.* 2009, Midulla *et al.* 2010, Miron *et al.* 2010, Nascimento *et al.* 2010, Antunes *et al.* 2010, Calvo *et al.* 2010), although other viruses such as human rhinovirus (HRV), human metapneumovirus (hMPV), and human bocavirus (HBoV) are increasingly recognized (Papadopoulos *et al.* 2002, Regamey *et al.* 2008, van der Zalm *et al.* 2009).

Susceptibility to severe bronchiolitis is multifactorial and involves a combination of factors, including those related to the host, to the environment and perhaps to the virus itself (Vicencio. 2010). Young infants, especially those aged <3 months, are at risk for a more severe disease (Simoes & Carbonell-Estrany. 2003). Infants with high birth weight are at risk of presenting with LRTI caused by RSV (Houben *et al.* 2011). Environmental factors such as exposure to tobacco smoke, crowded living conditions, and living with school-aged siblings have been associated with more severe disease (Simoes & Carbonell-Estrany. 2003). The severity of bronchiolitis can depend on the causative virus itself and the host response to infection can also vary depending on the viral agent. RSV has been shown to cause more severe disease than other viruses (Marguet *et al.* 2009, Calvo *et al.* 2010, Midulla *et al.* 2010, Singleton *et al.* 2010), but findings with of an equivalent clinical picture of RSV and HRV LRTI also exist (Korppi *et al.* 2004, van Leeuwen *et al.* 2012). Some properties of RSV contribute significantly to the infective cycle and interfere with the immune response of the host (Harris & Werling. 2003).

The severity of bronchiolitis can also be affected by multiple viral infections or co-infections with bacteria. The number of multiple viral infections is around 20% (Calvo *et al.* 2008a, Miron *et al.* 2010, Nascimento *et al.* 2010). There is no consensus on the effect of viral co-infection on disease severity (Aberle *et al.* 2005, Semple *et al.* 2005, Jartti *et al*. 2008a, Martin *et al.* 2011). Co-infections with bacteria, especially with *Bordetella pertussis*, need attention. *B. pertussis* has been verified in <1% (Siberry *et al.* 2006, Walsh *et al.* 2011) to 16% (Cosnes-Lambe *et al.* 2008) of unvaccinated infants with RSV infection, with varying impacts on the severity of bronchiolitis. In two studies, the clinical picture of bronchiolitis did not differ if *B. pertussis* involvement was present (Korppi & Hiltunen. 2007, Cosnes-Lambe *et al.* 2008). On the other hand, *B. pertussis* was identified as often as in 23% of infants hospitalized for severe respiratory manifestations like apnea and respiratory failure (Crowcroft *et al.* 2003).

Differences in cytokine production may be related to different susceptibilities to infections and different disease presentations (Bidwell *et al.* 1999). The pathogenesis of RSV bronchiolitis has been studied by characterizing host immune responses through measuring cytokine concentrations from peripheral blood (Chen *et al.* 2002, Castro *et al.* 2008) or from nasal wash specimens (Bennet *et al.* 2007) during bronchiolitis or from cultured cells obtained from umbilical cord blood (Juntti *et al.* 2009), supplemented by studies in cells from peripheral blood (Copenhaver *et al.* 2004, Gern *et al.* 2006). The results have been inconsistent varying from no association to significant association. The discrepancy may depend on the complexity of the cytokine network including redundant, synergistic and antagonistic molecules (Bueno *et al.* 2011).

Since the cytokine network is highly flexible with considerable overlap and redundancy between the function of individual cytokines (Haddad 2002), studies have focused on cytokine gene polymorphisms (Bidwell *et al*. 1999). By studying cytokine gene polymorphisms in infectious diseases, potential markers of susceptibility, severity, and clinical outcome can be identified (Bidwell *et al.* 1999). RSV susceptibility is complex, and is probably regulated by many genes. Polymorphisms in genes involved with either the adaptive or innate immune systems have been associated with severe RSV bronchiolitis, including, but not limited to, genes regulating interleukin (IL)-4, IL-8, and IL-10 production, toll-like receptor (TLR) 4 expression and surfactant protein D production (Hull *et al.* 2000, Choi *et al.* 2002, Lahti *et al.* 2002, Hoebee *et al.* 2003, Hoebee *et al.* 2004, Tal *et al.* 2004). Candidate gene studies need large study populations,

and the often weak associations obtain more power when supported by functional evidence of the effect of the polymorphism (Tregoning & Schwarze. 2010).

Viral bronchiolitis in infancy increases the risk of recurrent wheezing in later life. The relation between RSV bronchiolitis and subsequent wheezing illnesses in childhood has been shown in both clinical post-bronchiolitis follow-up and in birth cohort studies (Martinez *et al.* 1995, Stein *et al.* 1999, Kneyber *et al.* 2000, Schauer *et al.* 2002, Sigurs 2002, Korppi *et al.* 2004, Henderson *et al.* 2005, Kotaniemi-Syrjänen *et al.* 2005, Carroll *et al.* 2009). In recent years, due to improved viral diagnostics by polymerase chain reaction (PCR), the role of HRV in causing post-bronchiolitis wheezing has been significantly clarified (Kotaniemi-Syrjänen *et al.* 2003, Lemanske Jr *et al.* 2005, Kusel *et al.* 2007, Jackson *et al.* 2008, Jartti *et al.* 2009b). The fundamental question as to whether severe LRTI is the cause of asthma or whether susceptibility to asthma only predisposes children to severe LRTI in response to viral infection is still open (Singh *et al.* 2007). One potential determinant is the involvement of genetic susceptibility loci to asthma during viral bronchiolitis. Until now, only a few candidate genes have been replicated in studies, including the genes regulating IL-4, IL-13, and tumor necrosis factor (TNF) production, as well as IL-4 receptor (IL-4Rα) expression (Singh *et al.* 2007).

The connection between weight gain, overweight and post-bronchiolitis wheezing has been poorly studied. There is increasing evidence that obesity precedes asthma onset in children and is associated with both the persistence and intensity of symptoms (Noal *et al.* 2011). Changes in many adipose-derived inflammatory modulators, including TNF-α, leptin, and adiponectin, have the capacity to promote airway hyper-responsiveness (AHR), and thus may contribute to asthma in the obese (Flaherman & Rutherford. 2006, Peroni *et al.* 2010). In a recent study, wheezing in infancy from 3 to 12 months of age was associated with higher weight for length (WFL) Z-scores (Jee *et al.* 2010). In line with this, infants with higher WFL Z-scores at 6 months of age had a greater risk of recurrent wheezing by the age of 3 years, but the association was not seen at 1 or 2 years of age (Taveras *et al.* 2008). In a recent birth cohort from the UK, greater infant weight and adiposity gains were associated with both atopic and non-atopic wheezing (Pike *et al.* 2010).

In this thesis, genetic, microbial and other early-life factors were studied as risk factors for disease severity and subsequent wheezing in children hospitalized for bronchiolitis at <6 months of age and prospectively followed up until 1.5 years of age.

# 2. REVIEW OF THE LITERATURE

## 2.1 Definition of bronchiolitis

Since the 1940s, the term bronchiolitis has been used as a diagnosis for a specific clinical respiratory symptom complex affecting infants and young children. After a brief one to three day prodrome of upper respiratory symptoms including nasal obstruction and/ or rhinorrhea, typical signs of bronchiolitis occur characterized by wheezing, dyspnea, respiratory distress, poor feeding, tachypnea (>50/min) and radiologic evidence of hyperaeration of the lung. Fine crackles (crepitations) can often be heard by auscultation of the chest (Simoes. 1999, Hall. 2001, Panitch. 2001, Fitzgerald & Kilham. 2004, AAP. 2006, Smyth & Openshaw. 2006). The severity of infection varies from mild respiratory symptoms treated at home to severe respiratory distress and hypoxemia demanding hospitalization.

Because the diagnosis of bronchiolitis is clinical, the definitions and treatment practices have varied between different countries and even between different hospitals in the same country. In some studies, bronchiolitis has been restricted to RSV cases only (Schauer *et al.* 2002, Sigurs. 2002), or to cases occurring during RSV epidemics (Shay *et al.* 1999). According to current US and UK guidelines, the diagnosis of bronchiolitis includes wheezing children <24 months of age (AAP. 2006, SIGN. 2006). This age limit also has been used in most studies, especially in studies in the US. In some European studies, the age of bronchiolitis has been limited to <12 months (Schauer *et al.* 2002, Sigurs. 2002, Sznajder *et al.* 2005). The diagnosis of bronchiolitis is likely to include different disease entities with different immunological and pathogenic mechanisms according to viral etiology, wheezing phenotype and preceding inflammatory state (Papadopoulos *et al.* 2002, Kotaniemi-Syrjänen *et al.* 2003, Jartti *et al.* 2004, Allander *et al.* 2007, Kusel *et al.* 2007, Lehtinen *et al.* 2007, Mansbach *et al.* 2007, Jackson *et al.* 2008). Only children with their first episode of wheezing should be diagnosed as having bronchiolitis (Jartti *et al.* 2009a).

In the majority of patients suffering from bronchiolitis, the signs and symptoms resolve within a few days to a week after the onset of illness, but complete recovery of the lungs and airway function may take several weeks (Swingler *et al.* 2000).

## 2.2 Etiology of bronchiolitis

Bronchiolitis is the leading cause of infant hospitalizations in developed countries, especially in the age group of <6 months (Shay *et al.* 1999, Panitch. 2001, Stockman *et al.* 2012). The annual hospitalization rate for bronchiolitis among infants <1 year of is around 30 per 1000 in both the US and Europe (Shay *et al.* 1999, Simoes & Carbonell-Estrany. 2003, Stockman *et al.* 2012). On the other hand, severe bronchiolitis treated in the pediatric intensive care unit (PICU) is uncommon in previously healthy full-term infants <12 months of age (Popoff *et al.* 2011). RSV is the predominant causative viral agent at that age (Iwane *et al.* 2004, Mansbach *et al.* 2007, Calvo *et al.* 2008a, Hall *et al.* 2009, Marguet *et al.* 2009). Some other viruses, such as influenza A (IVA) and parainfluenza viruses (PIV), especially PIV type 3, have been identified in young children with bronchiolitis (Hall. 2001, Mackie. 2003). The use of PCR in viral diagnostics has revealed the role of certain viruses, such as HRV, hMPV, and HBoV (Korppi *et al.* 2004, Choi *et al.* 2006, Calvo *et al.* 2010, Midulla *et al.* 2010, Singleton *et al.* 2010). In addition, the use of PCR for viruses has confirmed the role of multiple viral infections among children with bronchiolitis (Jartti *et al*. 2008a, Calvo *et al.* 2008a, Miron *et al.* 2010, Nascimento *et al.* 2010). The connection between the clinical severity of LRTI and identification of multiple viruses is contradictory, and appears to be dependent on which viruses are involved in the co-infection. For example, when adenovirus was detected during RSV infection, there was no increase in severity (Aberle *et al.* 2005). Infections with multiple virus detection can be less (Martin *et al.* 2011) or more severe than single infections; for example, co-infection with hMPV increased the PICU admissions of RSV patients (Semple *et al.* 2005), while co-infection with HRV more often resulted in moderate-to-severe respiratory illnesses than single infections (Jartti *et al.* 2008a), and co-infection with HBoV increased the severity score of infection and the duration of hospitalization (Midulla *et al*. 2010) .

### 2.2.1 Respiratory syncytial virus

Since its identification and isolation from children with pulmonary disease in 1956 (Chanock 1957), RSV has been documented as the single most important virus causing acute respiratory infections in children. Almost all children are affected by RSV at least once by 2 years of age (Kim *et al.* 1973, Simoes. 1999) and re-infections are common, even during sequential years (Hall *et al.* 1991, Simoes. 1999, Wennergren & Kristjansson. 2001). Peak rates occur in infants aged 6 weeks to 6 months, particularly in those less than 3 months of age (Simoes 1999).

RSV is an enveloped RNA virus that belongs to the *Paramyxoviridae* family, within the *Pneumovirus* genus. Integral to immunity and pathogenesis are the large envelope glycoproteins, which consist of a fusion protein (F) and a second glycoprotein (G). Fusion of the viral envelope with host cell membranes and syncytium formation are essential stages in the RSV life cycle, and both processes require the F protein (Harris & Werling 2003). In murine and human *in vitro* experiments, the G protein has elicited predominantly Th2-oriented immune responses, whereas the F protein has produced predominantly Th1-oriented responses (Hussell *et al.* 1998). There are two major groups of RSV strains, A and B, which are distinguished mainly by variations within the G protein (Hall *et al.* 1991).

Depending on age, the definition of bronchiolitis and the study setting, RSV has been the causative agent in 28–77% of bronchiolitis cases requiring hospital treatment (Rakes *et al.* 1999, Papadopoulos *et al.* 2002, Jartti *et al.* 2004, Manoha *et al.* 2007, Jacques *et al.* 2008b, Marguet *et al.* 2009, Stempel *et al.* 2009, Calvo *et al.* 2010, Midulla *et al.* 2010). The infections follow a seasonal pattern with annual winter and spring epidemics in temperate climate (Kim *et al.* 1973, Wennergren & Kristjansson. 2001, Jartti *et al.* 2004). In Finland, RSV epidemics typically occur in two-year cycles, with a minor epidemic in spring preceding the major epidemic in autumn (Waris. 1991, Jartti *et al.* 2004).

Both host and environmental factors contribute to the severity of RSV bronchiolitis. In a birth cohort study, infants with birth weight >4000g (OR=2.24, CI 95% 1.1 to 4.6) and infants born in April to September (OR= 2.17, CI 95% 1.1 to 4.4) were at risk of presenting with LRTI when infected with RSV (Houben *et al.* 2011). Environmental factors such as exposure to tobacco smoke, crowded living conditions, and living with

school-age siblings have been associated with more severe disease (Simoes & Carbonell-Estrany. 2003).

### 2.2.2 Human rhinovirus

HRVs are single-stranded RNA viruses which belong to the *Picornaviridae* family. HRV was first isolated in 1956 (Price. 1956), and since then, more than 100 genotypically and serotypically diverse HRV strains have been identified (McErlean *et al.* 2007, Lu *et al.* 2008). HRVs are classified by genetic sequencing to three species called HRV-A, HRV-B, and HRV-C (Palmenberg *et al.* 2009). For a long time, HRVs were believed to cause mainly mild upper respiratory infections, but now they are known to be associated with serious LRTIs like pneumonia (Juvén *et al.* 2000, Michelow *et al.* 2004). Depending on age, rhinoviruses have been identified in 9–49% of LRTI cases requiring hospital treatment (Papadopoulos *et al.* 2002, Manoha *et al.* 2007, Louie *et al.* 2009, Piotrowska *et al.* 2009, Calvo *et al.* 2010, Midulla *et al.* 2010). In addition HRV-A and HRV-C species, but not HRV-B, have been associated with hospitalization because of LRTIs and serious illness outcomes in children <5 years (Iwane *et al.* 2011). On the other hand, in a birth cohort study, HRV was found to be the most common viral finding in infants <1 year old with acute respiratory infection not needing hospitalization (Kusel *et al.* 2006). Furthermore HRV has been detected by PCR in up to 15% in asymptomatic subjects (Jartti *et al*. 2008b), suggesting that detection may not always reflect a current illness.

### 2.2.3 Human metapneumovirus

hMPV is a single-stranded RNA virus and a member of the newly discovered *Metapneumovirus* genus of the *Paramyxoviridae* family that infects humans (van den Hoogen *et al.* 2001). The clinical symptoms in children infected with hMPV are similar to those infected with other viruses (Boivin *et al.* 2003, van den Hoogen *et al.* 2003, Williams *et al.* 2004). When reverse-transcription PCR (RT-PCR) has been used, the proportion of hMPV detected in nasopharyngeal aspirate samples of children with acute respiratory tract infection has varied from 6 to 12% (Jartti *et al.* 2002, Boivin *et al.* 2003, van den Hoogen *et al.* 2003, Williams *et al.* 2004).

### 2.2.4 Human bocavirus

HBoV is a single-stranded DNA virus and belongs to the *Bocavirus* genus of the family *Parvoviridae*. Since the detection of HBoV from respiratory tract samples from Swedish infants and children with LRTI (Allander *et al.* 2005), reports related to HBoV have been published around the world. HBoV has been associated with upper and lower respiratory infections and is detected year around, with rates of 0.5–25% in patients with LRTI (Foulonge *et al.* 2006, Manning *et al.* 2006, Allander *et al.* 2007, Calvo *et al.* 2008b, Jacques *et al.* 2008b, Söderlund-Venermo *et al.* 2009). However, HBoV pathogenicity is uncertain, as it is mainly detected as a co-infection (Allander. 2008, Longtin *et al.* 2008). Recently, increases in the antibody response were found between paired sera in children with pneumonia, which speaks for the etiological role of HBoV (Don *et al.* 2010). In addition, evidence is mounting to show that HBoV is an important cause of lower respiratory tract illness. The best currently available diagnostic approaches are quantitative PCR and serology (Jartti *et al.* 2011).

### 2.2.5 Influenza virus

Influenza virus belongs to the *Orthomyxoviridae* family. The RNA is protected by a closely associated nucleoprotein (NP) forming a helical structure called a nucleocapsid. Three known types of influenza virus (A, B, and C) currently circulate in the human population; types A and B are associated with clinically important respiratory illness (Wright. 2004). Previously healthy infants are at risk for hospitalization because of influenza infection, and the clinical presentation is most frequently high fever, cough, and rhinorrhea (Izurieta *et al.* 2000, Neuzil *et al.* 2000, Peltola *et al.* 2003).

### 2.2.6 Parainfluenza virus

PIVs are enveloped RNA viruses belonging to the *Pneumoviridae* genus of the *Paramyxoviridae* family (Hall. 2001). PIVs, classified as types 1-4, cause a spectrum of respiratory illnesses which seldom lead to hospitalization (Knott *et al.* 1994). On the other hand, especially PIV type 3 may cause bronchiolitis and other LRTIs in infants (Iwane *et al.* 2004, Aberle *et al.* 2005, Weinberg *et al.* 2009).

### 2.2.7 Age vs. viral etiology of bronchiolitis

Immune development in early life requires a multifaceted interaction of genetic, molecular and cellular components, which may act differently in different infections and within different age windows. The ability to respond to viral surface glycoproteins with protective antibody responses appears to develop gradually over the first 6–9 months of life, reflecting developmental maturation of the immune system present at birth. The youngest infants mount relatively poor antibody responses to microbes, particularly when they have transplacentally-acquired specific antibodies in low titers (Crowe Jr & Williams. 2003). This is one reason why all infants <6 months of age are at risk of developing bronchiolitis requiring hospitalization during respiratory viral infection (Voets *et al.* 2006).

Infants infected with HRV or RSV have presented with similar clinical characteristics, but children hospitalized with HRV have been older (Papadopoulos *et al.* 2002, Korppi *et al.* 2004, Jartti *et al.* 2006, Jartti *et al.* 2009b). In Finnish studies, the infants with HRV bronchiolitis were more likely to have atopic dermatitis and eosinophilia (Korppi *et al.* 2004, Jartti *et al*. 2006), and had more evidence of airway inflammation, higher levels of exhaled nitric oxide, and allergic sensitization to foods (Jartti *et al.* 2009b). These findings suggest that non-RSV bronchiolitis might be its own, separate disease entity, i.e. an acute viral illness resembling an asthma exacerbation (Papadopoulos *et al.* 2002). In addition both hMPV and HRV infection were independently significant predictive factors of later asthma (Manoha *et al.* 2007).

The majority of severe bronchiolitis cases occur in infants <2 months old and at that age, nearly all cases of bronchiolitis are caused by RSV. In two recent studies, children with RSV infection were younger than those with HBoV and hMPV infection (Calvo *et al.* 2010, Midulla *et al.* 2010). HRV seems to cause less severe bronchiolitis in older infants (Marguet *et al.* 2009).

Prospective bronchiolitis studies with etiological identifications available published in the 2000's are summarized in Table 1.

*Table 1.*

*Prospective studies on viral etiology of bronchiolitis versus age. Included studies were published in 2002-2010, study children were < 36 months of age, and at least RSV and HRV were assayed.*

| Author | Virus | Age (months) | Country | Viral tests |
|---|---|---|---|---|
| Papadopoulos *et al.* 2002 | RSV<br>HRV<br>RSV+RV | 3.2 *<br>5.2<br>5.2 | Greece | RT-PCR |
| Korppi *et al.* 2004 | RSV<br>HRV | 5 †<br>13 | Finland | In-house RT-PCR (HRV)<br>Antigen assay (TR-FIA) +<br>Antibody assay (CF) +<br>RT-PCR (RSV) |
| Manoha *et al.* 2007 | RSV<br>HRV<br>hMPV | 4 †<br>4<br>6 | France | Indirect IF (RSV),<br>RT-PCR (HRV, hMPV) |
| Mansbach *et al.* 2007 | RSV<br>HRV<br>RSV+HRV<br>Others ‡ | 4.9†<br>7.5<br>7.3<br>7.3 | USA | One-step RT-PCR |
| Jartti *et al.* 2009b | RSV<br>HRV | 9.4*<br>16.8 | Finland | Virus culture<br>PCR (RSV and HRV)<br>Viral antigens (RSV) |
| Marguet *et al.* 2009 | RSV<br>HRV<br>RSV+HRV<br>hMPV | 2.2†<br>1.7<br>2.7<br>2.6 | France | Multiplex RT-PCR +<br>Direct Immunofluorescence assay (DFA) |
| Calvo *et al.* 2010 | RSV<br>HRV<br>hMPV<br>HBoV | 4.1 *<br>7.6<br>5.9<br>9.6 | Spain | Nested RT-PCR |
| Midulla *et al.* 2010 | RSV<br>HRV<br>HBoV | 2.0 *<br>3.1<br>4.9 | Italy | RT-PCR, nested PCR |
| Singleton *et al.* 2010 | RSV<br>HRV<br>IVA | 9.5*<br>9.4<br>13.6 | USA | Single-plex real-time PCR |

RSV, respiratory syncytial virus; HRV, human rhinovirus; hMPV, human metapneumovirus; IVA, influenza A virus; HBoV, human bocavirus

* mean; †median; ‡hMPV, IVA, IVB

### 2.2.8 *Bordetella pertussis* in bronchiolitis

Infection with *B. pertussis* causes significant morbidity and mortality and, globally, is one of the least-controlled vaccine-preventable diseases (Ward *et al.* 2005, WHO. 2010). Mild pertussis infection may be unrecognized in adults, and such adults may act as a source of infection for unvaccinated or partially vaccinated infants at risk (Crowcroft *et al.* 2003, Senanayake. 2007). The simultaneous occurrence of RSV and *B. pertussis* infection was first documented over 20 years ago (Nelson *et al.* 1986). The development of PCR tests for RSV and for *B. pertussis* in particular, has allowed for confirmation of this observation (Crowcroft *et al.* 2003, Versteegh *et al.* 2006, Korppi & Hiltunen. 2007, Cosnes-Lambe *et al.* 2008). *B. pertussis* co-infection has been documented from <1% (Siberry *et al.* 2006, Walsh *et al.* 2011) to 16% (Cosnes-Lambe *et al.* 2008) in RSV infections in non-vaccinated infants. *B. pertussis* was identified in 23% of <5-month-old infants hospitalized for severe respiratory disorders like apnea and respiratory failure (Crowcroft *et al.* 2003). There are no significant differences in the clinical picture of *B.* pertussis-positive and -negative LRTI and the absence of cough does not rule out pertussis (Greenberg *et al.* 2007, Korppi & Hiltunen. 2007).

## 2.3 Genetics of bronchiolitis

Inherited differences in the DNA sequence contribute to phenotypic variation, influencing an individual's anthropometric characteristics, risk of disease, and response to the environment. A single-nucleotide polymorphism (SNP) is a DNA sequence variation occurring when a single nucleotide in the genome (or other shared sequence) differs between members of a biological species or paired chromosomes in an individual. Most human sequence variations are attributable to single nucleotide polymorphisms (SNPs), with the rest being caused by insertions and deletions of one or more bases, repeat length polymorphisms and rearrangements. SNPs are highly abundant and are estimated to occur at an average rate of 1 per 1000 bases in the common genome (The International SNP Map Working Group. 2001).

The rationale for studying cytokine and innate immunity gene polymorphisms in infectious diseases includes enhancing the understanding of the etiology and pathogenesis of diseases, identifying potential markers of infection susceptibility, infection severity and

clinical outcome, and discovering targets for therapeutic interventions (Bidwell *et al.* 1999).

### 2.3.1 Virus recognition

The goal of the immune response is to limit the spread of viruses and to clear the infection. A prompt immune response not only eliminates the infecting organism but also provokes symptoms like wheezing caused by inflammation. Innate immunity is an ancient form of host defence that relies on a small number of germ-line encoded receptors. These receptors are encoded in our genome and therefore are principally functional after they have been synthesized. The innate immune system contributes to the earliest phase of the host defence against foreign organisms and has both soluble and cellular pattern recognition receptors (PRR) for microbial products (Hallman *et al.* 2001). PRRs are germ-line encoded proteins distinguishing pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharides (LPS), mannans, glycans, and double-stranded RNA present in micro-organisms but not in the host (Hallman *et al.* 2001). Proteins derived from the micro-organisms are processed in the lysosomes of antigen-presenting cells to generate antigenic peptides, which form a complex with major histocompatibility complex (MHC) class II molecules on the surface of the macrophage. These peptides are recognized by T-cell receptors. In the case of the signaling class of PRRs, the recognition of PAMPs by TLRs leads to the activation of signaling pathways that induce the expression of cytokines, chemokines, and co-stimulatory molecules. Therefore, PRRs have a role in the generation of both the peptide-MHC molecule complex and the co-stimulation required for the activation of T cells (Medzhitov & Janeway Jr. 2000) (Figure 1). This allows the body to respond immediately to the microbial invasion before the development of acquired immunity (Hallman *et al.* 2001).

Since innate immune responses are considered particularly important for the course of RSV infection in infancy, the genes regulating these responses have been selected as candidate genes for the risk of severe RSV infection.

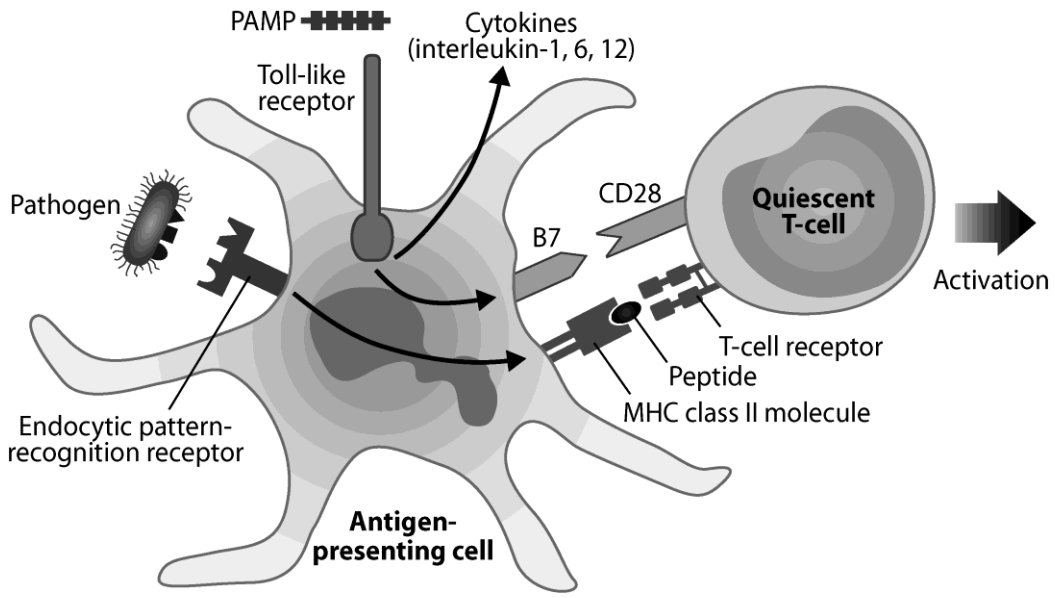


Figure 1. The receptors involved in the interplay of the innate and adaptive immune systems (according to Medzhitov & Janeway Jr. 2000).
Recognition of the pathogen-associated molecular pattern (PAMP) by pattern-recognition receptors (PRRs), such as the toll-like receptors (TLRs), generates signals that activate cytokine and/or interleukin production and the adaptive immune system. Endocytic pattern-recognition receptors, such as the mannose binding lectin (MBL), bind to microbial components and mediate the uptake and phagocytosis of pathogens by antigen-presenting macrophages and dendritic cells. Proteins derived from the microbes form a complex with major-histocompatibility-complex (MHC) class II molecules. These peptides are recognized by T-cell receptors.

### 2.3.2 Toll-like receptor 4

TLR4 is an 839–841 amino acid member of the TLR family of receptors which are type I transmembrane proteins (Medzhitov *et al.* 1997). TLRs, germ-line encoded PRRs required for normal immune responses, are of great importance for initiating (by PAMP recognition) and regulating immune signaling and subsequent adaptive immune responses (Hallman *et al.* 2001). TLR4 recognizes the F protein of RSV and is also part of a receptor complex involved in the immune response against RSV (Kurt-Jones *et al.* 2000, Haynes *et al.* 2001).

The *TLR4* gene is located on chromosome 9q32–33 (Rock *et al.* 1998). There are two co-segregating polymorphic sites, Asp299Gly (A/G) and Thr399Ile (T/I), which affect the extracellular protein domain and thus can alter the ability of the host to respond to pathogens (Arbour *et al.* 2000). The A/G change interrupts TLR4-mediated LPS signaling and causes those homozygous for the G allele to be hypo-responsive to LPS (Arbour *et al.* 2000). This mutation may be associated with delayed and/or attenuated triggering of the innate immune response to RSV, resulting in reduced activation of the intracellular NF-κB signaling pathway. The resulting weak innate immune responses may contribute to enhanced susceptibility to infection (Tulic *et al.* 2007).

Several studies have investigated whether the A/G (or T/I) alleles are linked to the severity of RSV infection with varying results (Tal *et al.* 2004, Mandelberg *et al.* 2006, Awomyi *et al.* 2007, Paulus *et al.* 2007, Tulic *et al.* 2007, Löfgren *et al.* 2010). In a study from Israel in 99 infants hospitalized for severe RSV bronchiolitis, both polymorphic *TLR4* loci were associated with severe RSV bronchiolitis (Tal *et al.* 2004). The same group confirmed this finding in another population, and they also found that hypo-responsiveness to LPS was associated with increased risk for more severe bronchiolitis (Mandelberg *et al.* 2006). In another study, the heterozygosity of A/G was strongly associated with the risk of symptomatic RSV among mainly preterm infants (Awomyi *et al.* 2007). On the other hand, Canadian and Finnish studies (Paulus *et al.* 2007, Löfgren *et al.* 2010) found no association between *TLR4* gene variants and RSV bronchiolitis, but a *post hoc* analysis of two different RSV epidemics separately revealed an association with the *TLR4* locus and severe bronchiolitis (Löfgren *et al.* 2010). While during one epidemic, the homozygotic G genotype was a risk factor, the same genotype protected against severe disease during another epidemic (Tal *et al.* 2004) .

According to these studies, the homozygous LPS hypo-responsive G allele increases the risk of severe RSV infection, but this effect seems to differ between RSV epidemics. As both poor innate immune responses via Toll signaling and excessive production of proinflammatory cytokines may lead to severe clinical symptoms, the homozygotic G genotype may be either a risk or protective factor, depending on the virulence of the virus (Rämet *et al.* 2011).

### 2.3.3 Mannose-binding lectin

MBL is a soluble pattern recognition protein that contributes to the killing of a broad range of pathogenic micro-organisms via the lectin complement pathway and through opsono-phagocytosis (Eisen & Minchinton. 2003). MBL is a liver-derived serum protein that binds to mannose and N-acetyl glucosamine residues commonly found in micro-organisms. The primary 32 kDa subunit of MBL consists of an N-terminal cross-linking region, a collagen-like domain, and a C-terminal carbohydrate-recognition domain. These identical subunits combine to form an MBL structural unit stabilized by N-terminal disulfide bonds (Turner. 1996). A single gene, *MBL2* located on chromosome 10, codes for human MBL (Sastry *et al.* 1989). Deficiency states arise from polymorphisms in the structural and promoter sequences of the *MBL2* gene. There are three polymorphisms in the structural gene *MBL2*, at codons 52, 54, and 57, and they are encoded for variant alleles referred to as D, B, and C, respectively, or collectively referred to as O. The wild-type genotype is referred to as A (Minchinton *et al.* 2002).

There is an association between the *MBL2* haplotype and MBL production and function. More than one third of the population will have haplotypes associated with reduced MBL serum levels, with very low levels being expected in 12% (Turner. 1996). It is not certain how many individuals with wild-type MBL genes have low or unmeasurable MBL levels and function (Minchinton *et al.* 2002). Casanova & Abel concluded in their review that only about 5% of individuals are homozygous or compound heterozygous for any of the three missense mutations and can be considered as "MBL deficient" (Casanova & Abel. 2004).

MBL status has been assessed in children with respiratory infections. In a prospective population-based study from Greenland, MBL insufficiency (genotype A/O or O/O) was associated with increased risk for acute respiratory infections particularly at the age from 6 to 17 months (Koch *et al.* 2001). Thus, MBL plays an important role in host defence, particularly during the vulnerable period in infancy, when antibodies of maternal origin have disappeared and the host’s adaptive immune system is immature (Koch *et al.* 2001, Super *et al.* 1989).

### 2.3.4 Cytokines

Cytokines represent a multi-diverse family of polypeptide regulators; they are relatively low molecular weight, pharmacologically active proteins that are secreted by one cell for the purpose of altering either its own functions (autocrine effect) or the functions of adjacent cells (paracrine effect) (Haddad. 2002, Akdis *et al.* 2011). Cytokines comprise interleukins (IL), lymphokines, monokines, interferons, colony-stimulating factors (CSF), chemokines, and variety of other proteins. In many instances, individual cytokines have multiple biological activities, contributing for example to innate and adaptive immunity, to inflammation and infection, to hematopoiesis, and to tissue repair (Haddad. 2002, Akdis *et al.* 2011). Cytokines activate target cells by specific receptors, and work in interaction as a complex network.

$CD4^+$ T helper (Th) cells are divided into distinct subsets according to cytokine profile (Akdis *et al.* 2011). The term Th1 cytokines refers to cytokines produced by type-1 helper cells and Th2 cytokines produced by type-2 helper cells. Th1 cells are responsible for delayed-type hypersensitivity reactions as well as autoimmune diseases and rejection of allograft (Romagnani. 2006). On the other hand, Th2 cells play a role in parasitic infections and in allergic inflammatory responses in the body (Romagnani. 2006). Regulatory T cells (Treg) are able to inhibit the development of both Th1 and Th2 cell responses by suppressing the secretion of Th1- and Th2-type cytokines. Th1 cytokines include, for instance, IL-2, IFN-γ, TNF-α and TNF-β, while Th2 cytokines include IL-4, IL-5, IL-9, and IL-13 (Ozdemir *et al.* 2009). In addition, Treg cytokines include IL-2, IL-10, and TGF-β (Ozdemir *et al.* 2009).

RSV first infects respiratory epithelial cells and macrophages, which respond by secreting cytokines. The severity of RSV disease may be associated with the characteristic expression patterns of cytokines (Miller *et al.* 2004). Studies on cytokines and their genetics suggest that some of the inter-individual differences in cytokine profiles can be explained by allelic polymorphisms within the regulatory or coding regions of cytokine genes (Bidwell *et al.* 1999). Studying the genetics of cytokines in RSV pathogenesis has been found to be age-dependent; the cytokine gene allele and genotype distribution among children hospitalized for RSV bronchiolitis at <6 months of age is significantly different from that among children hospitalized at >6 months of age (Hoebee *et al*. 2003, Hoebee *et al.* 2004).

### 2.3.4.1 Interferon-gamma

IFN-γ, a type II interferon, is produced by T cells and natural killer (NK) cells and has pleiotropic biological effects (Young & Hardy. 1995). The properties of IFN-γ include direct antiviral activity, as it assists in the generation and activation of cytotoxic T cells; as well, IFN-γ stimulates antigen presentation through the induction of MHC Class I and II molecule expression and activates NK cells (Boehm *et al.* 1997). IFN-γ has a role in the regulation of the switch of antibody isotopes, including the switch in expression from IgM to IgG2a producing B cells (Boehm *et al.* 1997). IFN-γ is considered to be a key cytokine in inducing protective responses against viral pathogens. IFN-γ, a pro-inflammatory cytokine associated with a Th1-like immune response, is important in recruiting other inflammatory mediators, and in addition, seems to have direct antiviral activity. Production of IFN-γ is genetically controlled; the A allele at +874 is associated with low expression of the *IFNG* gene leading to low IFN-γ production (Pravica *et al.* 2000).

Low IFN-γ levels measured in peripheral blood or in nasopharyngeal aspirates (NPA) have been associated with severe RSV disease (Aberle *et al.* 1999) requiring mechanical ventilation (Bont *et al.* 2001). In a mouse model, the role of IFN-γ in RSV infection was both protective in limiting viral replication and inflammatory responses, but contributed to worsening airway obstruction (van Schaik *et al.* 2000). In line with this, serum IFN-γ was higher in infants with RSV bronchiolitis than in those with non-RSV LRTI, suggesting a role for IFN-γ in airway obstruction (Chen *et al.* 2002). The protective role of IFN-γ together with IL-12 was observed in the same study in terms of diminishing viral replication; high IL-12 and IFN-γ levels in serum may work jointly and lessen the severity of viral infection (Chen *et al*. 2002). In another study, the *INFG* +874 genotypes were associated with clinical scores in infants with LRTI, but the low-production A/A genotypes were associated with a lower score and the high-production genotypes with a higher score (Gentile *et al.* 2003). However, the high-production genotypes were protective for acute otitis media (AOM) (Gentile *et al.* 2003). In a Finnish study, it was also observed that in acute HRV infection, the IFN-γ concentrations were 6-fold higher than in RSV infection and even 27-fold higher than in the control group (Jartti *et al.* 2009b). In addition, adults with a strong IFN-γ response produced fewer viruses during experimental rhinovirus infection (Gern *et al.* 2000, Message *et al*. 2008).

In a birth cohort at risk for allergy and asthma, an association was found between inducible cytokine responses at birth and infections in the first year of life, inversely relating IFN-γ responses to the number of symptomatic respiratory viral infections (Copenhaver *et al.* 2004). In the same birth cohort, children with more than 2 episodes of wheezing during the first year of life had lower inducible cord blood IFN-γ responses, but by 1 year of age, this trend had reversed, and responses were significantly higher compared with children who wheezed once or not at all (Gern *et al.* 2006). In an older study, lower IFN-γ production during bronchiolitis predicted lower pulmonary function and higher responsiveness to histamine after bronchiolitis and asthma later in childhood (Renzi *et al.* 1999).

Viral infections induce bronchial obstruction also by neural reflexes, either directly or indirectly mediated by IFN-γ production. A strong pro-inflammatory response, including high IFN-γ production, may augment wheezing triggered by viral infection. IFN-γ can induce or at least enhance bronchial obstruction by damaging the muscarinic acetylcholine M2 receptors, which limits the release of acetylcholine from vagal nerve endings (Bowerfind *et al.* 2002). This IFN-γ-mediated bronchial obstruction may be one of the mechanisms leading to recurrent wheeze during viral infections, particularly in children who react to viruses with strong IFN-γ responses.

#### 2.3.4.2 Interleukin-10

IL-10 is a multifunctional anti-inflammatory cytokine produced by activated immune cells, in particular monocytes, macrophages and T cell subsets including Treg and Th1 cells (Sabat *et al.* 2010). The principal routine function of IL-10 is to limit and ultimately terminate inflammatory responses (Moore *et al*. 2001). Above all, monocytes/ macrophages are the main target cells of the inhibitory effects of IL-10. IL-10 influences three important functions of monocytes/ macrophages: the release of immune mediators, antigen presentation, and phagocytosis. Simply put, IL-10 suppresses all functions of monocytes/ macrophages that are responsible for the positive role of these cells in both innate and adaptive immunity (Sabat *et al.* 2010). IL-10 antagonizes the LPS- or IFN-γ-induced production of pro-inflammatory cytokines, such as tumor necrosis factor (TNF)-α, IL-1, IL-6, IL-18, leukemia inhibitory factor, granulocyte-CSF, and granulocyte/ macrophage-CSF (Moore *et al.* 2001, Sabat *et al*. 2010) (Figure 2). Moreover, IL-10

inhibits the synthesis of IL-12, hampering the development of Th1 immunity (Sabat *et al*. 2010). Conversely, IL-10 enhances humoral immunity as it promotes the proliferation and differentiation of B cells, enhances MHC II expression, prevents apoptosis in B cells, and plays a positive role in immunoglobulin class switching (Rousset *et al*. 1995, Moore *et al.* 2001). In addition, IL-10 promotes tolerance and suppression by Treg cells (Thunberg *et al.* 2007); the development of a healthy or an allergic immune response seems to be determined by the ratio between Treg and Th2 cells (Akdis *et al.* 2004).

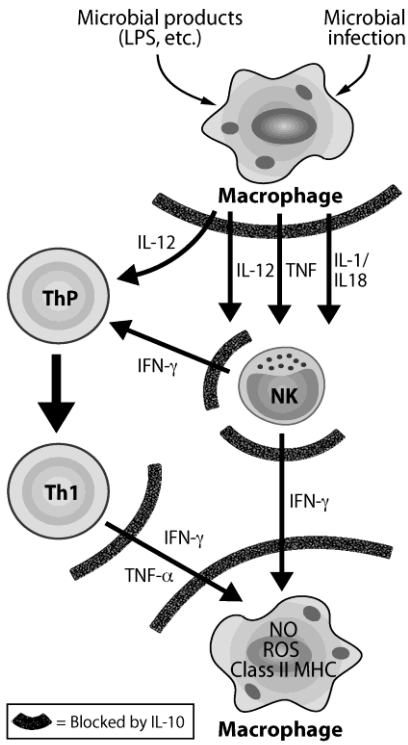


Figure 2. A schematic depiction of IL-10's roles in regulating the innate and adaptive immune responses to infection (according to Moore *et al*. 2001)

The *IL-10* gene is located on chromosome 1 at 1q31–32, and is composed of five exons and four introns (Reuss *et al.* 2002). The *IL-10* gene is highly polymorphic. In the promoter region, there are three SNPs at positions –592 (C/A), –819 (C/T), and –1082 (G/A).The SNPs are also in strong linkage disequilibrium, forming three haplotypes common in the Caucasian population: GCC, ACC, and ATA (Turner *et al.* 1997). The *IL-10* –1082 A/G polymorphism influences the level of *IL-10* production *in vitro* such that

the presence of the G allele is associated with higher IL-10 production (Turner *et al.* 1997).

IL-10 may be a causative mediator in virus-provoked asthma exacerbations, and the generation of IL-10 is a response to a greater degree of pre-existing airway inflammation in individuals predisposed to virus infection (Message *et al.* 2008). In a prospective Finnish cohort study, cord blood samples were collected from newborns and stimulated with LPS from *Escherichia coli*. The hospitalized patients with RSV infection showed lower IL-10 responses than those treated as outpatients (Juntti *et al.* 2009). On the other hand, IL-10 may be protective against hypoxia in infants with bronchiolitis (Bennet *et al.* 2007).

Differences in cytokine production during the first 12 months of life and promoter polymorphisms in cytokine genes may affect RSV disease severity. The *IL-10* –1082 A/A genotype, conferring to low production of IL-10, has been associated with pneumonia during RSV bronchiolitis (Gentile *et al.* 2003). In another study, where the study population consisted of 580 infants with severe RSV infection, *IL-10* gene polymorphisms at the G/G allele increased the risk of requiring mechanical ventilation (Wilson *et al.* 2005). The *IL-10* –592 A/C polymorphisms were underrepresented in infants with RSV bronchiolitis, which means that such heterozygous children might be protected against severe RSV bronchiolitis (Hoebee *et al.* 2004).

Patients with increased IL-10 responses could have increased risk for recurrent wheezing. During a one-year post-bronchiolitis follow-up, an increased monocyte IL-10 response to in *vitro* stimulation during the convalescent phase of RSV bronchiolitis was associated with subsequent recurrent episodes of wheezing (Bont *et al.* 2000). The same study found an association between an increased IL-10 response and physician-diagnosed asthma (Bont *et al.* 2000). Increased *IL-10* gene expression in airway cells may be a feature of virus-induced asthma in older patients with previously diagnosed asthma (Grissel *et al.* 2005). In line with this, it was found that children with HRV or RSV infection in the acute phase had higher IL-10 levels than controls or compared to the convalescent phase of the infection (Jartti *et al.* 2009b).

#### 2.3.4.3 Interleukin-18

IL-18 is a proinflammatory cytokine produced by a wide range of cells, including macrophages, neutrophils and airway epithelial cells. It induces IFN-γ production from T cells without any requirement for T-cell receptor (TCR) engagement, an effect that is enhanced by the presence of IL-12. Thus, IL-12 and IL-18 jointly enhance Th1-oriented responses (Xu *et al.* 1998). IL-18, when acting jointly with IL-12, has an inhibitory effect on IgE production by B cells (Yoshimoto *et al.* 1997). In the absence of IL-12, IL-18 can induce the production of IL-4, IL-10, and IL-13 from NK and T cells and thus promotes Th2-oriented lymphocyte differentiation (Hoshino *et al.* 1999). The *IL-18* gene is located on chromosome 11q22.2–22.3 (Nolan *et al.* 1998). The expression of IL-18 is influenced by *IL-18* promoter gene polymorphisms (Giedraitis *et al.* 2001).

IL-18 may play an immunoregulatory role in allergic and autoimmune diseases, and decreased expression of IL-18 may shift the immune responses to both Th1- and Th2-mediated pathways (Cebeci *et al.* 2006). In bronchial asthma and atopic dermatitis, IL-18 secretion has been significantly higher than in non-allergic patients (El-Mezzein *et al.* 2001). RSV-induced bronchiolitis has been shown to be accompanied by an increased nasal IL-18 response (van Benten *et al.* 2005) explaining the eosinophilia observed in bronchiolitis. The *IL-18* gene polymorphism –133 C/G has been associated with severe RSV infection (Puthothu *et al.* 2007).

## 2.4 Short-term prognosis of bronchiolitis

Bronchiolitis in infancy has been associated with recurrent wheezing, airway hyper-responsiveness, and asthma in later childhood (Wennergren & Kristjansson. 2001, Pérez-Yarza *et al.* 2007, Bont & Ramilo. 2011). Children with a history of bronchiolitis suffer from wheezing or asthma, although in other children, wheezing tends to diminish by adolescence (Stein *et al.* 1999, Sigurs *et al.* 2005). Infants are susceptible to viral infections, and in addition, pulmonary alveolar multiplication and airway remodeling of the airways to accommodate growth takes place in infancy. Therefore, viral infections can adversely affect lung development, and inherited abnormalities in pulmonary function predispose infants to more severe pulmonary disorders (Young *et al.* 1995, Gern *et al.* 2005) (Figure 3).

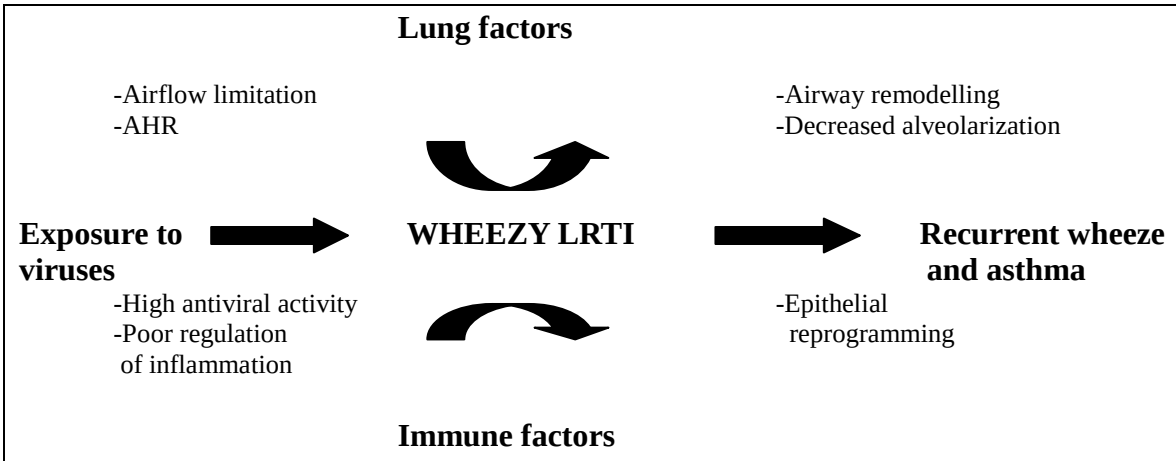


Figure 3. Interactions between lung and immune factors in determining the severity of viral infections and long-term outcomes; AHR, airway hyper-responsiveness (according to Gern *et al.* 2005)

It is not known whether viral bronchiolitis contributes to asthma inception or simply identifies infants who are at increased risk for subsequent wheezing (Martinez *et al.* 1995, Sigurs *et al.* 2005, Piippo-Savolainen & Korppi. 2008) due to asthmatic predisposition or pre-existing abnormal lung function (Singh *et al.* 2007). Three clinical respiratory phenotypes have been identified: (1) transient wheezers, who have poor lung function at birth and wheeze during the first 3 years of life but do not wheeze at 6 years of age, (2) non-atopic wheezers, who have normal lung function at birth and acquire risk factors other than atopy by 11 years of age, and (3) atopic wheezers, who have IgE-associated atopy and tend to develop persistent asthma (Stein. 2009).

Cytokine dysregulation, or a Th1/Th2 imbalance, plays an important role in the development of asthma and allergic diseases. Viral infection in infancy may alter the subsequent pattern of Th1/Th2 immune responses (Legg *et al.* 2003) and early wheezing in infants is associated with a Th2-oriented response (Renzi *et al.* 1997, Martinez *et al.* 1998). On the other hand, the Th2 phenotype is not associated with subsequent asthma, eczema or allergic sensitization after severe RSV infection early in life, suggesting that Th2 orientation is not unique to RSV infection (Castro *et al*. 2008).

### 2.4.1 Role of viruses

The occurrence of recurrent wheezing after RSV bronchiolitis or LRTI has been recognized for decades. Children hospitalized for RSV bronchiolitis during infancy are more likely to have subsequent episodes of recurrent wheezing or childhood asthma during the first decade of life compared with children without a history of bronchiolitis hospitalization during infancy (Sigurs *et al.* 1995, Kneyber *et al.* 2000, Sigurs *et al.* 2000, Schauer *et al.* 2002, Korppi *et al.* 2004, Henderson *et al.* 2005, Kotaniemi-Syrjänen *et al.* 2005, Sigurs *et al.* 2005, Carroll *et al.* 2009). Recurrent wheezing and asthma are common for some years after hospitalization for RSV bronchiolitis, but thereafter decrease by preschool and school age. However, an increased risk of asthma continues at least until early adulthood (Stein *et al.* 1999, Hyvärinen *et al.* 2005, Goksör *et al.* 2008, Piippo-Savolainen & Korppi. 2008).

RSV has been the most studied virus causing bronchiolitis and inducing post-bronchiolitis wheezing, but the development of PCR methodology has facilitated the diagnosis of viral infections. Certain viruses with antigen or antibody assays not available, such as HRVs, have also proved to be important in bronchiolitis. HRV-induced wheezing in infancy is an important predictor of recurrent wheezing (Kotaniemi-Syrjänen *et al.* 2003, Korppi *et al.* 2004, Lemanske Jr *et al.* 2005, Kusel *et al.* 2007, Lehtinen *et al.* 2007, Jartti *et al.* 2008, Koponen *et al.* 2011). HRV bronchiolitis is associated with an increased prevalence of asthma at early and late school age; asthma was present in 24–58% of former rhinovirus bronchiolitis patients and only in 4–20% of former RSV bronchiolitis patients (Kotaniemi-Syrjänen *et al.* 2003, Hyvärinen *et al.* 2005, Koponen *et al.* 2011).

Recurrent wheezing episodes are common until 4 years of age after bronchiolitis in infancy. RSV, HRV, and PIV, have been associated with post-bronchiolitis wheezing (Table 2), although the focus has been on RSV bronchiolitis (Schauer *et al.* 2002Cifuentes *et al.* 2003, Henderson *et al.* 2005). An RSV etiology of bronchiolitis increases the risk of post-bronchiolitis wheezing compared to children without respiratory infections in infancy (Schauer *et al.* 2002), compared to children with bronchiolitis caused by some other virus (Cifuentes *et al.* 2003) or to non-selected children from a birth cohort (Henderson *et al.* 2005). However, HRV was not analyzed in these studies.

HRV bronchiolitis remarkably increases the risk of post-bronchiolitis wheezing (Lemanske Jr *et al.* 2005, Valkonen *et al.* 2009), but in one study, picornavirus infection

including both HRV and enteroviruses was protective (Lee *et al.* 2007). Enteroviruses can cause bronchiolitis (Jartti *et al.* 2004, Jacques *et al.* 2008a, van der Zalm *et al.* 2009, Nascimento *et al.* 2010), though they usually cause milder respiratory infections (Jacques *et al.* 2008a) and induce wheezing in >3 years old children (van der Zalm *et al.* 2009). There are no studies on subsequent wheezing specifically associated with enterovirus bronchiolitis. In a birth cohort consisting of children at risk for asthma and allergy, HRV infection with wheezing in infancy was the strongest predictor of wheezing at 2-3 years of age and increased the risk to 3-fold compared with RSV infection with wheezing (Lemanske Jr *et al.* 2005). In a Finnish retrospective study of post-bronchiolitis wheezing, children with non-RSV infection had a 4-fold risk of subsequent recurrent wheezing compared with laboratory-confirmed RSV infection (Valkonen *et al.* 2009).

Data on allergy or atopic sensitization are available in most post-bronchiolitis studies (Schauer *et al.* 2002, Cifuentes *et al.* 2003, Lemanske Jr *et al.* 2005, Lee *et al.* 2007). RSV bronchiolitis has been strongly associated with food allergy (Schauer *et al.* 2002) and with atopic sensitization in children at risk (Lemanske Jr *et al.* 2005), but two studies were negative (Cifuentes *et al.* 2003, Lee et al. 2007).

*Table 2. Studies on association between viral etiology of bronchiolitis and subsequent wheezing. Included studies were published in 2002-2010, study children were < 12 months of age, at least RSV and HRV were assayed and follow-up time was 6-36 months*

| Author | Study design | Virus | Patients/controls | Follow-up time * | Age at bronchiolitis | Outcome | Measure of association |
|---|---|---|---|---|---|---|---|
| Schauer | Prospective | RSV | 42/84 | 6-9 | 16 weeks ** | Reported wheezing | OR=12.1, p<0.01 |
| *et al*. 2002 | case-control study | | | | | IgE (food) | OR=56.4, p<0.01 |
| | | | | | | IgE (pneumoallergen) | OR=6.4, NS |
| Cifuentes | Prospective | RSV vs. | 36 | 12 | 3.5 months** | Recurrent wheezing | aRR=1.41 (CI 1.03,1.93), p=0.03 |
| *et al*. 2003 | cohort study | non-RSV | 41 | | 4.2 months** | Eczema | aRR=0.93 (CI 0.67,1.31), p=0.68 |
| | | | | | | Allergic rhinitis | aRR=1.46 (CI 0.99,2.15), p=0.050 |
| Henderson | Longitudinal | RSV | 150/9826 | 42 | | Reported wheezing | OR= 2.3 (CI 1.3,3.9), p=0.002 |
| *et al.* 2005 | birth cohort study | | | | | | |
| Lemanske Jr | Prospective cohort study of "high risk" children | Any | 87 | 36 | | Third year wheezing | OR=10 (CI 3.0,44) |
| *et al*. 2005 | | RSV | 50 | | | | OR=3.0 (CI 1.6,5.8) |
| | | HRV | 43 | | | | OR=10 (CI 4.7,23) |
| | | Other | 47 | | | | OR=3.9 (CI 0.96,3.2) |
| | | | | | | Food allergies at 1 year | OR=2.0 (CI 1.1,3.6) |
| Lee | Randomized prospective cohort study of "high risk" children | RSV | 182/248 | 24 | | Persistent/transient asthma | OR=1.3 (CI 0.51,3.30), p=0.574 |
| *et al.* 2007 | | PIV | 214/215 | | | | OR=2.82(CI 1.10,7.25), p=0.059 |
| | | Picornavirus | 140/304 | | | | OR=0.72 (CI 0.28,1.85), p=0.765 |
| Valkonen | Retrospective analysis | RSV | 199 | 36 | 0.34 years† | Recurrent wheezing | |
| *et al.* 2009 | | non-RSV | 217 | | 0.92 years† | | RR=3.4 (CI 2.0,5.7), p<0.001 |

*months **mean ; †median

CI, 95% confidence Interval; OR, odds ratio; aRR, adjusted relative risk; RR, relative risk; Other, other than RSV or HRV

### 2.4.2 The role of atopy

RSV bronchiolitis during the first year increased risk of the later allergy and asthma (Sigurs *et al.* 2000, Oddy *et al.* 2002). HRV infections in infancy seem to interact with atopy, promoting later allergic asthma (Kusel *et al.* 2007). Atopic dermatitis in infancy and atopic sensitization to aeroallergens and certain foods in early childhood are well-known allergic risk factors for asthma at school age (Martinez *et al.* 1995, Sigurs *et al.* 2000, Oddy *et al.* 2002, Lehtinen *et al.* 2007, Jackson *et al.* 2008, Kotaniemi-Syrjänen *et al.* 2008). Viral infection and atopic inflammation may interact synergistically, contributing to asthma pathogenesis (Martinez *et al.* 1998, Oddy *et al.* 2002, Kusel *et al.* 2007). The age of the child may play an important role; atopic sensitization during the first two years of life seems to increase asthma risk more than later sensitization (Kusel *et al.* 2007). HRV, but not other viruses, has been associated with aeroallergen sensitization (OR 4.18, CI 95% 2.00 to 8.72), total IgE levels (OR 2.06, CI 95% 1.32 to 3.21), and food allergen sensitization (OR 2.02, CI 95% 1.08 to 3.78) (Jartti *et al.* 2010).

There are also studies with negative results. Atopy at school age was not associated with RSV bronchiolitis (Henderson *et al.* 2005), and atopic dermatitis was not associated with recurrent wheezing or asthma at preschool age after RSV or hMPV bronchiolitis (Carcía-García *et al.* 2007). In most studies, a family history of atopy has not been associated with recurrent wheezing and asthma after bronchiolitis (Legg *et al.* 2003, Bont *et al.* 2004, Lemanske Jr *et al.* 2005, Jackson *et al.* 2008). On the other hand, in many studies, a family history of asthma, especially maternal asthma, has been a risk factor for asthma after bronchiolitis (Martinez *et al.* 1995, Cifuentes *et al.* 2003, Lehtinen *et al.* 2007, Carroll *et al.* 2009, Koponen *et al.* 2011).

### 2.4.3 Role of genetics

It has been discussed that RSV bronchiolitis and asthma have shared genetic determinants. Several polymorphisms in cytokine genes linked to asthma have been found as significant in studies of RSV severity. Compared to asthma, the number of candidate gene associations related to bronchiolitis has thus far been limited. Among

the 29 genes strongly linked to asthma, only nine have been studied as candidate genes for severe RSV infection (Singh *et al.* 2007). Polymorphisms in the *IL-10*, chemokine receptor 5(*CCR5*), transforming growth factor-β1 (*TGFB1*), *TLR4*, *IL-13*, *IL-4*, and *IL-4Rα* genes have been associated with RSV severity, whereas polymorphisms in the monocyte *CD14* and *TNF* genes have not had such an association (Choi *et al.* 2002, Hoebee *et al.* 2003, Hull *et al.* 2003, Hoebee *et al.* 2004, Tal *et al.* 2004, Wilson *et al.* 2005). In addition, polymorphisms in the promoter region of the *IL-8* gene may be associated with repeated wheezing after bronchiolitis (Goethebuer *et al.* 2004). The *IL-4–IL-13* locus seems to contribute to both the severity of RSV bronchiolitis and the risk of atopy (Forton *et al.* 2009).

The *IL-10* promoter polymorphism at –1082 A/G, –819 C/T, and –592 C/A have been connected to asthma, atopic dermatitis, IgE levels, and eosinophil counts in asthma (Lim *et al.* 1998, Karjalainen *et al.* 2003, Chatterjee *et al.* 2005). The –1082A allele has been shown to be more common in individuals with severe asthma (Chatterjee *et al.* 2005, Lim *et al.* 1998).

MBL-deficient polymorphisms have been found in children prone to respiratory infections (Koch *et al.* 2001, Cedzynski *et al.* 2004, Kristensen *et al.* 2004). Results on the association between serum MBL levels or *MBL2* gene polymorphisms and allergy or asthma in children are conflicting (Uguz *et al.* 2005, Leung *et al.* 2006, Müller *et al.* 2007). The expectation is that high serum MBL levels, rather than low levels, would be associated with allergy, wheezing and asthma, as was seen in 72 Turkish schoolchildren with asthma (Uguz *et al.* 2005). One *MBL2* gene polymorphism has been found to be associated with increased serum MBL levels, eosinophilia, and asthma with allergic bronchopulmonary aspergillosis (Kaur *et al.* 2006). However, large population-based studies performed in blood donors, school children and adults have failed to confirm any association between *MBL2* gene polymorphisms and asthma or allergy (Leung *et al.* 2006, Müller *et al.* 2007, Wang *et al.* 2007, Rantala *et al.* 2008, Cardinale *et al.* 2008). In a birth cohort study from the UK, MBL levels did not differ between atopic and non-atopic children or between wheezing and non-wheezing children (Staley *et al.* 2007). In wheezing children, however, MBL levels were associated with the severity of wheezing assessed by the required treatment; serum MBL was higher in children treated with inhaled corticosteroids compared with untreated wheezing children and with healthy children (Staley *et al.* 2007).

Viral respiratory infections predispose children to AOM. New otitis media episodes coincident with evidence of RSV and HRV infection were significantly more frequent in children with high production *IL-10* phenotypes (Alper *et al.* 2009). Innate immunity presumably mediates the initial host response to and defense against infection, because very few lymphocytes are found in the healthy middle ear (Ryan *et al.* 1986). The involvement of the innate immunity mediated by TLRs in AOM has been implicated primarily in cell lines and in association studies of innate immune gene polymorphisms and AOM prevalence (Leichtle *et al.* 2011). Both TLR2 and TLR4 signaling were critical to the regulation of infection in AOM caused by non-typeable *Haemophilus influenzae* in a mouse model (Leichtle *et al.* 2009).

### 2.4.4 Role of weight gain

The prevalence of obesity has reached epidemic proportions concomitantly with the asthma and allergy epidemics. A recent systematic review proposed that obesity precedes asthma in children and is associated with the persistence and intensity of symptoms (Noal *et al.* 2011). The pathogenic link between asthma and obesity is difficult to establish. Changes in many adipose-derived inflammatory mediators, including TNF-α, leptin, and adiponectin, have the capacity to promote airway reactivity and may thus contribute to AHR and asthma in the obese (Flaherman & Rutherford. 2006, Peroni *et al.* 2010). The association between weight gain and early childhood wheezing is less studied than is the association between overweight and childhood asthma (Korppi. 2010).

In Denmark, a registry-based study of 8280 twin pairs showed that low birth weight is a risk factor for asthma in children 3–9 years old, independently of gestational age, sex, and birth length (Kindlund *et al.* 2010). In a Finnish nation-wide, register-based, nested case-control study a low ponderal index was an independently significant risk factor for asthma in children less than 3 years of age (Metsälä *et al.* 2008). In line with this, lower birth weight in children born at term was associated with wheezing at 2–6 years of age, but not with earlier wheezing (Caudri *et al.* 2007).

High birth weight has also been connected to asthma and atopy in childhood (Yuan *et al.* 2002, Sin *et al.* 2004, Remes *et al.* 2008). In a Finnish birth cohort followed up

until 16 years of age, a significant association was found between high birth weight (>4500 g) and asthma among atopic subjects (Remes *et al.* 2008).

The association between weight gain and wheezing in early childhood has been poorly studied. In a retrospective study, wheezing at 3–12 months of age was associated with higher WFL Z-scores (Jee *et al.* 2010). In two birth cohort studies, infants with higher WFL Z-scores at 6 months of age had a greater risk of recurrent wheezing by 3 years of age, but not at 1 or 2 years of age (Taveras *et al.* 2008), and greater infant weight and adiposity gains assessed by subscapular skinfold thickness at 0–6 and 6–12 months were associated with both atopic and non-atopic wheeze (Pike *et al.* 2010).

# 3. AIMS OF THIS STUDY

The principal aim of this thesis was to study the etiology, genetic risk factors, and short-term outcome of bronchiolitis in infants aged <6 months. The specific aims were to evaluate:

1. The viral etiology of bronchiolitis in infants hospitalized for bronchiolitis at <6 months of age.
2. The occurrence of the polymorphisms *IL-10* –1082 G/A, *IL-18*–137 G/C, *TLR4* +896 A/G, and *IFNG* +874 T/A in infants with bronchiolitis, compared with unselected blood donors, and their associations with the viral etiology and clinical characteristics of bronchiolitis.
3. The connection between the polymorphisms *IL-10* –1082 G/A, *IL-18* –137 G/C, *TLR4* +896 A/G, and *IFNG* +874 T/A and subsequent infections and post-bronchiolitis wheezing until the age of 18 months.
4. The occurrence *MBL2* gene polymorphisms in infants with bronchiolitis and the associations between polymorphisms and clinical characteristics and the outcome of bronchiolitis.
5. The prevalence and clinical picture of co-infections with *Bordetella pertussis* in viral bronchiolitis, in relation to vaccination status.
6. Birth weight, weight gain in infancy and overweight assessed by weight for height at 1–2 years of age as risk factors for wheezing after hospitalization for bronchiolitis.

# 4. MATERIALS AND METHODS

## 4.1 Study design

The study was conducted at the Department of Pediatrics, Tampere University Hospital between December 1st 2001 and May 31st 2002 and between October 28th 2002 and May 31st 2004. The study periods covered three winter seasons, including two RSV outbreaks from December 2001 to March 2002 and from November 2003 to March 2004. The follow-up visits for children treated during the first season were organized from May 5th to June 13th 2003 (69 children attended), for children treated during the second season from May 3rd to May 7th 2004 (22 children attended) and for children treated during the third season from May 30th to June 8th 2005 (38 children attended).

Infants <6 months of age with the first LRTI needing hospitalization were eligible for the study. On admission, the patients underwent normal clinical evaluation by doctors on duty including medical history and physical examination.

### 4.1.1 Patients

During the two study periods in total 325 infants <6 months of age were hospitalized because of LRTI with discharge diagnosis J21.0 or J21.9 (WHO ICD-10, 1992). From December 1st 2001 to May 31st 2002 there were 126 admissions and from October 28th 2002 to May 31st 2004 there were 197 admissions. Upon admission, 203 infants with bronchiolitis were recruited, and 187 infants were enrolled in the study. Exclusion criteria included prematurity in 13 infants, severe and chronic underlying disease in two infants, verified bacterial infection (caused by *B. pertussis* in two cases and *Chlamydia trachomatis* one case) and readmission (11 cases) during the study period (Figure 4).

Bronchiolitis was defined as an acute LRTI characterized by rhinorrhea, cough, tachypnea, and diffuse wheeze or widespread inspiratory crackles, and/or apnea, and/or

feeding problems (AAP. *et al.* 2006, Fitzgerald & Kilham. 2004, Simoes. 1999, Smyth & Openshaw. 2006).

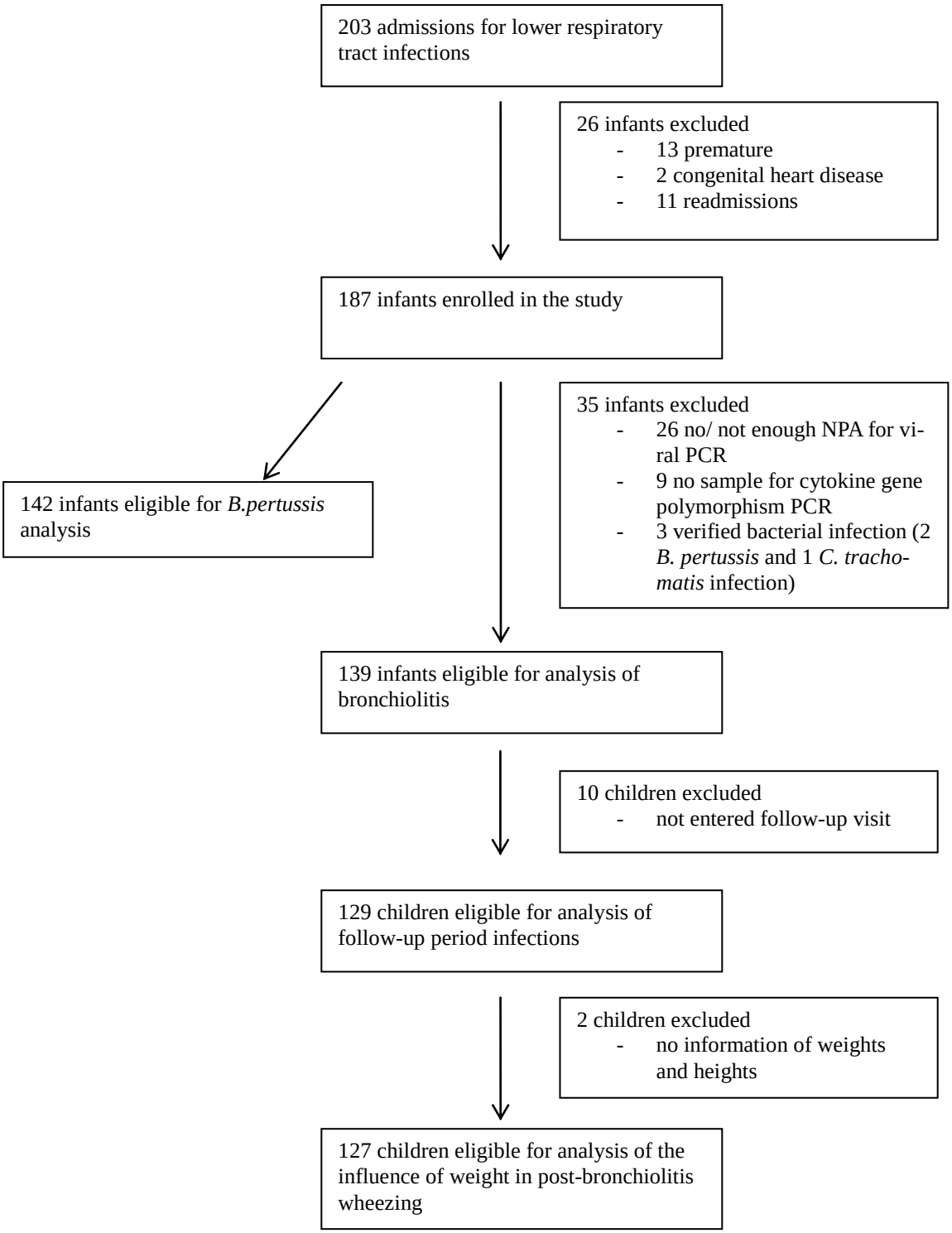


Figure 4. The study subjects

### 4.1.2 Follow-up

Upon departure from the hospital, follow-up diaries were given to the parents, and they were trained on how to record information in the diaries. The information consisted of illness histories of respiratory infections, also including otitis media and wheezing episodes during the follow-up period.

The children were invited to attend the follow-up visit about 12 months after hospitalization at an average age of 18 months. At the follow-up visit, the diaries filled in by the parents were checked, the parents were interviewed, and structured questionnaires were filled in by the doctor. A physical examination was performed on the children, the weights and heights were measured, and blood samples were taken for IgE measurements and genotyping of cytokine gene polymorphisms. Atopy was defined by elevated total serum immunoglobulin E (IgE) or by the presence of atopic dermatitis diagnosed by a dermatologist. Serum IgE was considered to be elevated if the concentration was more than +2 SD above the mean for non-atopic Finnish children (>60 kU/l) (Saarinen *et al.* 1982). A questionnaire regarding illness histories and demographic data was sent to the study patients who did not come in for the follow-up visit.

## 4.2 Demographic data

The mean age of the 139 infants attending the study was 10.1 weeks (SD 6.9, range 1–25). There were 69 (49.6%) boys and 70 girls. The mean duration of hospital stay was 4.7 days (SD 3.2, range 0–28), and none needed intensive care. About a year later, 129 children attended the follow-up visit at a mean age of 18.1 months (SD 2.3, range 13–25). At follow-up, there were 63 (48.8%) boys and 66 girls (Table 3).

*Table 3.*

*Basic data of the study patients*

| | **Hospital** | **Follow-up** |
|---|---|---|
| Patients (N) | 139 | 129 |
| Male gender, N (%) | 69 (49.6) | 63 (48.8) |
| Age (mean, SD, range) | 10.1 w ± 6.9 (1-25) | 18.1 mo ± 2.3 (13-25) |
| Breastfeeding, N (%) | 101 (72.6) | 92 (71.3) |
| No. siblings (mean, SD, range) | 2.3 ± 1.4 (1-10) | 2.5 ± 1.5 (1-10) |
| Pet in the family, N (%) | - | 36 (27.9)* |
| Smoking in the family, N (%) | - | 58 (45.0)* |
| Allergy in a first degree relative, N (%) | - | 84 (65.1)* |
| Length of hospital stay, days (mean, SD, range) | 4.7 ± 3.2 (0-28) | 4.6 ± 3.1 (0-22) |
| Feeding support, N (%) | 48 (34.5) | 44 (34.1) |
| Required oxygen, N (%) | 26 (18.7) | 23 (17.7) |

w, weeks; mo, months

*no statistical significance between RSV and non-RSV groups, Chi-square test

## 4.3 Microbiological methods

Nasopharyngeal aspirates (NPA) were taken upon admission and all NPAs were studied by direct immunofluorescence (IF) at the Laboratory Center of Tampere University Hospital. NPAs were collected with a disposable catheter inserted in the nostril to the depth of 5–7 cm and drawn back while applying gentle suction with a mechanic suction device. The specimens were transported at room temperature to the laboratory, where antigen detection tests were done on the same day or the following day. The rest of the sample was frozen at –70 °C for subsequent PCR studies. Since the IF and PCR tests gave no contradictory results for the viruses studied by both methods, and PCR identified more cases over IF, only PCR results have been reported.

### 4.3.1 Viruses

The viruses studied in fresh samples by IF antigen detection were RSV, influenza A (IVA) and B (IVB) virus, parainfluenza (PIV) types 1, 2 and 3 and adenovirus [PathoDx Respiratory Virus Panel (RVP) kit, Remel products, Thermo Fisher Scientific, Lenexa, KS, USA].

In frozen samples, multiplex nested RT-PCR was used for RSV and IVA detection (Stockton *et al.* 1998) and nested RT-PCR was used for the detection of PIV type 3 (Echeverria *et al.* 1998). For adenovirus detection, PCR was used as described earlier (Allard *et al.* 1992). For human rhinovirus (HRV) detection, a separate RT reaction was performed (Pang *et al.* 2005) followed by real-time PCR (Deffernez *et al.* 2004). One-step PCR detection was used for human metapneumovirus (hMPV) (Peret *et al.* 2002) and human bocavirus (HBoV) (Sloots *et al.* 2006).

Viral RNA extracted from RSV, PIV type 3, hMPV, HRV, and adenovirus cell culture samples were used as positive controls. RSV A and B virus stocks were received from the Department of Virology, University of Turku. A positive hMPV control stock #26583 was received from the Centre de recherché en infectiologie, Québec, Canada. PIV type 3 and adenovirus strain B and C stocks were from the Global Bioresource Center, ATCC number VR-93, VR-3, and VR-846, respectively. An HRV cell culture stock sample was received from the University of Tampere Medical School, Department of Virology. PIV type 3 and hMPV were cultured in the LLC-$MK_2$ cell line and RSV B in the A549 cell line. RSV A was cultured in HeLa cells from a positive patient sample.

All amplification products were visualized under UV illumination (AlphaDigiDoc® image analyzer) by electrophoresis on ethidium bromide-containing agarose gel. Confirmation of the positive PCR finding was done by sequencing using an ABI PRISM® 10 Genetic Analyzer and comparing the results to published sequences in GenBank. Phylogenetic analyses of hMPV sequences were done by CLUSTALW using the multiple-sequence alignment method.

### 4.3.2 *Bordetella pertussis*

In 2009, good-quality frozen NPA samples were available for *B. pertussis* PCR from 142 children hospitalized for bronchiolitis 5 to 8 years earlier. NPAs were transported frozen to the Pertussis Laboratory at the National Institute for Health and Welfare (THL), Turku and the Department of Medical Microbiology and Immunology, University of Turku.

The diagnostic PCR for the detection of *B. pertussis* was done using an in-house PCR method, as described previously (He *et al.* 1993). The High Pure PCR Template Preparation Kit (Roche Diagnostics GmbH, Mannheim, Germany) was used for DNA extraction from NPAs according to the instructions from the manufacturer. Five microliters of extracted DNA were used for PCR amplification.

The primers were designed according to insertion sequence 481 of *B. pertussis* genome (5, 12) (Sigma Aldrich, Haverhill, UK). DNA extracted from the prototype strain of *B. pertussis* at a concentration of 5 ng/µl was used as the positive control. All reagents, devoid of added DNA, were included in the negative control tube in each PCR run. Ten microliters of the PCR products were run on a 1.5% agarose gel. After staining with ethidium bromide and destaining with water, PCR products were visualized and photographed under UV light.

## 4.4 Genetic methods

The gene polymorphisms of four cytokines that have been shown to influence the clinical picture of bronchiolitis and/or susceptibility to asthma development were selected for this study (Kurt-Jones *et al.* 2000, Nagarkatti *et al.* 2002, Gentile *et al.* 2003, Tal *et al.* 2004, Chatterjee *et al.* 2005, Wilson *et al.* 2005, Puthothu *et al.* 2007). A venous sample for genotyping *IL-18* –137 G/C, *IL-10* –1082 G/A, *TLR 4* +896 A/G and *IFNG* +874 T/A was drawn during hospitalization for bronchiolitis, if venous samples were needed for clinical purposes. Forty-three samples were collected during the hospitalization period and 96 at the follow-up visit. The samples were transported to the Laboratory Center of Tampere University Hospital and frozen at –70°C until they were analyzed together.

### 4.4.1 Cytokine and toll-like receptor 4 gene polymorphisms

DNA was extracted from whole blood and the buffy coat using a commercially available kit (QIAGEN Inc., USA) according to the manufacturer's instructions at the Laboratory Center of Tampere University Hospital. The rest of the genomic DNA was frozen at –70°C.

Genotyping of the *IL-18* –137 G/C (rs187238), *IL-10* –1082 G/A (rs1800896) and *TLR 4* +896 A/G (rs4986790) gene polymorphism was performed using the ABI PRISM® 7000 Sequence Detection System for both PCR and allelic discrimination (Applied Biosystems, CA, USA). For *IL-18* –137 G/C and *TLR4* +896 A/G, the nucleotide sequences of the primers and fluorogenic allele-specific oligonucleotide probes were deduced from the published sequence deposited in the GenBank database and were chosen and synthesized in conjunction with Applied Biosystems (Assay by Design). For *IL-10* –1082 G/A, a commercial kit was used (Assay on Demand, C_1747360_10 IL10). The universal PCR thermal cycling conditions from ABI were followed: 50ºC for 2 min, then 95ºC for 10 min, and then 40 cycles at 95ºC for 15 s and 60ºC for 1 min. The PCR reaction was done in a 25 µl reaction 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.

The *IFNG* +874 T/A (rs2430561) polymorphism was analyzed by the amplification refractory mutational system-PCR method described earlier (Pravica *et al.* 2000, Raitala *et al.* 2005). Genomic DNA was amplified using Thermoprime$^{PLUS}$ DNA polymerase (Abgene, Surrey, UK) in two different PCR reactions; each reaction employed a generic antisense primer and one of the two allele-specific sense primers. To assess the success of PCR amplification, one internal control of 426 bp was amplified using a pair of primers designed from the nucleotide sequence of human growth hormone. PCR was performed in a 25 µl volume and conditions were as follows: 95ºC for 1 min, then 10 cycles of 95ºC for 15 s, 62ºC for 50 s and 72ºC for 40 s, followed by 20 cycles of 95ºC for 20 s, 56ºC for 50 s and 72ºC for 50 s. The amplified products were separated by electrophoresis on a 2% agarose gel stained with ethidium bromide.

### 4.4.2 Control samples

Four hundred control samples were obtained from unselected blood donors at the Finnish Red Cross (FRC) Blood Transfusion Center, Tampere. The blood samples donated for the study contained no donor identification labels. The control samples were analyzed in the same way as the study samples at the Laboratory Center of Tampere University Hospital.

### 4.4.3 Mannose-binding lectin 2 gene polymorphism

The genotyping of *MBL2* was performed from previously extracted DNA. Genomic DNA was extracted at the Laboratory Center of Tampere University Hospital and the samples were transported frozen to the Laboratory Department of THL, Turku, where the analysis was performed in 2010.

The genotyping of *MBL2* gene mutations was performed by pyrosequencing for simultaneous detection of three SNPs in codons 52, 54, and 57, in the Pertussis Laboratory of THL, Turku and the Department of Medical Microbiology and Immunology, University of Turku.

Genomic DNA was purified from peripheral blood with the Qiagen QiAmp DNA Blood Mini Kit 250 (Qiagen, Hilden, Germany) according to the instructions from the manufacturer. Five microliters of isolated DNA were used for the amplification of the first exon of the *MBL2* gene by PCR. The PCR product was used for pyrosequencing as described earlier (Roos *et al.* 2006) and with modifications made by the Pertussis Laboratory. Sequential addition of NTPs was done in the following order: CTCTGTGTCATCACAGC. The reaction was performed at 28ºC.

The close proximity of three functionally important SNPs in exon 1 of the *MBL2* gene allows their detection in one single pyrosequencing reaction. This procedure resulted in pyrograms with unique and easily identified patters for each allele combination of the three *MBL* SNPs.

## 4.5 Determination of total serum immunoglobulin E

A venous sample was drawn from study patients during the follow-up visit for the determination of serum total IgE. The samples were transported to the Laboratory Center of Tampere University Hospital and frozen at –70ºC. All the samples were collected and assessed together by electro-chemiluminescence (ECLIA, Roche Diagnostics GmbH, Mannheim, Germany) according to routine practice.

## 4.6 Outcome data collection

### 4.6.1 Clinical findings during hospitalization

Symptoms of respiratory tract infection before hospitalization were recorded upon admission, including the presence and duration of cough, fever, apnea, dyspnea, rhinorrhea, and eating problems. Demographic data included age in weeks, gender, number of siblings, history of breastfeeding, smoking habits in the families, and allergies/ asthma in the families.

Clinical and laboratory data were recorded on departure from hospital. Clinical data included the length of hospitalization in days, highest fever and duration of fever >37.5ºC, the need and duration of oxygen support, the lowest oxygen saturation recorded, and the type and duration of eating support if needed. The need, doses and duration of the use of inhaled drugs and the route of drug administration (spacer or nebulizer) were recorded. Systemic drugs such as antibiotics or glucocorticoids were also recorded. If mechanical support for respiration (artificial ventilation, continuous positive airway pressure) or intensive care was needed, the data were recorded.

In 2009, patient cards were reviewed retrospectively and cough symptoms on admission were classified into three categories: cough with spells, cough without spells and no cough.

### 4.6.2 Diseases during follow-up

In all, 129 children attended the follow-up visit. The mean follow-up time of the children after the bronchiolitis episode was 15.6 ± 1.7 months (SD) (range 11.3–19.5).

At the follow-up visit, the parents returned the diaries with data on wheezing, infections other illnesses including the date, diagnosis, and treatment. Demographic data were collected by using a structured questionnaire (Appendix 1). The data in the diaries were supplemented by interviewing the parents, with special attention to doctor-diagnosed wheezing episodes, food allergy and/or atopic dermatitis in the study children, as well as the smoking habits, pets, and allergies in the families. The use of oral and inhaled corticosteroids was registered at the follow-up visit, and data were supplemented from the medical records of the hospital. The use of daily inhaled corticosteroids or the need for oral or inhaled corticosteroids during wheezing episodes were recorded separately. In the analyses, the use of corticosteroids was analyzed as combined. The number of children using inhaled corticosteroids was 17/25 (68%) and the number of children using one or more systemic corticosteroid courses was 8/25.

### 4.6.3 Weight status

The birth weights of the infants were obtained from the medical records of the hospital during the control visit. Birth weights were classified into three groups: <3000 g, 3000-4000 g and >4000 g. A birth weight of 3000 g approximates the $5^{th}$ percentile and 4000 g approximates the $95^{th}$ percentile of birth weights in Finnish newborns (Pihkala *et al.* 1989).

On admission, the weights of the infants were measured using calibrated scales and registered. The rate of weight gain was calculated as the weight on admission minus the birth weight divided by age in weeks upon admission and was expressed as gram per week. Weight gain was regarded as poor if the mean weight gain was <70 g/ week (<10 g/ day).

At the follow-up visit the children were measured using standardized techniques and calibrated scales and stadiometers. Length was measured to the nearest 0.1 cm in a fully extended supine position with the heels in contact with a baseboard. Children were weighed without clothing, and the weight was rounded to the nearest 0.1 kg. The

WFLs were calculated using the Finnish national population-based gender-specific growth charts as references (Sorva *et al.* 1990a, Sorva *et al.* 1990b). WFLs were categorized by the limits of 110% (overweight) and 120% (obesity). Underweight was defined in two ways, WFL <90% and WFL <80% (Cole *et al.* 2000).

## 4.7 Statistical analyses

All analyses were performed using the Statistical Package for Social Science versions 16.0–19.0 (SPSS Inc., Chicago, IL, USA and IBM SPSS Co., NY, USA). The Chi-square and Fisher's exact tests were used for categorical variables, and Student's t-test was used for normally distributed data and the Mann-Whitney U-test was used for non-normally distributed continuous variables.

The allele carrier status and allele and genotype frequencies were compared between infants with different viral etiology of bronchiolitis and the blood donor with the Chi-square test. Kruskall-Wallis ANOVA was used to evaluate the associations between the genotype and the measures of bronchiolitis severity such as the duration of hospitalization, feeding support, and oxygen treatment, and to evaluate the associations between genotype and post-bronchiolitis wheezing, recurrent infections, and prescribed antibiotic courses.

Hardy-Weinberg equilibrium (HWE) analysis was performed for the *IL-10*, *IL-18*, *TLR4*, and *IFNG* polymorphic sites.

Logistic regression with adjustments for sex and allergy was used to analyze the associations between weight parameters and post-bronchiolitis wheezing. Logistic regression analysis, adjusted for allergy (atopic dermatitis and/or food allergy), was used to analyze the association between carrying the *IFNG* A allele and the use of corticosteroids.

Two-tailed tests were used in all analyses, and the statistical significance was estimated by p-values or by odds ratios (OR), adjusted ORs (aOR), and their 95% confidence intervals (95% CI). A result was considered to be statistically significant if the p-value was <0.05, or if the upper or lower limit of the 95% CIs of ORs or aORs did not include the value 1.0.

## 4.8 Ethics

The study protocol was approved by the Ethics Committee of the Tampere University Hospital District. Informed consent was obtained from at least one parent of the child before enrolling the children in the study. The personal data of the study subjects were not given to the laboratories which performed the genetic studies.

# 5. RESULTS

## 5.1 Etiology and genetics of bronchiolitis

### 5.1.1 Viral findings (I, II, III)

In 2005, when the PCR was done for the first time, the viruses studied were RSV, influenza A, PIV type 3, HRV, and adenovirus. In 2006 the virological studies were verified and supplemented by PCR for hMPV. The results were available for original articles I and II. When the PCR for HBoV was done in 2009, all viral findings were combined, and these final results were available for original articles III and IV. Viral findings by IF antigen detection were done in fresh samples upon admission for RSV, IVA, IVB, PIV types 1, 2, and 3, and adenoviruses. When reporting viral results, only findings by PCR were reported, because there were no differing results by IF.

A potential causative virus was detected by PCR in 121/139 (87.1%) samples (I), 112/129 (86.8%) samples (II, hMPV included), and in 115/129 (89.1%) samples (III and IV, HBoV included). IF was positive in 99/139 (91 RSV positive, 6 IVA positive, 2 adenovirus positive and 0 PIV type 1, 2 or 3 positive). The respective figures by PCR were 103 for RSV (97 single and 6 mixed), 6 for IVA, 5 for adenoviruses (1 single and 4 mixed) and 0 for PIV type 3. There were no contradictory results between IF and PCR, but PCR identified some additional cases. HRV, hMPV, and HBoV were studied only by PCR. As seen in Table 4, the most common virus detected was RSV; the proportion varied from 62.8% to 69.8%. HRV was the second most common virus; the proportion varied from 6.5% to 7.0%. In the final results, mixed viral infection caused by two viruses was detected in 13.2%. Finally, there were only 14 (10.9%) NPA samples with no virus detected. HBoV was detected only with other viruses: with RSV in 4 cases and with HRV in 3 cases (Table 4).

*Table 4.*

*Causative virus in infants with bronchiolitis*

| **Causative virus** | **Original article I** | **Original article III** |
|---|---|---|
| | N (%) | N (%) |
| *Single infections* | | |
| Respiratory syncytial virus (RSV) | 97 (69.8) | 81 (62.8) |
| Human rhinovirus (HRV) | 9 (6.5) | 9 (7.0) |
| Influenza A virus (IVA) | 6 (4.3) | 6 (4.7) |
| Human metapneumovirus (hMPV) | 2 (1.4) | 2 (1.6) |
| Adenovirus | 1 (0.7) | 0 |
| Human bocavirus (HBoV) | ND | 0 |
| *Mixed infections* | | |
| RSV+ HBoV | ND | 4 (3.1) |
| RSV+ HRV | 2 (1.4) | 6 (4.7) |
| HRV+ HBoV | ND | 3 (2.3) |
| Adenovirus+ IVA | 0 | 1 (0.8) |
| Adenovirus+ RSV | 4 (2.9) | 3 (2.3) |
| No virus detected | 18 (12.9) | 14 (10.9) |
| All | 139 (100) | 129 (100) |

ND, not determined

During hospitalization for bronchiolitis, the mean age of the 90 infants with RSV infection was 9.05 weeks (SD 6.6), of those 17 with mixed infection the mean age was 13.56 weeks (SD 7.3, p=0.026 vs. RSV cases), and of those 15 with no virus findings the mean age was 12.93 weeks (SD 7.1, p=0.043 vs. RSV cases) (Table 5). There were no significant differences in the clinical characteristics, such as the need for supplemental oxygen, the need for feeding support, the length of hospital stay, or the presence of AOM, between the RSV and HRV bronchiolitis cases.

*Table 5.*

*Age of the infants hospitalized for bronchiolitis in relation to viral findings*

| **Virus** | **N (%)** | **Age weeks mean ± SD (range)** | **p-value *** |
|---|---|---|---|
| RSV | 90 (64.8) | 9.05 ± 6.6 (1-25) | |
| HRV | 9 (6.5) | 9.11± 6.9 (2-22) | 0.99 |
| IVA | 6 (4.3) | 11.0± 7.2 (4-23) | 0.49 |
| hMPV | 2 (1.4) | 10.0± 4.2 (7-13) | 0.84 |
| mixed virus infection | 17 (12.2) | 13.56± 7.3 (3-25) | 0.026 |
| no virus detected | 15 (10.8) | 12.93± 7.1 (3-25) | 0.043 |

* compared to RSV cases, Student's t-test

### 5.1.2 Gene polymorphisms in bronchiolitis (I)

There were no differences in the genotype distributions, allele frequencies or allele carrier status of *IL-10* –1082 G/A, *IL-18* –137 G/C, *TLR4* +896 A/G, and *IFNG* +874 T/A between the entire bronchiolitis group or those with RSV bronchiolitis, when compared to blood donors. However, a significant difference was found in the allele carrier status and allele frequency of *IL-10* –1082 G/A when infants infected with a single virus other than RSV (non-RSV) were compared to blood donors. As seen in Table 6, the infants hospitalized with non-RSV bronchiolitis were more often *IL-10* –1082 G allele non-carriers, that is, homozygous for the A allele at –1082 of *IL-10*, than were blood donors ($p<0.0001$). When infants with HRV bronchiolitis alone were compared to blood donors, they were also less often G allele carriers at –1082 of *IL-10* (2/9, $p<0.001$). Further, patients with non-RSV (22.2%) or HRV infection (16.7%) also had a lower frequency of the G allele than blood donors (46.1%, $p<0.005$ vs. non-RSV and $p<0.004$ vs. HRV). However, infants with non-RSV and/or HRV infection did not differ for the polymorphisms *IL-18* –137 G/C, *TLR4* +896 A/G, or *IFNG* +874 T/A when compared to blood donors. No deviation from HWE was observed in any of the analyzed polymorphic sites.

*Table 6.*

*Genotypes and allele frequencies of IL-10 –1082 A/G polymorphisms in infants with bronchiolitis*

| **Cytokine polymorphism** | **RSV infected infants** N (%) | **Non-RSV infected infants** N (%)* | **Blood donors** N (%) ** |
|---|---|---|---|
| ***IL-10* –1082 A/G** | | | |
| AA | 26 (26.8) | 12 (66.7) | 112 (28.0) |
| AG | 49 (50.5) | 4 (22.2) | 207 (51.8) |
| GG | 22 (22.7) | 2 (11.1) | 81 (20.3) |
| G allele carrier (GG and AG) | 71 (73.2) | 6 (33.3) † | 288 (72.0) |
| G allele non-carrier (AA) | 26 (26.8) | 12 (66.7) ‡ | 112 (28.0) |
| Frequency of G allele (%) | 47.9 | 22.2 | 46.1 |

*HRV (n=9), IVA (n=6), hMPV (n=2), adenovirus (n=1); **Samples from 400 blood donors were analyzed for *IL-10* –1082 A/G polymorphisms

†p< 0.0001 (after Bonferroni correction p< 0.0004) non-RSV infected vs. blood donors, Chi-square test;

‡p<0.005 (after Bonferroni correction p<0.02) non-RSV infected vs. blood donors, Chi-square test

### 5.1.3 Mannose-binding lectin 2 gene polymorphisms (III)

Two-thirds (68.2%) of the 129 children had the A/A genotype, 28.7% had A/O and 3.1% had the O/O genotype. The A allele, either homozygous or heterozygous, was present in nearly all (96.9%) children. Thus, the A allele comprised 82.6% of all the 258 alleles. The respective figures were 10.5%, 0.4% and 5.8% for alleles B, C, and D. The 41 children with variant genotypes were more often infected by multiple viruses (21.9%, p=0.047) than children with the wild-type A/A genotype (9.1%) (Table 7).

The MBL genotypes or allele frequencies had no significant associations with clinical characteristics of bronchiolitis. No significant associations were found between the MBL genotypes and disease severity.

*Table 7.*

*MBL genetics in 129 children with bronchiolitis in relation to viral findings*

| **Genotypes and alleles** | **Total** | **Single RSV infections** | **Other single infections**[‡] | **Two or more viruses**[†] **detected** | **No viruses detected** |
|---|---|---|---|---|---|
| | N (%) | N (%) | N (%) | N (%) | N (%) |
| *Genotypes* | 129 (100) | 81 (100) | 17 (100) | 17 (100) | 14 (100) |
| AA | 88 (68.2) | 60 (74.1) | 10 (58.8) | 8(47.1) | 10 (71.4) |
| AO | 37 (28.7) | 17 (21.0) | 7 (41.2) | 9 (52.9)[*] | 4 (28.6) |
| OO | 4 (3.1) | 4 (4.9) | 0 | 0 | 0 |
| *Alleles* | | | | | |
| A | 125(96.9) | 77 (95.1) | 17 (100) | 17 (100) | 14 (100) |
| B | 27 (20.9) | 13 (16.0) | 6 (35.3) | 4 (23.5) | 4 (28.6) |
| C | 1 (0.8) | 0 | 1 (5.9) | 0 | 0 |
| D | 15 (11.6) | 10 (12.3) | 0 | 5 (29.4)[**] | 0 |

[‡]Human rhinovirus (HRV) in 9 cases, human metapneumovirus (hMPV) in 2 cases, influenza A virus (IVA) in 6 cases; [†] RSV and HRV in 6 cases, RSV and adenovirus in 3 cases, RSV and human bocavirus (HBoV) in 4 cases, HRV and HBoV in 3 cases, IVA and adenovirus in 1 case; [*] Fisher's exact test, p=0.047 vs. single infections; p=0.034 vs. single RSV infections; [**] Fisher's exact test, p=0.046 vs. single infections

## 5.2 *Bordetella pertussis* co-infection (IV)

The patients were chosen based on good-quality frozen NPA samples and they were in part different patients (no need for cytokine polymorphism studies and no exclusion because of *B. pertussis* infection suspected and verified on a clinical basis). There were 142 infants eligible for this study. The number of *B. pertussis* positive infants was 12 (8.5%). Their mean age was 10.4 ± 5.1 weeks and all infants with *B. pertussis* involvement were <20 weeks old, which means that they were unvaccinated or only partially vaccinated. *B. pertussis* positive infants were not more likely to have older siblings than infants with viral bronchiolitis alone (Table 8).

*Table 8.*

*Demographic data in relation to the presence or absence of Bordetella pertussis by polymerase chain reaction*

| **Findings** | ***B. pertussis* positive** (N=12) (%) | ***B. pertussis* negative** (N=130) (%) |
|---|---|---|
| Boys | 4 (33.3) | 67 (51.5) |
| Older siblings | 8 (66.7) | 91(70.0) |
| Breastfeeding | 6 (50.0) | 91 (70.0) |
| Age <4 weeks | 1 (8.3) | 31 (23.8) |
| Age 5-8 weeks | 4 (33.3) | 33 (25.4) |
| Age 9-16 weeks | 5 (41.7) | 42 (32.3) |
| Age 17–24 weeks | 2 (16.7) | 24 (18.5) |

No statistical significant differences, Fisher's exact test

### 5.2.1 Viruses associated with *B. pertussis* (IV)

*B. pertussis* was associated with RSV in 6 cases, with RSV and another virus in 2 cases, and with viruses other than RSV in an additional 3 cases (Table 9). Thus, *B. pertussis* was identified in 8 (7.6%) of all RSV infections. There was only one single *B. pertussis* infection (Table 9).

*Table 9.*

*Viral findings in relation to the presence or absence of Bordetella pertussis by PCR*

| | ***B. pertussis* positive** (N=12) (%) | ***B. pertussis* negative** (N=130) (%) |
|---|---|---|
| Viral infection | 11 (91.7) | 120 (92.3) |
| Respiratory syncytial virus (RSV) | 8 (66.7)* | 97 (74.6) |
| Human rhinovirus (HRV) | 1 (8.3) | 7 (5.4) |
| Influenza A (IVA) | 1 (8.3) | 7 (5.4) |
| Other viruses | 1 (8.3)† | 9 (6.9) |

* RSV with HBoV in 1 case and RSV with adenovirus in 1 case; † One case with PIV type 3 virus
No statistically significant differences, Fisher's exact test

### 5.2.2 Clinical findings in *B. pertussis* co-infection (IV)

There were no significant differences in the clinical findings between infants with viral infection alone and infants with *B. pertussis* co-infection. The need for or the duration of supplementary oxygen and feeding support and the duration of hospital treatment were rather similar. One infant with *B. pertussis* co-infection needed intensive care but was not mechanically ventilated. Seven (58.3%) infants with *B. pertussis* versus 51 (39.2%) *B. pertussis* negative infants were treated with antibiotics. Only two infants with *B. pertussis* (16.7%) were treated with macrolides.

Overall, 135 (95.1%) infants presented with cough; 24 had coughing spells and 111 had cough with no spells (Table 10). Coughing spells were present in five (41.7%) *B. pertussis* positive infants compared with 19 (14.6%) *B. pertussis* negative infants (p= 0.038). The single infant with *B. pertussis* alone presented with coughing spells.

*Table 10.*

*Respiratory syncytial virus (RSV) and Bordetella pertussis findings in infants in relation to the presence of cough*

| **Microbe findings** | **Group 1 (Coughing spells)** N=24 | **Group 2 (Cough, no spells)** N=111 | **Group 3 (No cough)** N=7 |
|---|---|---|---|
| *B. pertussis* + RSV – | 2 | 2 | 0 |
| *B. pertussis* + RSV + | 3 | 5 | 0 |
| *B. pertussis* – RSV + | 15 | 77 | 5 |
| *B. pertussis* – RSV – | 4 | 27 | 2 |
| All *B. pertussis* + | 5 (20.8%)* | 7 (6.3%)† | 0 (0%) |

* p= 0.038 vs. Group 2, Fisher's exact test; † p= 0.644 vs. Group 3, Fisher's exact test

## 5.3 Short-term prognosis (II, III, V)

Among the 129 participants, 113 children (87.6%) had visited a doctor because of respiratory infection during the follow-up period, and 107/129 (82.9%) had received at least one course of antibiotics. There were altogether 410 AOM cases in 102 children during the follow-up period. Forty-seven (36.4%) children had suffered from doctor-diagnosed wheezing; in 91.5% wheezing was induced by infection and in only four children by an allergic trigger. In all, allergy had been diagnosed by a physician in 32 (24.8%) children; 16 (50.0%) of them, compared with 31 (32.0%) non-allergic children, had suffered from wheezing (p=0.07). Atopic disease was diagnosed by a doctor during the follow-up period in 24 (18.6%) children. When measured at the follow-up visit, the mean concentration of total serum IgE was 44.1 ± 97.2 (SD) (range 1−621), and serum IgE was elevated (> 60 IU/L) in 21 children.

### 5.3.1. Viral findings vs. short term prognosis (II, III)

There was no significant difference in the short-term outcome between the groups of children with bronchiolitis due to RSV or HRV infection. Of the 81 RSV positive children, 23 (28.4%) had suffered from wheezing, compared to 4/9 (44.4%, p=0.26) HRV positive children. The figures for repeated (two or more) wheezing episodes were 12/81 (14.8%) and 3/9 (33.3%, p=0.35), respectively. The causative viral agent during the initial bronchiolitis episode, the place of residence, breastfeeding, parental smoking, pets in the family, form of day-care, allergy in the family or number of siblings had no significant association with the number of infections, number of AOM episodes, use of antibiotics, placement of tympanostomy tubes, number of wheezing episodes or use of corticosteroids. Children with doctor-diagnosed allergy had more wheezing episodes per patient month than non-allergic children [0.10±0.16 (SD) vs. 0.04±0.07 (SD), p=0.02] and were more likely to receive corticosteroids [11/32 (34.4%) vs. 13/97 (13.4%), p=0.008].

### 5.3.2 Gene polymorphisms vs. short term prognosis (II, III)

The gene polymorphisms of *IFNG*, *IL-10*, *IL-18*, *TLR4*, and *MBL2* showed no association with doctor-diagnosed allergy, total serum IgE or the wheezing history of the child. However, the *IFNG* polymorphism at +874 A/T seemed to be associated with infection susceptibility (Table 11). The number of infections per patient month and the number of antibiotic courses were significantly associated with the *IFNG* genotype, such that the A allele had a protective effect. The number of AOM episodes per patient month was also less, although not significantly, in children with the A allele (p=0.07). The studied gene polymorphisms of the *IL-10*, *IL-18*, *TLR4*, and *MBL2* genes were not associated with the infection or wheezing histories of the children.

The carriers of the *IFNG* A allele were less likely to have used corticosteroids [10/83 (12.0%) vs. 14/46 (30.4%), p=0.010], though no association was found with total numbers or numbers per patient month of wheezing episodes (Table 11). In multivariate logistic regression, the use of corticosteroids was associated with both allergy [aOR 4.6 (95% CI 1.2 to 18.1)] and carrying the *IFNG* A allele [aOR 0.23 (95% CI 0.07 to 0.83)], with no interaction between the three variables (p= 0.88). The *MBL2*

genotype A/O was associated with the need for treatment with corticosteroids for post-bronchiolitis wheezing. Children with the A/O genotype had used corticosteroids more often (13/41; 31.7%) vs. 12/88 (13.6%) children with the A/A genotype (p= 0.016).

*Table 11.*

*Associations of genotypes and allele carrier status of interferon gamma (IFNG) +874 T/A with the number and number per patient month of infections, presence of acute otitis media, use of antibiotics and wheezing episodes in children hospitalized for bronchiolitis*

| **Cytokine Polymorphism** | **No. Infections per patient month** | **No. Otitis media per patient month** | **No. Antibiotic courses per patient month** | **No. Wheezing episodes per patient month** |
|---|---|---|---|---|
| ***IFNG* +874 T/A** | | | | |
| AA (n=14) | 0.14 ± 0.12 (0-0.44) | 0.11 ± 0.12 (0-0.44) | 0.11 ± 0.12 (0-0.44) | 0.03 ± 0.06 (0-0.18) |
| TA (n=69) | 0.21 ± 0.17 (0-0.67) | 0.16 ± 0.15 (0-0.63) | 0.18 ± 0.15 (0-0.63) | 0.05 ± 0.11 (0-0.67) |
| TT (n=46) | 0.30 ± 0.26 (0-0.94)* | 0.23 ± 0.22 (0-0.93) † | 0.26 ± 0.25 (0-0.93) ** | 0.06 ± 0.1 (0-0.35) |
| A allele carrier (AA and AT) (n=83) | 0.20 ± 0.16 (0-0.67) ‡ | 0.15 ± 0.15 (0-0.65) ‡ | 0.17 ± 0.15 (0-0.63) ‡ | 0.05 ± 0.11 (0-0.67) |
| A allele non-carrier (TT) (n=46) | 0.30 ± 0.26 (0-0.94) | 0.23 ± 0.22 (0-0.93) | 0.26 ± 0.25 (0-0.93) | 0.06 ± 0.10 (0-0.35) |

Mean ± SD (range); *p=0.05, Kruskall-Wallis test; †p=0.07, Kruskall-Wallis test; **p=0.05, Kruskall-Wallis test; ‡p=0.06, Mann-Whitney U-test

Children who were carriers of the *IFNG* A allele, were less likely to have tympanostomy tubes inserted than non-carriers [11/83 (13.3%) vs. 13/46 (28.3%), p= 0.037]. Carriers of the *TLR4* +896 A/G G allele or the *IL-10* –1082 A/G G allele were more likely to have tympanostomy tubes inserted than non-carriers [8/21 (38.1%) vs. 16/108 (14.8%), p=0.012, and 21/89 (23.6%) vs. 3/40 (7.5%), p=0.030, respectively].

### 5.3.3 Birth weight vs. post- bronchiolitis wheezing (V)

The mean (SD, 95% CI) birth weight of the 127 children was 3525 g (543, 3430 to 3620), ranging from 1635 g to 5455g. There were only four children with birth weight <2500 g, 19 children with birth weight <3000 g and 23 children with birth weight >4000 g.

Weight gain per week from birth to hospitalization depended on the birth weight; the mean (SD, 95% CI) was 202.2 g/week (86.3, 185.4 to 219.0) if the birth weight was <4000 g, and 170.7 g (66.0, 142.2 to 199.2) if the birth weight was >4000 g (p=0.06).

The use of antibiotics and the presence of AOM between hospitalization for bronchiolitis and the control visit were not associated with birth weight (Table 12).
Both occurrence and recurrence of post-bronchiolitis wheezing was associated with birth weight >4000 g vs. birth weight <4000 g (Table 12). No corresponding associations were seen for low birth weight <3000 g.

*Table 12.*

*Post-bronchiolitis use of antibiotic courses, presence of otitis media, wheezing episodes and use of corticosteroids in relation to birth weight*

| **Outcome** | **Birth weight <3000 g** (N=19) (%) | **Birth weight 3000-4000 g** (N=85) (%) | **Birth weight >4000 g** (N=23) (%) |
|---|---|---|---|
| Use of antibiotics | 15 (78.9) p=0.39* | 71 (83.5) | 20 (87.0) p=0.44** |
| History of otitis media | 14 (73.7) p=0.34* | 69 (81.2) | 18 (78.3) p=0.53** |
| One or more subsequent wheezing episodes | 6 (31.6) p=0.46* | 25 (29.4) | 13 (56.5) p=0.015** |
| Two or more subsequent wheezing episodes | 2 (10.5) p=0.25* | 13 (15.3) | 8 (34.8) p=0.028** |
| Corticosteroid use | 2 (10.5) p=0.20* | 17 (20.0) | 6 (26.1) p=0.28** |

* Compared with 108 children with birth weight >3000g, Fisher's exact test; ** Compared with 104 children with birth weight <4000g, Fisher's exact test

### 5.3.4 Weight gain and weight for length vs. post-bronchiolitis wheezing (V)

The mean WFL was –1.0% (SD 9.8, 95% CI –2.8 to +0.7), ranging from –20% to +53%. WFL was >110% (overweight) in 11 children and >120% (obesity) in only three children. WFL was <90% in 13 children and <80% in only one child. Only four (17.4%) of the 23 children with birth weight >4000 g had WFL >110% 18 months later (p=0.11) and, correspondingly, only four (21.1%) of the 19 children with birth weight <3000 g had WFL <90% (p=0.68).

The use of antibiotics and the presence of AOM between hospitalization for bronchiolitis and the control visit were not associated with overweight at the control visit. The recurrence of post-bronchiolitis wheezing was associated with WFL >110% [5/11 (45%) children, p=0.028)] at control visit vs. WFL <110% [18/116 (16%)]. No corresponding associations were seen for low WFL (<90%).

There was no significant association between weight gain and post-bronchiolitis wheezing. When the analyses were done separately in the three birth weight groups, there was a significant association between weight gain before bronchiolitis and wheezing after bronchiolitis if the birth weight was >4000 g (p=0.042). Poor weight gain (<70 g/week) was present in only 10 (7.9%) children; two had suffered from wheezing but none from recurrent wheezing.

Post-bronchiolitis wheezing tended to differ but not significantly between boys and girls: for ≥1 episode, the figures were 28/61 (45.9%) in boys vs. 19/66 (28.8%) in girls (p=0.07), and for ≥2 episodes 24.6% (boys) vs. 12.1% (girls) (p=0.11), respectively. All analyses were repeated separately for the 88 infants hospitalized for bronchiolitis at <3 months of age. The association between the occurrence and recurrence of post-bronchiolitis wheezing and birth weight >4000 g lost statistical significance, but the associations between WFL >110% and recurrent wheezing remained significant.

Since doctor-diagnosed allergy in children (II), birth weight >4000 g and WFL >110% at age 1.5 years were associated with an increased risk of post-bronchiolitis wheezing, the analyses were continued with adjusted logistic regression. Allergy and high birth weight >4000 g were significant risk factors for both the occurrence and recurrence of wheezing, and WFL >110% was a significant risk factor only for the recurrence of wheezing (Table 13).

*Table 13.*

*Logistic regression: association of birth weight >4000 g and weight for length (WFL) >110 % with post-bronchiolitis wheezing*

| **Variables** | **Adjusted odds ratio** | **95% confidence interval** |
|---|---|---|
| *One or more post-bronchiolitis wheezing episodes* | | |
| Birth weight >4000 g | 3.11 | 1.19 − 8.143 |
| WFL >110% | 2.19 | 0.59 − 8.20 |
| Allergy* | 2.76 | 1.15 − 6.67 |
| Gender (males) | 1.66 | 0.76 − 5.10 |
| *Two or more post-bronchiolitis wheezing episodes* | | |
| Birth weight >4000 g | 3.21 | 1.06 − 9.78 |
| WFL >110% | 4.63 | 1.14 − 18.89 |
| Allergy* | 4.39 | 1.53 − 12.66 |
| Gender (males) | 1.87 | 0.20 − 1.46 |

* Parent-reported doctor-diagnosed atopic dermatitis or food allergy

# 6. DISCUSSION

## 6.1 Etiology of bronchiolitis

In this study the most common viral etiology in bronchiolitis was RSV (62.8–69.8%), as expected. This result is in line with other studies on the viral etiology of bronchiolitis, when the age of the patients, less than 6 months, is taken into account (Calvo et al. 2010, Jacques et al. 2008b, Papadopoulos et al. 2002, Rakes et al. 1999, Stempel et al. 2009). The second most common single viral finding was HRV (6.5–7.0%), in accordance with a proportion of 7.2% found in another study in infants less than 6 months of age (Marguet et al. 2009). In studies also including older infants, HRV has been found more frequently in 17–44% of cases (Calvo et al. 2010, Manoha et al. 2007, Papadopoulos et al. 2002, Singleton et al. 2010). Thus, age is an important factor, and the proportion of HRV infections increases in relation to the age of wheezing children (Papadopoulos *et al.* 2002, Korppi *et al.* 2004, Jartti *et al.* 2009a). The availability of PCR for viral diagnosis has modified our knowledge, revealing first the role of HRV and then the role of some new viruses such as hMPV and HBoV. In the present study the rate of bronchiolitis caused solely by these new viruses was low, but they were rather often involved in co-infections with other viruses, in line with other studies (Calvo *et al.* 2008a, Miron *et al.* 2010, Nascimento *et al.* 2010). The clinical characteristics of bronchiolitis were similar with no dependence on viral etiology in this study. The results vary in other studies where children with RSV infection had more severe disease (Papadopoulos *et al.* 2002, Marguet *et al.* 2009, Calvo *et al.* 2010, Midulla *et al.* 2010, Singleton *et al.* 2010), while others have not found such differences with RSV and HRV infections (Korppi *et al*. 2004, van Leeuwen *et al.* 2012). The inconsistent results of these studies may be due to differences in the severity of infection, in the age, and other characteristics of the study populations, or due to different criteria for hospitalization. Young age is a crucial risk factor for hospitalization regardless of viral findings (Voets *et al.* 2006). In addition, bronchiolitis caused by RSV and some other virus may be more severe than RSV infection alone

(Papadopoulos *et al.* 2002, Semple *et al*. 2005, Jartti *et al*. 2008a, Midulla *et al.* 2010).

### 6.1.1 *Bordetella pertussis*

*B. pertussis* diagnosed by PCR was common (8.6%) in non-vaccinated infants hospitalized for bronchiolitis in this study. Two-thirds of the pertussis cases were mixed infections with RSV. The findings were similar to another Finnish study (Korppi & Hiltunen. 2007), in which 8% of infants less than 6 months old with bronchiolitis had *B. pertussis* identified by PCR during an RSV epidemic. In line with this, more than 20% of infants requiring PICU treatment during an RSV epidemic had *B. pertussis* infection, and 33% were co-infected with RSV (Crowcroft *et al.* 2003). Although all infants in this study improved despite not using antibiotics effective against *B. pertussis*, it is important to diagnose *B. pertussis* cases because of the morbidity and mortality of pertussis (Ward *et al*. 2005) and to prevent epidemics (WHO. 2010).

RSV and *B. pertussis* cases could not be separated by the presence of cough, in agreement with observations made more than 20 years earlier when *B. pertussis* was identified by culture (Nelson *et al.* 1986), as well as with later studies using PCR (Crowcroft *et al.* 2003, Greenberg *et al.* 2007, Korppi & Hiltunen. 2007). The *B. pertussis* positive group, like the respective groups in other studies (Greenberg *et al.* 2007, Korppi & Hiltunen. 2007, Cosnes-Lambe *et al.* 2008) was quite small, and was underpowered to reveal small albeit real differences between the groups. Despite this, preliminary evidence was found that coughing spells, but not cough without spells, were associated with *B. pertussis* positive cases.

## 6.2 Genetics of bronchiolitis

### 6.2.1 Interleukin-10

The results of this study suggest that the *IL-10* polymorphism (homozygosity for A at –1082) is associated with bronchiolitis but only when the causative virus is other than RSV, rhinoviruses in particular. Thus, HRV infections in infants with that

genotype may, more likely than in other infants, present with lower respiratory tract infection severe enough to be treated in hospital.

Until now, most studies on cytokine gene polymorphisms in infants with bronchiolitis have focused on patients with RSV infection, because RSV is the single most important cause of the disease. When the same polymorphic site of *IL-10* (–1082 A/G) investigated in the present study, was analyzed in relation to the severity of RSV bronchiolitis, an association with the requirement for mechanical ventilation was found (Wilson *et al.* 2005). Likewise, *IL-10* –1082 and *IFNG* +874 polymorphisms were associated with greater clinical severity (74% needed oxygen therapy) and a higher risk of complications associated with RSV bronchiolitis (Gentile *et al.* 2003). In the present study, however, no association was found between disease severity and *IL-10* polymorphisms, and the result was similar when all bronchiolitis cases or when only RSV bronchiolitis cases were included. It is possible that the overall disease severity was lower since no infants needed PICU treatment or mechanical ventilation, and only 18.7% needed oxygen therapy.

The results of this study suggest that carrying the G allele at position –1082 of the *IL-10* gene promoter protects infants from severe HRV or other non-RSV bronchiolitis and subsequent hospitalization, whereas homozygotes for the A allele are more likely to develop bronchiolitis when infected with HRV. In line with previous studies, infants homozygous for the *IL-10* low producer A allele (AA) at position –1082 may be at risk for later asthma (Chatterjee *et al.* 2005, Karjalainen *et al.* 2003, Lim *et al.* 1998), at least if they present with bronchiolitis when infected with HRV.

### 6.2.2 Toll-like receptor 4

Polymorphisms at *TLR4* +896 A/G were not associated with the etiology or clinical characteristics of bronchiolitis in this study, although mutations at this site have been previously associated with severe RSV bronchiolitis (Tal *et al.* 2004). Despite the fact that the patients in both studies had a rather similar clinical picture, the age distributions were different.

### 6.2.3 Mannose-binding lectin

The distribution of the three *MBL2* genotypes A/A (68.2 vs. 65%), A/O (28.7 vs. 30%) and O/O (3.1 vs. 5%) was nearly identical with that in the Finnish population (Aittoniemi *et al.* 2008, Huttunen *et al.* 2008, Rantala *et al.* 2008). *MBL2* genotypes were not associated with the need for hospitalizations with RSV infections, in line with a case-control study in 168 infants with serum MBL concentrations also available (Kristensen *et al.* 2004). In a study from Brazil, however, 81 children less than 5 years old with RSV infections had lower MBL levels than were observed in 40 healthy controls, and in particular, patients with severe disease requiring hospitalization had the lowest levels (Ribeiro *et al.* 2008). In another recent study MBL deficiency assessed by low serum concentration was associated with the risk of acute respiratory tract infections particularly at the age of 6 through 17 months, when antibodies of maternal origin have disappeared and the host's adaptive immune system is immature (Koch *et al.* 2001). Many other factors may explain the differences between the studies, including disease severity, infections by different viral strains or different serotypes of the same virus, other concomitant infections and differences in MBL haplotypes which vary worldwide (Casanova & Abel. 2004).

## 6.3 Outcome after bronchiolitis

In the present study, there were no significant associations between the RSV etiology of bronchiolitis and subsequent wheezing or infections, allergic manifestations, and the use of antibiotics or corticosteroids during the one-year follow-up period. HRV bronchiolitis has been associated with subsequent wheezing in many studies (Kotaniemi-Syrjänen *et al.* 2003, Korppi *et al.* 2004, Lemanske Jr *et al.* 2005, Kusel *et al.* 2007, Lehtinen *et al.* 2007, Jartti *et al.* 2008, Koponen *et al.* 2011), but in the present study, the number of HRV cases was so small that the study was underpowered for that specific purpose. In the great majority of cases, wheezing at this age is induced by respiratory virus infection. In line with this, only 3% of the children had wheezing triggered by an allergen in this study. Children with doctor-diagnosed allergy had more wheezing episodes per patient month than children without allergy. In accordance with these results, the detection of allergen-specific IgE to either food

(Schauer *et al.* 2002, Kotaniemi-Syrjänen *et al.* 2003, Lemanske Jr *et al.* 2005, Jartti *et al.* 2010) or inhaled allergens (Kotaniemi-Syrjänen *et al.* 2003, Jartti *et al*. 2010) was strongly associated with post-bronchiolitis wheezing.

Bronchiolitis in infancy is a risk factor for subsequent wheezing, later airway hyper-reactivity and even asthma in childhood (Wennergren & Kristjansson. 2001, Pérez-Yarza *et al.* 2007, Bont & Ramilo. 2011). In recent studies, recurrent wheezing has been common especially after rhinovirus-induced wheezing in infancy (Kotaniemi-Syrjänen *et al.* 2003, Lemanske Jr *et al.* 2005, Jartti *et al.* 2009a). In the present study, no differences in the short-term outcome were observed after bronchiolitis caused by different viruses. The results are in line with other studies with short follow-up times of less than 4 years after infancy, when wheezing and especially recurrent wheezing has been common in different etiological groups of bronchiolitis in infancy (Schauer *et al.* 2002, Cifuentes *et al.* 2003, Henderson *et al.* 2005, Lemanske Jr *et al.* 2005, Lee *et al.* 2007, Valkonen *et al.* 2009). The risk of post-bronchiolitis wheezing has been associated with RSV bronchiolitis (Schauer *et al.* 2002, Cifuentes *et al.* 2003, Henderson *et al.* 2005, Lemanske Jr *et al.* 2005, Lee *et al.* 2007, Valkonen *et al.* 2009), with HRV bronchiolitis (Cifuentes *et al.* 2003, Lemanske Jr *et al.* 2005, Lee *et al.* 2007, Valkonen *et al.* 2009) and also with PIV bronchiolitis (Lee *et al.* 2007). In long-term follow-up studies, however, persistent wheezing and asthma have been strongly associated with bronchiolitis caused by HRV (Korppi *et al.* 2004, Kotaniemi-Syrjänen *et al.* 2003, Lemanske Jr *et al.* 2005, Kusel *et al.* 2007, Lehtinen *et al.* 2007, Jartti *et al.* 2008).

## 6.4 Genetic risk factors

In the present study, some associations were found between the polymorphisms *IFNG* +874 T/A, *TLR4* +896 A/G, and *MBL2* A/O and post-bronchiolitis wheezing or repeated infections. The number of patients was small for genetic outcome studies with a risk of underpowering, but on the other hand, the large number of statistical analyses including different outcomes, different polymorphisms and numerous other risk factors, may have caused multi-testing problems with false positive findings.

### 6.4.1 Interferon-gamma

The *IFNG* polymorphism seemed to influence the susceptibility to respiratory infections, such that the A allele at +874 decreased the likelihood of recurrent infections and wheezing tendency after bronchiolitis. Specifically, carrying the A allele decreased the need for treatment by corticosteroids. Allergy is a well-known factor associated with an increased need for corticosteroids, and in this study, 34.4% of allergic and 18.6% of non-allergic children had used corticosteroids. IFN-γ-mediated bronchial obstruction may be one of the mechanisms leading to recurrent wheezing during viral infections, especially in individuals who have a heightened IFN-γ response. The finding that the *IFNG* A allele at +874, which is associated with low IFN-γ production, decreased the need for corticosteroids could be explained by a more controlled inflammatory reaction leading to less wheezing. In logistic regression, this finding was robust to the adjustment for allergy, suggesting that carrying the *IFNG* A allele is an independent protective factor against recurrent wheezing after bronchiolitis.

This finding, that low IFN-γ production is protective against recurrent wheezing, is in line with results from animal models where IFN-γ has induced or at least enhanced bronchial obstruction by damaging the M2 receptors which limits the release of acetylcholine from vagal nerve endings (Bowerfind *et al.* 2002) and observations where high levels of IFN-γ are both protective and contributory in worsening the airway obstruction (Chen *et al*. 2002). On the other hand, lower IFN-γ production during bronchiolitis at the mean age of 8 months has been associated with lower pulmonary function and higher responsiveness to histamine after bronchiolitis, and the subsequent development of asthma after two years or more (Renzi *et al.* 1999). IFN-γ responses measured *ex vivo* in peripheral blood cells from infants at risk for allergy and/or asthma revealed bidirectional interactions between IFN-γ responses and respiratory infections. It was found that recurrent wheezing was associated with lower inducible IFN-γ responses at birth, but by 1 year of age, this trend was reversed, and wheezing children had higher responses (Gern *et al.* 2006).

The finding of this study that the *IFNG* A allele at +874 is associated with less need for tympanostomy tubes can be explained by lower IFN-γ production and therefore by a less severe local inflammatory reaction in AOM. However, high IFN-γ production may be protective against AOM during RSV bronchiolitis (Gentile *et al.* 2003).

### 6.4.2 Toll-like receptor 4 and interleukin-10

The polymorphism of *TLR4* at +896 was associated more strongly than the *IFNG* polymorphism with the need of tympanostomy tubes, such that carriers of the G allele were more likely to require these tubes. The A/G polymorphism at +896 of *TLR4* has been associated with the severity of RSV infection, as well as with susceptibility to severe Gram-negative infections, such that carrying the G allele has increased both susceptibilities (Lorenz *et al.* 2002, Mandelberg *et al.* 2006, Karoly *et al.* 2007). Therefore, the G allele at +896 of *TLR4* could predispose children to more severe RSV infection with pronounced inflammatory reaction and to recurrent or chronic otitis media caused by Gram-negative bacteria such as *H. influenzae*. In line with this, TLR4-mediated induction of TLR2 signaling is critical in the resolution of AOM caused by *H. influenzae* (Leichtle *et al.* 2009).

The carriers of the *IL-10* –1082 A/G G allele were more likely to have tympanostomy tubes than non-carriers. This is in line with mouse studies where remarkable increase in middle ear IL-10 expression was observed after *H. influenza* inoculation and failure in AOM recovery (Leichtle *et al.* 2009).

Some authors suggest that the Bonferroni correction should be used when more than one polymorphism is included in the analysis. If the p-values of the present study were multiplied by the number of analyzed cytokine gene polymorphisms, only the *TLR4* +896A/G polymorphism would have a statistically significant association with only one infection marker, the need for tympanostomy tube placement.

### 6.4.3 Mannose-binding lectin

In the present study, preliminary evidence was found that the variant *MBL2* genotypes A/O or O/O may be associated with more severe post-bronchiolitis wheezing requiring treatment with corticosteroids. This observation may reflect long-term or chronic airway inflammation or only vulnerability to repeated infections. Thus far, there are no previous studies on the association between MBL and bronchiolitis or other early childhood wheezing.

Some studies have documented an association between *MBL2* gene polymorphisms and repeated respiratory infections in children (Cedzynski *et al.* 2004, Chen *et al.*

2009). *MBL2* gene mutations and low serum MBL levels have been independent risk factors for recurrent infections in children (Cedzynski *et al.* 2004). In a large population-based study in China, low serum MBL levels and the presence of allele B in the *MBL2* gene were associated with recurrent respiratory infections (Chen *et al.* 2009). In the present study, no association was found between *MBL2* gene polymorphisms and the presence of AOM or the use of antibiotics until 18 months of age; both parameters reflect susceptibility to respiratory infections.

## 6.5 Weight as a risk factor

There were three main results on weight as a risk factor for post-bronchiolitis wheezing. First, a high birth weight of >4000 g was associated with post-bronchiolitis wheezing; second, overweight (WFL >110%) at 1.5 years of age was associated with recurrent post-bronchiolitis wheezing; third, the rate of weight gain during the first months of life was associated with post-bronchiolitis wheezing if the birth weight was >4000 g.

A high birth of > 4000 g was associated with post-bronchiolitis wheezing at 0.5-1.5 years of age, but low birth weight <3000 g was not. Also, earlier register-based studies (Sin *et al.* 2004, Yuan *et al.* 2002) and a recent birth cohort study (Remes *et al.* 2008) have demonstrated that high birth weight (>4500 g) may be associated with an increased risk of childhood asthma.

The finding that overweight (WFL >110%) at 1.5 years of age was associated with recurrent post-bronchiolitis wheezing is in line with three other studies applying length-related weight gains for infants (Taveras *et al.* 2008, Jee *et al.* 2010, Pike *et al.* 2010). Children with low birth weight and rapid postnatal weight gain have an increased risk of having impaired lung function in infancy (Lucas *et al.* 2004) and overweight and obesity in adolescence (Mai *et al.* 2005). In a prospective cohort study from the US, infants with higher WFL at 6 months of age had a greater risk of recurrent wheezing by age 3 years, but not with wheezing at 1 or 2 years of age (Taveras *et al.* 2008).

Some evidence was found that excessive weight gain is associated with post-bronchiolitis wheezing if the birth weight was >4000 g. There is increasing evidence

that overweight and obesity may increase asthma risk in children of school age (Flaherman & Rutherford. 2006, Peroni *et al.* 2010). Excessive weight gain seems to be associated with asthma symptoms, treatments and diagnoses (Peroni *et al.* 2010), and even with decreased lung function (Sidoroff *et al.* 2011), but not with bronchial hyper-responsiveness (Peroni *et al.* 2010). An association between postnatal adiposity, weight gain and wheezing may even reflect prenatal influences (Pike *et al.* 2010).

From the age of two years onwards, overweight and obesity have usually been assessed by calculating body mass index (BMI) adjusted for age (Cole *et al.* 2000), but BMI is not necessarily the best method in infants. In addition, Finnish age- and sex-specific BMI references are not available for children <24 months of age (Saari *et al.* 2011). Like the three other thus far published studies on weight status and wheezing in infancy (Taveras *et al.* 2008, Jee *et al.* 2010, Pike *et al.* 2010), we used WFL calculated on the basis of national sex-specific age-related growth references.

## 6.6 Methodological aspects

### 6.6.1 Strengths of the study

The main strengths of the present study are the prospective design and homogeneity of the patients consisting of young infants hospitalized for bronchiolitis at less than 6 months of age. The study population originates from a rather small area with only a Finnish population forming a homogenous group suitable for genetic studies. Only a few previous studies have evaluated weight status and genetic markers in bronchiolitis and none of them have investigated MBL genetics. The present study is so far the only one focusing on bronchiolitis at less than 6 months of age.

The virological testing panel was extensive including RSV, HRV, adenovirus, PIV type 3, and the new viruses, hMPV and HBoV. Both antigen detection and PCR were performed with no major differences in the results.

The clinical data after hospitalization were collected by diaries which the parents filled in prospectively, supplemented by interviews and hospital record data. Only diagnoses made by a physician were accepted. Thus, the clinical data were collected prospectively and carefully, offering basic data for multivariate analyses when needed.

### 6.6.2 Shortcomings of the study

The main shortcoming of the study is the small number of patients infected with viruses other than RSV. By focusing the study on infants less than 6 months of age hospitalized for bronchiolitis, we encountered mostly RSV patients (Manoha *et al.* 2007, Jacques *et al.* 2008b, Marguet *et al.* 2009, Calvo *et al.* 2010, Midulla *et al.* 2010, Stempel *et al.* 2009). When designing the study, the main focus was on RSV bronchiolitis, but during the study, the knowledge on HRV bronchiolitis increased rapidly and the influence of HRV on post-bronchiolitis wheezing and asthma became evident (Papadopoulos *et al.* 2002, Kotaniemi-Syrjänen *et al.* 2003, Korppi *et al.* 2004, Lemanske Jr *et al.* 2005). Even the new viruses hMPV and HBoV were found (van den Hoogen *et al.* 2001, Allander *et al.* 2005). The lack of a control group without bronchiolitis is an obvious shortcoming, but even in the presence of control groups, the comparisons of outcomes between different viral groups must be done within the study population (Kotaniemi-Syrjänen *et al.* 2003, Lemanske Jr *et al.* 2005, Carcía-García *et al.* 2007, Lee *et al.* 2007).

In the genetic studies on the cytokine gene polymorphisms *IL-10* –1082 A/G, *IFNG* +874 T/A, and *IL-18* –137 G/C in the control group consisted of healthy Finnish blood donors from the same area. These blood donors form a rather homogenous control group and, in addition, all adults have a high probability of having had an RSV infection in infancy (Simoes. 1999), with a lower than 3% risk of having been hospitalized for bronchiolitis in infancy (Simoes & Carbonell-Estrany. 2003). In genetic studies, there is always a risk that a potentially positive association is biologically irrelevant because of population admixture (Bidwell *et al.* 1999). When cases and controls are optimally matched, the sample sizes are easily too small with insufficient statistical power to detect small or moderate gene effects. In the study of *MBL2* gene polymorphisms, no healthy controls were available; however, the distribution of *MBL* genotypes and different alleles is well-studied in the Finnish population, which is rather homogenous throughout the country (Aittoniemi *et al.* 2008, Huttunen *et al.* 2008, Rantala *et al.* 2008).

In the analysis of co-infections with *B. pertussis*, the patients and samples were collected prospectively, but data on cough history was collected retrospectively. The results are nearly identical with other corresponding studies where the cough history

was collected retrospectively (Greenberg *et al.* 2007, Korppi & Hiltunen. 2007). The analysis of the association of birth weight and weight gain with post-bronchiolitis wheezing was a secondary analysis from the data collected prospectively for genetic analyses. However, all the data for this study, such as weights, heights, and illness histories, were collected prospectively, but because of the study design, the results can be applied to post-bronchiolitis wheezing only.

### 6.6.3 Genetic methods

The genetic panel of the present study included gene polymorphisms of four cytokines that, based on previous studies, have influenced the clinical picture of bronchiolitis and/or susceptibility to asthma development (Kurt-Jones *et al.* 2000, Nagarkatti *et al.* 2002, Gentile *et al.* 2003, Tal *et al.* 2004, Chatterjee *et al.* 2005, Wilson *et al.* 2005, Puthothu *et al.* 2007). However, many other gene polymorphisms have been associated with susceptibility to asthma (Bidwell *et al.* 1999, Singh *et al.* 2007, Tregoning & Schwarze. 2010). In addition, a limited number of polymorphisms were determined within each gene in the present study. A biologically significant allele may be associated with a chromosome that carries a specific haplotype rather than restricted to a single SNP (Bidwell *et al.* 1999, Choi *et al.* 2002). The presence of linkage disequilibrium between polymorphic variants may affect the functional effects of cytokine genes (Reich *et al.* 2001). In addition, specific combinations of cytokine genotypes, rather than single genotypes, may predispose to disease susceptibility or contribute to the outcome (Choi *et al.* 2002, Hoebee *et al.* 2004).

# 7. CONCLUSIONS

The viral etiology of bronchiolitis in young infants is most often RSV, but also other viruses such as HRV, IVA, hMPV and especially mixed viral infections can also be found. The clinical characteristics of bronchiolitis caused by different viruses are rather similar, but outcomes may be different. Therefore, at least RSV and HRV should be determined in infants hospitalized for bronchiolitis.

Infants with severe bronchiolitis not caused by RSV, and especially if caused by HRV, were likely to be homozygous for the A allele and unlikely to carry the G allele at position –1082 of *IL-10*. This result emphasizes the importance of both genetic properties and causative viruses in infants with severe bronchiolitis.

The polymorphisms of *TLR4* at +896 and *IL-10* at –1082 were associated with the need of tympanostomy tubes such that carriers of the G allele were more likely to require the tubes. These results suggest that abnormalities in innate immunity may contribute to the development and resolution of AOM.

Preliminary evidence was found that in young infants hospitalized for bronchiolitis, variant non-AA *MBL2* genotypes may be associated with repeated or severe post-bronchiolitis wheezing. The MBL pathway of innate immunity is important during the period when the adaptive immune system is immature and deficiency in that pathway may increase susceptibility to recurrent severe infections.

*Bordetella pertussis* co-infection with RSV is common in unvaccinated or partially vaccinated infants. *B. pertussis* is underdiagnosed in infants with respiratory tract infection and the diagnosis cannot be based on the presence or absence of cough, but coughing spells may be suggestive of pertussis.

Some evidence was found that high birth weight may be associated with post-bronchiolitis wheezing at age 0.5 to 1.5 years and that overweight at age 1.5 years was associated with recurrent post-bronchiolitis wheezing by age 1.5 years. This result emphasizes the importance of normal weight gain and the avoidance of overweight in infancy.

The understanding of the interaction between cytokine genes and viruses offers the potential of identifying at-risk infants, in whom preventing infections or tailoring anti-inflammatory therapy could alter later outcomes. Progress in understanding innate immunity and the mechanisms involved will help to improve treatment strategies and to develop new drugs and vaccines.

# ACKNOWLEDGEMENTS

The prospective study and follow-up were carried out in the Department of Pediatrics, Tampere University Hospital, Finland from 2001-2005. I express my sincere thanks to my colleagues and collaborators I worked with to reach this goal.

I owe my deepest gratitude to Professor Matti Korppi, my supervisor for introducing me into the world of scientific thinking and working. His endless encouragement, patience and trust in me over these years have supported me in my scientific work. His clear advice, immediate responses, and ability to simplify complicated matters have provided practical guidance through this mysterious immunological and "wheezy world". He has been a great example of an advanced, enthusiastic scientist and skillful clinician.

I am very grateful to Docent Merja Helminen, my principal supervisor, for introducing me into the world of scientific work. She encouraged me to start this study and to continue working despite of ups and downs during that time. Furthermore, when working together in the clinic, she has taught me in practice how to make prompt clinical diagnoses and decisions regarding treatment in pediatric infectious diseases.

I thank Docent Ville Peltola and Professor Johannes Savolainen, the official reviewers of this thesis, for their constructive criticisms and expert advice during the preparation of the final manuscript.

I am grateful to Professor Markku Mäki, the head of the Department of Pediatrics for creating an inspiring atmosphere and excellent working facilities in the Pediatric Research Center. I want to express my thanks to Professor Timo Vesikari, the head of the Department of Virology, and Professor Mikko Hurme, the head of the Laboratory Department of Tampere University Hospital, for their supportive attitudes at the beginning of this study, for providing possibilities for laboratory co-operation and for constructive comments and advice in writing articles.

I am very grateful to my co-authors Miia Virta, who also helped me to understand genetic methods and candidate gene approaches, and Petri Koponen, who also helped

me in collecting the data on cough history. I also thank Anne Halkosalo and Qiushui He for their excellent laboratory work with viruses, bacteria, and innate immunity genes, and for their participation in writing articles.

I warmly thank Heini Nieminen and Mari Koski, the nurses I worked with to arrange and perform the follow-up visits. I also thank all the nurses at the Infectious Department of the Pediatric Clinic of Tampere University hospital, as they helped me to enroll the study patients. I want to express my sincere thanks to all the small patients and their parents as they voluntarily and willingly participated in the study.

I am very grateful to all my colleagues in the Pediatric Clinic, at Seinäjoki Central Hospital for their flexibility in arranging work lists and for their supportive attitudes during this study. I especially thank my friends and colleagues Tiina Petäjä and Riitta Marttila for their understanding and encouragement despite the ups and downs of scientific work. Thanks also to my friends and colleagues Tarja Laamanen, Mervi Linna, and Eija Yli-Rahnasto for their pleasant companionship at the regular “twelve o, clock” coffee break over the years.

I owe my warm gratitude to my parents Veikko and Eeva Luostarinen, and to my father-in-law Matti and his wife Tuula Nuolivirta for their support and practical help in everyday life taking care of our children while I was working at Tampere.

Most of all, I owe my loving gratitude to my family. I am thankful to my husband Timo for his love and patience, and for providing the opportunities for me to work on this study over the years. I also thank our marvelous children Aino, Eini, Eero and Anni for always reminding me of the wonders in life other than the scientific ones and for giving me the happiest moments in my life.

This study was financially supported by Tampere University Hospital, Finland, Seinäjoki Central Hospital, Finland and the Foundation for Pediatric Research, Finland.

Seinäjoki, December 2011

Kirsi Nuolivirta

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Appendix 1.

**RSV-STUDY**
**Tampere University Hospital, Department of Paediatrics**
1.12.2001

**Study period**_____

**Follow- up visit** Date_____

**Name** _____

**Date of birth**_____

DI _____ Weight _____ kg Length _____cm

**Location of residence** □ city □ country side

**Number of siblings**_____

**Age of siblings'** _____

**Doctor diagnosed allergy** □ yes □ no

Allergens _____

Symptoms □ wheezing □ cough □ rhinorrhea □ eczema

**Doctor diagnosed atopy** □ yes □ no

**Allergy** in siblings □ yes □ no
allergens _____

mother □ yes □ no
allergens _____

father □ yes □ no
allergens _____

**Atopy** in siblings □ yes □ no

mother □ yes □ no

father □ yes □ no

**Asthma** in siblings □ yes □ no

mother □ yes □ no

father □ yes □ no

**Smoking** mother □ yes □ no at home □ outside □

father □ yes □ no at home □ outside □

**Pets at home** □ yes □ no

Type of animal □ cat □ dog some else_____

**Infections during the follow-up period**

□yes □ no

**1. Infection** date_____
□ wheezing
□ rhinorrhea
□ otitis
□ cough
□ fever
□ pneumonia
□ other_____

Treatment
□ antibiotics
□ inhaled bronchodilatator
□ p.o bronchodilatator
□ inhaled corticosteroid
□ p.o/i.m corticosteroid
□ other_____

**2. Infection** date_____
□ wheezing
□ rhinorrhea
□ otitis
□ cough
□ fever
□ pneumonia
□ other _____

Treatment
□ antibiotics
□ inhaled bronchodilatator
□ p.o bronchodilatator
□ inhaled corticosteroid
□ p.o/i.m corticosteroid
□ other_____

**3. Infection** date_____
□ wheezing
□ rhinorrhea
□ otitis
□ cough
□ fever
□ pneumonia
□ other_____

Treatment
□ antibiotics
□ inhaled bronchodilatator
□ p.o bronchodilatator
□ inhaled corticosteroid
□ p.o/i.m corticosteroid
□ other_____

**4. Infection** date_____
□ wheezing
□ rhinorrhea
□ otitis
□ cough
□ fever
□ pneumonia
□ other _____

Treatment
□ antibiotics
□ inhaled bronchodilatator
□ p.o bronchodilatator
□ inhaled corticosteroid
□ p.o/i.m corticosteroid
□ other_____

**5. Infection** date_____
□ wheezing
□ rhinorrhea
□ otitis
□ cough
□ fever
□ pneumonia
□ other_____

Treatment
□ antibiotics
□ inhaled bronchodilatator
□ p.o bronchodilatator
□ inhaled corticosteroid
□ p.o/i.m corticosteroid
□ other_____

**6. Infection** date_____
□ wheezing
□ rhinorrhea
□ otitis
□ cough
□ fever
□ pneumonia
□ other _____

Treatment
□ antibiotics
□ inhaled bronchodilatator
□ p.o bronchodilatator
□ inhaled corticosteroid
□ p.o/i.m corticosteroid
□ other_____

**7. Infection** date_____
□ wheezing
□ rhinorrhea
□ otitis
□ cough
□ fever
□ pneumonia
□ other_____

Treatment
□ antibiotics
□ inhaled bronchodilatator
□ p.o bronchodilatator
□ inhaled corticosteroid
□ p.o/i.m corticosteroid
□ other_____

**8. Infection** date_____
□ wheezing
□ rhinorrhea
□ otitis
□ cough
□ fever
□ pneumonia
□ other _____

Treatment
□ antibiotics
□ inhaled bronchodilatator
□ p.o bronchodilatator
□ inhaled corticosteroid
□ p.o/i.m corticosteroid
□ other_____

**9. Infection** date_____
□ wheezing
□ rhinorrhea
□ otitis
□ cough
□ fever
□ pneumonia
□ other_____

Treatment
□ antibiotics
□ inhaled bronchodilatator
□ p.o bronchodilatator
□ inhaled corticosteroid
□ p.o/i.m corticosteroid
□ other_____

**10. Infection** date_____
□ wheezing
□ rhinorrhea
□ otitis
□ cough
□ fever
□ pneumonia
□ other _____

Treatment
□ antibiotics
□ inhaled bronchodilatator
□ p.o bronchodilatator
□ inhaled corticosteroid
□ p.o/i.m corticosteroid
□ other_____

**Hospital admissions during follow-up period**

yes □ no□

**1. Hospital admission** date_____
reason
□ wheezing disease
□ pneumonia
□ other_____

**2. Hospital admission** date_____
reason
□ wheezing disease
□ pneumonia
□ other_____

**3. Hospital admission** date_____
reason
□ wheezing disease
□ pneumonia
□ other_____

**Hospital outpatient visits during follow-up period**

yes □ no□

**1. Visit** date_____ **2.Visit** date _____
reason reason
□ wheezing disease □ wheezing disease

□ other_____ □ other_____

**3.Visit**
□ wheezing disease
□ other_____

**Medication in use at follow-up visit**

yes □ no□

□ antibiotics

□ bronchodilatator □ inhaled □ mixture
□ everyday □ during respiratory infections

□ corticosteroid
□ everyday □ during respiratory infections

□ corticosteroid-long-acting bronchodilatator

□ everyday □ during respiratory infections

**Tympanostomy tubes**

□ yes □ no

tympanostomy tubes inserted date_____

**Other diseases**_____

**Other medications**_____

**Measures at birth** _____g/_____cm/_____cm

**Gestational weeks** _____

**Problems at birth** yes □ no □

What problems_____

**Status**

| | | | |
|---|---|---|---|
| **Auscultation** | □ normal | □ rales | □ wheezing |
| **Ears** | □ normal | □ infection | □ tubes |
| **Skin** | □ normal | □ eczema | |

# LIST OF ORIGINAL PUBLICATIONS

I. Helminen M, Nuolivirta K, Virta M, Halkosalo A, Korppi M, Vesikari T, Hurme M.
*IL-10* gene polymorphism at –1082 A/G is associated with severe rhinovirus bronchiolitis in infants.
Pediatric Pulmonology 2008;43(4):391-395.

II. Nuolivirta K, Hurme M, Halkosalo A, Koponen P, Korppi M, Vesikari T, Helminen M.

Gene polymorphism of *IFNG* +874 T/A and *TLR4* +896 A/G and recurrent infections and wheezing in toddlers with history of bronchiolitis.

The Pediatric Infectious Disease Journal 2009;28(12):1121-1123.

III. Nuolivirta K, He Q, Gröndahl-Yli-Hannuksela K, Koponen P, Helminen M, Korppi M.
Mannose-binding lectin gene polymorphisms in infants with bronchiolitis and post-bronchiolitis wheezing.
Allergology International 2012, accepted for publication.

IV. 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.

The Pediatric Infectious Disease Journal 2010;29(11):1013-1015.

V. Nuolivirta K, Koponen P, Helminen M, Korppi M.
Weight gain in infancy and post-bronchiolitis wheezing.
Acta Paediatrica 2012;101(1):38-42.

Pediatric Pulmonology 43:391–395 (2008)

# IL-10 Gene Polymorphism at −1082 A/G Is Associated With Severe Rhinovirus Bronchiolitis in Infants

Merja Helminen, MD,[1]* Kirsi Nuolivirta, MD,[2] Miia Virta, MD,[3] Anne Halkosalo, PhD,[4] Matti Korppi, MD,[1] Timo Vesikari, MD,[4] and Mikko Hurme, MD[3]

**Summary.** We analyzed polymorphisms of *IL-10* −1082 G/A, *IL-18* −137 G/C, *TLR4* +896 A/G, and *IFNG* +874 T/A in 139 infants under 6 months of age hospitalized with bronchiolitis and 400 unselected blood donors. Causative viruses were determined by PCR. Infants with bronchiolitis associated with a virus other than respiratory syncytial virus (N = 18), were more often IL-10 −1082 allele G non-carriers, that is, homozygous for allele A (AA) than controls (66.7% vs. 28.0%, $P < 0.0001$). Infants with RSV bronchiolitis did not differ from controls. This finding suggests a different pathogenic mechanism for RSV bronchiolitis as compared with wheezing associated with other viral infections, for example, rhinovirus in infants under 6 months of age. **Pediatr Pulmonol. 2008; 43:391–395.** © 2008 Wiley-Liss, Inc.

**Key words: wheezing; cytokine polymorphism; child.**

## INTRODUCTION

Acute bronchiolitis is a common cause for hospital admission in infants less than 6 months of age. Bronchiolitis is characterized by wheezing and rales on auscultation, feeding difficulties and often need of oxygen. Respiratory syncytial virus (RSV) is the classic causative agent of this condition but recent observations mainly by polymerase chain reaction (PCR) have stressed the role of other viruses, such as human metapneumovirus (hMPV), influenza virus, and rhinovirus.[1] Most infants with viral respiratory tract infection do not require hospital treatment or even medical evaluation. The specific mechanisms leading to severe bronchiolitis and also to virus-induced asthma exacerbations are under intensive study. Several recent studies provide evidence that genetic factors and the immune response to viral infections are connected with the pathogenesis of these conditions.[2–4] The viral etiology of bronchiolitis also seems to predict future asthma symptoms. There is evidence that wheezing during RSV and even more during RV infection in infancy is connected to subsequent asthma development.[5,6] In previous studies interleukin (IL)-10, IL-18, Toll-like receptor-4 (TLR4) and gamma-interferon (IFNG) have been important regulators in viral infections, bronchiolitis and asthma exacerbations.[2–4] IL-10 is a key anti-inflammatory and IL-18 a proinflammatory cytokine which mediates its action through IFNG. Both of these cytokines have been shown to influence severity of RSV bronchiolitis.[4,7] TLR4 polymorphism was studied because TLR4 is a key receptor for RSV and its polymorphism has also been shown to moderate the severity of RSV bronchiolitis.[3,8] The polymorphic sites that were analyzed in this study, *IL-10* −1082 A/G, *IL-18* −137 G/C, and *IFNG* +874 T/A, have been most intensively studied also previously because of their association with the level of protein production.[7,9,10]

Our hypothesis was that infants with bronchiolitis caused by RSV, RV and other viruses may have different genetic backgrounds. The aim of the study was to evaluate if the polymorphism of *IL-10 −1082 G/A*, *IL-18 −137 G/C*, *TLR4 +896 A/G*, and *IFNG +874 T/A* associates with the presence of bronchiolitis, compared with unselected

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Grant sponsor: University Hospital of Tampere, Finland; Grant number: MB0165.

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Received 13 September 2007; Revised 4 December 2007; Accepted 7 January 2008.

DOI 10.1002/ppul.20793
Published online in Wiley InterScience (www.interscience.wiley.com).

blood donors, or with the viral etiology of bronchiolitis, in hospitalized infants younger than 6 months of age.

## PATIENTS AND METHODS

Healthy full-term infants less than 6 months of age that were hospitalized because of bronchiolitis between December 1st, 2001 and May 31st, 2002 and between October 28th, 2002 and May 31st, 2004, were included in the study. The Ethics Committee of the Tampere University Hospital District approved the study. Informed consent was obtained from parents before enrolling the children. Bronchiolitis was defined as an acute lower respiratory illness characterized by rhinorrhea, cough, and diffuse wheezes and rales. Nasopharyngeal aspirates were obtained from all infants for antigen detection by indirect immunofluoresence (IF) method for RSV, Influenza A and B virus, adenovirus and parainfluenzavirus (PIV) 1, 2, and 3, according to routine practice at the hospital laboratory. For greater sensitivity, reverse transcriptase-PCR (RT-PCR) was used for detection of the same viruses and, in addition, rhinovirus and hMPV.[11–14] For the present analysis, combined results of IF and RT-PCR were used.

### Genotyping

DNA was extracted from whole blood using standard methods. Genotyping of *IL-10* −1082 G/A (rs1800896), *IL-18* −137 G/C (rs187238), and *TLR4* +896 A/G (rs4986790) gene polymorphisms were performed using the ABI PRISM 7000 Sequence Detection System for both PCR and allelic discrimination (Applied Biosystems, CA). For *IL-18* −137 G/C and *TLR4* +896 A/G the nucleotide sequences of the primers and fluorogenic allele-specific oligonucleotide probes were deduced from published sequence deposited in the GeneBank database and were chosen and synthesized in conjunction with Applied Biosystems. For *IL-10* −1082 G/A a commercial kit was used (Assay On Demand, C_1747360_10 IL10). *IFNG* +874T/A (rs2430561) polymorphism was analyzed by the amplification refractory mutational system-PCR method described earlier.[15] Four hundred control samples were obtained from unselected blood donors by the Finnish Red Cross (FRC) Blood Transfusion Centre, Tampere. The study was approved by the Ethics Committee of the FRC Blood Transfusion Centre. The blood samples donated for the study contained no donor identification labels.

### Statistical Analyses

The allele carrier status and allele and genotype frequencies were compared between infants with different viral etiology of bronchiolitis (all bronchiolitis cases, and separately RSV positive, rhinovirus positive and influenzavirus cases) and the blood donor population with the $\chi^2$-test. In the analyses the infants with multiple virus infection and the cases who had no virus detected were included in the group ''all bronchiolitis cases'' and they were excluded when single virus infections were analyzed. Kruskall–Wallis ANOVA was used to evaluate the associations between genotype and measures of bronchiolitis severity including duration of hospitalization, feeding support and oxygen treatment of the infants. The test was considered significant at $P$-value $\leq 0.05$. All analyses were performed using the Statistical Package of Social Science (SPSS).

## RESULTS

Altogether 139 infants were enrolled into the study. Their clinical characteristics and the causative pathogens of bronchiolitis are shown in Table 1. There were no differences in the genotype distributions, allele frequencies or allele carrier status of *IL-10* −1082 G/A, *IL-18* −137 G/C, *TLR4* +896 A/G, and *IFNG* +874 T/A between the entire patient group or those with RSV compared with the blood donors. Furthermore, infants with and without need of oxygen treatment, feeding support or otitis media did not differ for these markers.

However, a significant difference was found in the allele carrier status and allele frequency of *IL-10* −1082 G/A when infants infected with a single virus other than RSV (non-RSV) were compared to blood donors. As seen in Table 2, the infants hospitalized with non-RSV bronchiolitis were more likely to be *IL-10* −1082 allele G non-carriers, that is, homozygous for the allele A at −1082 of *IL-10* than were blood donors ($P < 0.0001$). When infants with rhinovirus bronchiolitis alone were compared to blood donors, they also were less likely to carry the allele G at −1082 of *IL-10* (2/9 [22.2%]; [$P < 0.001$]). Further, patients with non-RSV (including infants with rhinovirus infection) and rhinovirus infection also had a lower frequency of allele G than blood donors (22.2% vs. 46.1%,

**TABLE 1—Clinical Characteristics of Infants With Bronchiolitis and Viral Etiology of Infection**

| | |
|---|---|
| Patients, n | 139 |
| Age, months, median (range) | 9.0 (1–25) |
| Boys, n (%) | 69 (50) |
| Required oxygen, n (%) | 26 (18.7) |
| Feeding support, n (%) | 48 (34.5) |
| Length of hospital stay, days, median (range) | 4.0 (0–28) |
| Causative agent, n (%) | |
| Respiratory syncytial virus (RSV) | 97 (69.8) |
| Other single virus infections[1] | 18 (13.0) |
| Multiple viruses[2] | 6 (4.3) |
| No virus detected | 18 (12.9) |

[1]Rhinovirus (n = 9), influenza virus (n = 6), human metapneumovirus (n = 2), adenovirus (n = 1).
[2]Adenovirus + RSV (n = 4), rhinovirus + RSV (n = 2).

**TABLE 2— Genotype and Allele Frequencies and Allele Carriage of Interleukin *(IL)-10* −1082 A/G, *IL-18* −137 G/C, Toll-Receptor *(TLR)4* +896 A/G and Gamma-Interferon (*IFNG*) +874 T/A Polymorphisms in Infants With Bronchiolitis**

| Cytokine polymorphism | RSV infected infants n (%) | Non-RSV infected infants n (%)[1] | Blood donors n (%)[2] |
|---|---|---|---|
| *IL-10* −1082 A/G | | | |
| AA | 26 (26.8) | 12 (66.7) | 112 (28.0) |
| AG | 49 (50.5) | 4 (22.2) | 207 (51.8) |
| GG | 22 (22.7) | 2 (11.1) | 81 (20.3) |
| Allele G carrier (GG and AG) | 71 (73.2) | 6 (33.3)[3] | 288 (72.0) |
| Allele G non-carrier (AA) | 26 (26.8) | 12 (66.7) | 112 (28.0) |
| Frequency of allele G (%) | 47.9 | 22.2[4] | 46.1 |
| *IL-18* −137 G/C | | | |
| GG | 7 (7.2) | 2 (11.1) | 24 (6.0) |
| GC | 43 (44.3) | 9 (50.0) | 165 (41.3) |
| CC | 47 (48.5) | 7 (38.9) | 211 (52.7) |
| Allele G carrier (GG and GC) | 50 (51.5) | 11 (61.1) | 189 (47.3) |
| Allele G non-carrier (CC) | 47 (48.5) | 7 (38.9) | 211 (52.7) |
| Frequency of allele G (%) | 29.4 | 36.1 | 26.6 |
| *TLR4* +896 A/G | | | |
| AA | 80 (82.5) | 15 (83.3) | 327 (82.2) |
| GA | 17 (17.5) | 3 (16.7) | 66 (16.6) |
| GG | 0 (0) | 0 (0) | 5 (1.3) |
| Allele G carrier (GG and GA) | 17 (17.5) | 3 (16.7) | 71 (17.8) |
| Allele G non-carrier (AA) | 80 (82.5) | 15 (83.3) | 327 (82.2) |
| Frequency of allele G (%) | 8.8 | 8.3 | 9.5 |
| *IFNG* +874 T/A | | | |
| TT | 36 (37.1) | 6 (33.3) | 153 (38.3) |
| TA | 50 (51.6) | 9 (50.0) | 183 (45.9) |
| AA | 11 (11.3) | 3 (16.7) | 63 (15.8) |
| Allele A carrier (AA and TA) | 61 (62.9) | 12 (66.7) | 246 (61.7) |
| Allele A non-carrier (TT) | 36 (37.1) | 6 (33.3) | 153 (38.3) |
| Frequency of allele A (%) | 37.1 | 41.7 | 38.7 |

[1]Rhinovirus (n = 9), influenza virus (n = 6), human metapneumovirus (n = 2), adenovirus (n = 1).
[2]Samples from 400 blood donors were analyzed for *IL-10* and *IL-18*, 398 for *TLR4* and 399 for *IFNG* polymorphisms.
[3]$P < 0.0001$ (after Bonferroni correction $P < 0.0004$) non-RSV infected vs. blood donors, by $\chi^2$-test.
[4]$P < 0.005$ (after Bonferroni correction $P < 0.02$) non-RSV infected versus blood donors, by $\chi^2$-test.

$P < 0.005$ and 16.7% vs. 46.1%, $P < 0.004$). However, infants with non-RSV and/or rhinovirus infection did not differ for polymorphisms of *IL-18* −137 G/C, *TLR4* +896 A/G, and *IFNG* +874 T/A when compared to blood donors. When the six infants with influenza infection were compared to blood donors no difference in the polymorphism of *IL-10* *−1082* or any of the other polymorphisms studied could be shown (data not shown) No deviation from the Hardy–Weinberg equilibrium was observed in none of the polymorphic sites analyzed ($P > 0.05$).

## DISCUSSION

The results of this study suggest that *IL-10* polymorphism (homozygosity for A at −1082) is associated with bronchiolitis but only when the causative virus is other than RSV, rhinovirus in particular. Thus, rhinovirus infections in infants with that genotype may, more likely than in other infants, present with lower respiratory tract infection severe enough to be treated in hospital. In this study, unselected Finnish blood donors were used as a control population, with less than 3% risk having been hospitalized for bronchiolitis in infancy. Until now, most studies on cytokine gene polymorphism in infants with bronchiolitis have focused on patients with RSV infection because RSV is the single most important cause of the disease. Recently, the development of RT-PCR methods has facilitated the specific viral diagnosis of infections, and other viruses, like rhinoviruses, have also been shown to be important in bronchiolitis.[1] Wilson et al.[2] analyzed the same polymorphic site of *IL-10* (−1082 A/G) as in this study and was able to show a connection between need of mechanical ventilation in RSV bronchiolitis and *IL-10* polymorphism. We could not show any connection between the duration and need of hospitalization, the need of feeding support or supplementary oxygen, or the development of otitis media and the *IL-10* polymorphism,

when all infants with bronchiolitis and infants with RSV bronchiolitis were compared to controls. None of the infants in this study needed mechanical ventilation. Gentile et al.[4] studied the influence of cytokine polymorphism upon the clinical picture of RSV bronchiolitis in young infants and observed that both *IL-10* (−1082) and *IFNG* (+874) polymorphisms influenced the clinical severity and complications of the disease. In their study the clinical picture of the disease was more severe, because 74.0% needed oxygen therapy versus 18.7% in our study. Tal et al.[3] found that *TLR4* mutation at +896 A/G was associated with severe RSV bronchiolitis. The results of the present study do not support their finding. Though the patients in both studies had a rather similar clinical picture, Tal et al. included also older infants than ours did, which can at least partly explain the discrepancy between the results.

The results of this study suggest that carriage of the G allele at position −1082 of the *IL-10* promoter protects infants with rhinovirus, and possibly, with other viruses but RSV, from severe bronchiolitis and subsequent hospitalization whereas homozygotes for allele A are more likely to develop bronchiolitis when infected with rhinoviruses. IL-10 is an anti-inflammatory cytokine, and the *IL-10* promoter polymorphism has been connected to asthma, atopic dermatitis, IgE level and eosinophil count in asthma.[16–18] The allele −1082A has been shown to be more common in individuals with severe asthma.[16,17] The *IL-10* −1082 A/G polymorphism has also been shown to influence the level of IL-10 production in vitro, so that the presence of allele G is associated with higher IL-10 production.[10] These previous results together with the findings in this study suggest that infants homozygous for the IL-10 low producer allele A (AA) at position −1082 of *IL-10* may be at risk for later asthma, at least if they present with bronchiolitis when infected with rhinovirus.

Rhinovirus is the most common viral trigger of asthma exacerbation, and as documented recently, capable to infect lower airways and cause bronchiolitis in infants.[5] There are no clinical characteristics by which RSV and rhinovirus bronchiolitis can be separated. In recent studies, the risk of subsequent wheezing and later asthma has been higher after rhinovirus bronchiolitis than after RSV bronchiolitis.[5,6] Whether RSV or rhinovirus cause permanent airway damage or permanently modify inflammatory responses leading to asthma, is under intensive research. The main result of the present study was that infants with severe bronchiolitis not caused by RSV, and especially if caused by rhinovirus, were likely to be homozygous for the allele A and unlikely to carry the allele G at position −1082 of *IL-10*. Since the presence of allele A at this position is associated with asthma and allergy, our observations suggest that rhinovirus infection even at this young age can cause symptoms seen in asthma, at least in children with a genetic predisposition. The results are in line with the recent observations stressing the role of rhinoviruses in bronchiolitis, subsequent wheezing and development of later asthma.

The patients of the present study, infants less than 6 months of age treated in hospital for bronchiolitis and originating from a rather small area with only Finnish population, form a homogenous group suitable for genetic studies. In addition, advanced virological methods were available allowing virus-specific diagnoses of the most important causative agents of bronchiolitis, including also rhinoviruses. The main short-coming of the study is the small number of other than RSV patients. In addition, a limited number of polymorphisms were assessed within each gene. Therefore, the findings are preliminary, and should be confirmed by larger studies. Our results emphasize the importance of both genetic properties and causative viruses in infants with severe bronchiolitis. The understanding of the interaction between genes and viruses offers the potential of identifying at-risk infants, in whom preventing infections or tailoring anti-inflammatory therapy could alter the expression of asthma.

## ACKNOWLEDGMENTS

The authors have no commercial or other association that might pose a conflict of interest. The manuscript or its contents has not been presented in public previously. The authors have received financial support from the University Hospital of Tampere, Finland (nr MB0165) (Merja Helminen).

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# GENE POLYMORPHISM OF *IFNG* +874 T/A AND *TLR4* +896 A/G AND RECURRENT INFECTIONS AND WHEEZING IN TODDLERS WITH HISTORY OF BRONCHIOLITIS

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**Abstract:** Cytokine and TLR4 polymorphisms and their association with the infection history of 129 children hospitalized for bronchiolitis during the first 6 months of life were analyzed. The carriers of *IFNG +874 T/A* allele A had fewer infections and use of inhaled corticosteroids and the carriers of *TLR4+896 A/G* allele G were more likely to need tympanostomy than noncarriers.

**Key Words:** bronchiolitis, cytokine polymorphism, TLR4, recurrent infections

Accepted for publication May 19, 2009.

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Supported by the University Hospital of Tampere, Finland (nr MB0165) (to M.H.) and by the Finnish Paediatric Society (to K.N.).

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DOI: 10.1097/INF.0b013e3181af37ee10.1097/INF.0b013e3181af37ee

The link from bronchiolitis to subsequent wheezing and asthma has frequently been reported, but the mechanisms are not known. Viruses, such as rhinovirus, and host immunity have been suggested as contributing factors.[1–4] We recently found that *IL10* polymorphism at −1082 A/G is associated with bronchiolitis requiring hospitalization in infants under 6 months of age but only when the causative agent is not respiratory syncytial virus (RSV).[2] The aim of the present study was to evaluate the connection between polymorphisms of *IL10 −1082 A/G*, *IFNG +874 T/A*, *IL18 −137 G/C*, and *TLR4 +896 A/G*, and recurrent infections and wheezing in these children during the first 1½ years of life.

## PATIENTS AND METHODS

Healthy, full-term infants hospitalized for bronchiolitis at less than 6 months of age between December 1st, 2001 and May 31st, 2002 and between October 28th, 2002 and May 31st, 2004 were recruited in the study. Bronchiolitis was defined as acute lower respiratory illness characterized by rhinorrhea, cough, and diffuse wheezes and rales. The viral etiology of bronchiolitis was determined as described recently.[2] Children were invited to a follow-up visit between May 2003 and June 2005. The parents had recorded prospectively the illness history of the child during the follow-up period. At the follow-up visit, a questionnaire was used to obtain data on hospitalizations, breast-feeding, smoking and pets in the family, location of residence, form of day-care, number of siblings, history of allergy in the family, and the allergy status of the child (if diagnosed by the family doctor). Genotyping of *IFNG +874 T/A* (rs 2430561), *IL10 −1082 A/G* (rs1800896), *IL18 −137 G/C* (rs 187238), and *TLR4 +896 A/G* (rs4986790) gene polymorphisms has been described previously.[2] Statistical analyses were performed using the Statistical Package of Social Science. The Ethics Committee of Tampere University Hospital District approved the study. Informed consent was obtained from parents before enrolling the children.

## RESULTS

*Study Population.* In all, 129 of the 203 children (63.5%) who were enrolled in the initial study participated in the follow-up study. The mean follow-up time of the children after the bronchiolitis episode was 15.6 ± 1.7 months (SD) (range: 11.3–19.5 months).The basic characteristics and the causative agent of the initial bronchiolitis episode of the children are shown in Table 1. The causative virus, place of residence, breast-feeding, parental smoking, keeping of pets in the family, form of day-care, allergy in the family, or number of siblings had no significant association with the number of infections, number of ear infections, use of antibiotics, placement of tympanostomy tubes, number of wheezing episodes, or use of corticosteroids (data not shown). Children with doctor-diagnosed allergy had more wheezing episodes per patient month than nonallergic children (0.10 ± 0.16 [SD] vs. 0.04 ± 0.07 [SD], $P = 0.02$) and were more likely to receive steroids than nonallergic children (11/32 [34.4%] vs. 13/97 [13.4%], $P = 0.008$).

**TABLE 1.** The Demographic and Clinical Characteristics of the Study Children

| | No (%) (n = 129) | Mean ± SD (Median, Range) |
|---|---|---|
| Male | 63 (48.8) | |
| Age (mo) | | 18.1 ± 2.4 (18.0, 13–25) |
| Country-side residence | 33 (25.6) | |
| Day-care | 36 (27.9) | |
| Breastfeeding during infancy | 92 (71.3) | |
| Smoking in the family | 58 (45.0) | |
| Pet in the family | 36 (27.9) | |
| Allergy in a first-degree relative | 84 (65.1) | |
| No. siblings | | 2.5 ± 1.5 (2.0, 1–10) |
| IgE (IU/mL) | | 44.1 ± 97.2 (13.0, 1–621) |
| Tympanostomy tubes | 24 (18.6) | |
| Infection | 113 (87.6) | |
| No | | 4.3 ± 3.8 (4.0, 0–19) |
| Per patient month | | 0.24 ± 0.21 (0.19, 0–0.94) |
| Antibiotics | 107 (82.9) | |
| No | | 3.7 ± 3.5 (2.0, 0–18) |
| Per patient month | | 0.20 ± 0.19 (0.13, 0–0.93) |
| Ear infections | 102 (79.1) | |
| No | | 3.3 ± 3.2 (2.0, 0–15) |
| Per patient month | | 0.18 ± 0.18 (0.11, 0–0.93) |
| Allergy in the child | 32 (24.8) | |
| Food allergy | 27 (84.4) | |
| Atopic dermatitis | 27 (84.4) | |
| Wheezing episodes | 47 (36.4) | |
| No | | 0.97 ± 2.0 (0, 0–14) |
| Per patient month | | 0.05 ± 0.10 (0, 0–0.67) |
| Steroid therapy | 24 (18.6) | |
| Causative virus during bronchiolitis | | |
| RSV | 88 (68.2) | |
| Other virus* | 18 (14.0) | |
| Mixed infection† | 6 (4.6) | |
| No virus identified | 17 (13.2) | |

*Rhinovirus (n = 9), Influenzavirus (n = 6), human metapneumovirus (n = 2), adenovirus (n = 1).

†RSV + RV (n = 3), RSV + adenovirus (n = 3).

**TABLE 2.** Association of Genotype Frequencies and Allele Carrier Status of Gamma-Interferon (*IFNG*) +874 T/A With the Number and Number Per Patient Month of Infections, Ear Infections, Use of Antibiotics, and Wheezing Episodes in Children With History of Severe Bronchiolitis

| Cytokine Polymorphism | No. Infections Per Patient Month | No. Ear Infections Per Patient Month | No. Antibiotic Courses Per Patient Month | No. Wheezing Episodes Per Patient Month |
|---|---|---|---|---|
| *IFNG +874 T/A* | | | | |
| AA (n = 14) | 0.14 ± 0.12 (0–0.44) | 0.11 ± 0.12 (0–0.44) | 0.11 ± 0.12 (0–0.44) | 0.03 ± 0.06 (0.18) |
| TA (n = 69) | 0.21 ± 0.17 (0–0.67) | 0.16 ± 0.15 (0–0.63) | 0.18 ± 0.15 (0–0.63) | 0.05 ± 0.11 (0–0.67) |
| TT (n = 46) | 0.30 ± 0.26 (0–0.94)* | 0.23 ± 0.22 (0–0.93)† | 0.26 ± 0.25 (0–0.93)§ | 0.06 ± 0.1 (0–0.35) |
| Allele A carrier (AA and AT) (n = 83) | 0.20 ± 0.16 (0–0.67)‡ | 0.15 ± 0.15 (0–0.65)‡ | 0.17 ± 0.15 (0–0.63)‡ | 0.05 ± 0.11 (0–0.67) |
| Allele A noncarrier (TT) (n = 46) | 0.30 ± 0.26 (0–0.94) | 0.23 ± 0.22 (0–0.93) | 0.26 ± 0.25 (0–0.93) | 0.06 ± 0.10 (0–0.35) |

mean± SD (range).
*$P = 0.05$, Kruskal-Wallis test.
†$P = 0.07$, Kruskal-Wallis test.
§$P = 0.05$, Kruskal-Wallis test.
‡$P = 0.06$, Mann-Whitney $U$ test.

*Gene Polymorphisms and Infection, Wheezing and Use of Corticosteroids.* The polymorphisms of *IFNG*, *IL10*, *IL18*, and *TLR4* analyzed showed no association with doctor-diagnosed allergy, total serum IgE, or wheezing history of the child (data not shown). However, *IFNG* polymorphism at +874 T/A seemed to be associated with susceptibility to infections, so that allele A seemed to have a protective effect (Table 2). Children who were carriers of *IFNG* allele A were less likely to have tympanostomy tubes inserted than noncarriers (11/83 [13.3%] vs. 13/46 [28.3%], $P = 0.037$, $\chi^2$ test). The number of ear infections per patient month was also less, although not significantly, in children with the allele A ($P = 0.07$). The polymorphisms of *IL10*, *IL18*, and *TLR4* studied were not associated with the infection histories (data not shown). However, carriers of *TLR4+896 A/G* allele G or *IL10 −1082 A/G* allele G were more likely to have tympanostomy tubes than noncarriers (8/21 [38.1%] vs. 16/108 [14.8%], $P = 0.012$, and 21/89 [23.6%] vs. 3/40 [7.5%], $P = 0.030$, respectively, $\chi^2$ test). The carriers of *IFNG* allele A were also less likely to have used corticosteroids for wheezing (10/83 [12.0%] vs. 14/46 [30.4%], $P = 0.010$, $\chi^2$ test), though no association was found with the numbers per patient month of wheezing episodes (Table 2). In multivariate logistic regression, the use of corticosteroids was associated with both allergy (aOR: 4.6 [95% CI: 1.2–18.1]) and *IFNG* allele A carriage (aOR: 0.23 [95% CI: 0.07–0.83]), with no interaction between the 3 variables ($P = 0.88$).

## DISCUSSION

The aim of this postbronchiolitis follow-up was to evaluate the association between cytokine gene polymorphisms and subsequent respiratory infections and wheezing during the first 1.5 years of life. We selected the gene polymorphisms of 4 cytokines that have been shown to influence the clinical presentation of bronchiolitis and/or susceptibility to asthma development. Recently, we have shown that infants who were hospitalized for bronchiolitis caused by other viruses than RSV, especially by rhinoviruses, were more likely than controls to be *IL10 −1082* allele G noncarriers.[2]

In recent studies, recurrent wheezing has been common especially after rhinovirus-induced wheezing in infancy.[1] In this study, we could not show any significant associations between the causative virus of bronchiolitis and subsequent infections or wheezing, allergic manifestations, or use of antibiotics or corticosteroids during the 1-year follow-up period. However, *IFNG* polymorphism at +874 seemed to influence the susceptibility to respiratory infections, and *TLR4* polymorphism at +896 influenced the need of tympanostomy tube replacement. *IFNG* allele A at +874 also decreased the likelihood that corticosteroids were needed for postbronchiolitis wheezing.

IFNG is a pro-inflammatory cytokine which also seems to have direct antiviral activity. Production of IFNG is genetically controlled so that allele A at +874 is associated with low expression of IFNG.[5] In earlier studies, *IFNG* allele A at +874 has been shown to decrease the risk of rejection and lung fibrosis and to increase the risk of infections, such as malaria.[6,7] In addition, allele T at +874 has been shown to confer protection against chronic hepatitis B infection.[8]

In this study, allele A at +874 seemed to protect from respiratory infections and significantly decreased the need for corticosteroids used to prevent or treat postbronchiolitis wheezing. In logistic regression, this finding was robust to the adjustment for allergy, suggesting that, since steroids are used for recurrent wheezing, *IFNG* allele A carriage is an independent protective factor for recurrent wheezing after bronchiolitis. The finding that *IFNG* allele A at +874, which is associated with low IFNG production, decreased the need for corticosteroids, could be explained by a more controlled inflammatory reaction leading to less wheezing. Strong immune responses not only eliminate infecting organisms but also provoke inflammatory symptoms like wheezing. In addition, viral infections induce bronchial obstruction by neural reflexes,. In animal studies, IFNG has induced or at least enhanced bronchial obstruction by damaging the M2 receptors, which limit the release of acetylcholine from vagal nerve endings.[9] This IFNG-mediated bronchial obstruction may be one of the mechanisms leading to recurrent wheezing during viral infections, especially in individuals who have a heightened IFNG response. Therefore, individuals with a more controlled, lower IFNG response may be protected, not from viral infections, but merely from repeated wheezing episodes.

Polymorphism of *TLR4* at +896 was associated with the need of tympanostomy tubes so that carriers of allele G were more likely to require the tubes. TLR4 is a transmembrane protein which triggers the innate immune response to endotoxin.[4,10] *TLR4* polymorphism was analyzed in this study, because TLR4 is also part of a receptor complex involved in the immune response against RSV, a major pathogen in bronchiolitis.[4] The A/G polymorphism at +896 of *TLR4* has been associated with the severity of RSV infection, as well as with lipopolysaccharide responsiveness in septic infections, so that allele G seems to increase susceptibility to severe RSV and gram-negative bacterial infections.[4,10] Therefore,

the allele G at +896 of *TLR4* could predispose children to recurrent or chronic ear infections caused by gram-negative bacteria and also to more severe RSV infections and inflammatory reactions, further predisposing the child to recurrent otitis media and tympanostomy tube insertion. In this study, *IFNG* allele A at +874 was associated with less need for tympanostomy tubes.

Our results offer preliminary evidence that polymorphisms of *IFNG* +874 T/A, and of *TLR4*+896A/G had a significant association with the numbers and presentations of early-life respiratory infections in children with bronchiolitis at less than 6 months of age. Polymorphism at *IFNG* +874 T/A had no association with the number of postbronchiolitis wheezing episodes, but allele A carriage decreased the risk of repeated wheezing requiring treatment by corticosteroids. This finding was confirmed by multivariate analyses adjusted for allergy. The findings of this study have to be interpreted cautiously, since only 64% of the patients eligible for the study attended the follow-up visit, and only indirect outcome measures, the use of corticosteroids reflecting severe or repeated wheezing and the insertion of tympanostomy tubes reflecting repeated otitis media complicating respiratory infections, gave indisputably statistically significant results.

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# Mannose-Binding Lectin Gene Polymorphisms in Infants with Bronchiolitis and Post-Bronchiolitis Wheezing

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Supported by the Central Hospital of Seinäjoki.

**Running head**: MBL genetics and bronchiolitis

**Abstract**

**Background.** Mannose-binding lectin (MBL) encoded by the *MBL2* gene, is an important component of the innate immunity. Low levels have been linked with respiratory infections and both high and low levels with allergy and asthma. The aims of the study were to evaluate the connection between polymorphisms of the *MBL2* gene and viral findings, clinical characteristics and subsequent wheezing in young infants with bronchiolitis.

**Methods.** In all, 129 full-term infants hospitalized for bronchiolitis at age less than 6 months have been followed-up until the mean age of 1.5 years. The genotyping of the *MBL2* gene mutations was made by pyrosequencing for a simultaneous detection of three single nucleotide polymorphisms (SNP).

**Results.** The MBL genotypes or allele frequencies had no significant associations with clinical characteristics of bronchiolitis. The 41 children with variant genotypes were more often infected by multiple viruses (21.9%, p=0.047) than children with wild-type A/A genotypes (9.1%). In addition, more children with variant genotypes (31.7%, p=0.016) had used corticosteroids because of post-bronchiolitis wheezing, compared to those with wild-type A/A genotypes (13.6%). No other significant associations with viral findings or post-bronchiolitis outcomes were found.

**Conclusion.** Preliminary evidence was found that the variant non-A/A genotypes may be associated with susceptibility to multiple viral infections and more severe post-bronchiolitis wheezing requiring treatment with corticosteroids.

**Key words**: Bronchiolitis, Gene, Mannose-binding lectin, Wheezing, Virus

## Introduction

Mannose-binding lectin (MBL), encoded by the *MBL2* gene, plays a central role in the innate immunity, which is important in early infancy before an establishment of adaptive immunity. MBL responds as an acute-phase reactant and promotes phagocytosis directly and via the lectin complement pathway [1]. MBL deficiency due to mutations in the MBL gene is rather common and seems to predispose to respiratory infections [2-4]. However, data on the association with asthma and allergy in children are conflicting [5-7]. The results vary from no association with *MBL2* gene polymorphisms and asthma or atopy [6,7] to significant association [5]. Low serum MBL levels have been associated with single nucleotide polymorphisms (SNP) in the MBL structural gene coding region and in the promoter regions [1].

Point mutations in exon 1 of MBL structural gene, designated as dominant B, C, and D alleles, lead to lowered serum MBL levels. The wild-type allele is referred to as A with variants commonly designated collectively as O. Wild-type homozygotes are referred to as A/A, those heterozygous for a coding mutation as A/O and those homozygous or compound heterozygous as O/O [8].

We have prospectively enrolled and followed-up a group of children hospitalized for bronchiolitis at age less than 6 months [9,10]. Our recent studies have provided preliminary evidence that polymorphisms of *IL-10*, *IFNG* and *TLR4* genes may have a significant association with the aetiology, number and presentations of early-life respiratory infections [10,11].

The aim of the present study was to evaluate whether the polymorphisms of the *MBL2* gene associate with the presence, characteristics and viral aetiology of bronchiolitis in early infancy. In

addition, we evaluated if there are any associations between the polymorphisms and post-bronchiolitis outcomes including subsequent infections, use of antibiotics, subsequent wheezing and need of corticosteroid treatments by 1.5 years of age.

## Methods

Previously healthy, full-term infants hospitalized for bronchiolitis at less than 6 months of age between December 1st, 2001 and May 31st, 2002 and between October 28th, 2002 and May 31st, 2004 were enrolled in the study. Bronchiolitis was defined as an acute lower respiratory illness characterized by rhinorrhea, cough, and diffuse wheezes and crackles [12]. The microbial aetiology of bronchiolitis was studied with antigen detection assays and polymerase chain reactions as described recently [9,10]. Data on disease severity like the need of additional oxygen, feeding support and length of hospital stay were recorded during the inpatient care [9]. One hundred and thirty-nine children were invited to a follow-up visit at on average 18 months of age, and 129 (92.8%) attended [11]. At the follow-up visit, the parents were interviewed and a questionnaire on the presence of otitis media, the use of antibiotics and the presence of wheezing, repeated wheezing and use of corticosteroids for wheezing after hospitalization for bronchiolitis, was fulfilled [11]. The corticosteroid treatment included peroral steroid courses as well as maintenance treatment with inhaled corticosteroid. Repeated wheezing was defined when there were 2 or more wheezing episodes during follow-up time. Fresh or frozen whole blood samples were available for MBL genotyping from all 129 children. The Ethics Committee of the Tampere University Hospital District approved the study. Informed consent was obtained from parents before enrolling the children.

Genotyping of *MBL2* gene polymorphism

The genotyping of *MBL2* gene mutations was performed with pyrosequencing for simultaneous detection of three SNPs in codons 52, 54 and 57. In short: the genomic DNA was purified from peripheral blood with Qiagen QiAmp DNA Blood Mini Kit 250 (Qiagen, Hilden, Germany) according to the instructions of the manufacturer. Five microliters of isolated DNA were used for the amplification of the first exon of the *MBL2* gene by PCR. The PCR product was used for pyrosequencing described by Roos et al.[13]. Sequential addition of NTP was used in the following order: CTCTGTGTCATCACAGC. The reaction was performed at 28ºC. The close proximity of three functionally important SNPs in exon 1 of the *MBL2* gene allows their detection in a single pyrosequencing reaction. This procedure resulted in pyrograms with unique and easily identified patterns for each allele combination of the three *MBL2* SNPs.

Statistical analyses

Data were analysed by the SPSS package version 19.0 (IBM Corp. NY, USA). Chi-square and Fisher's exact tests were used for categorized and student's t-test for normally distributed and Mann-Whitney test for non-normally distributed continuous variables. The results are expressed as frequencies, proportional frequencies, means, medians and standard deviations (SD).

The personal data of the study subject were not given to the laboratory which performed the genetic studies (National Institute of Health and Welfare, Turku, Finland).

## Results

Two-thirds (68%) of the 129 patients had the A/A genotype, 29% had A/O and 3% had the O/O genotype. The A allele, as either homozygous or heterozygous, was present in nearly all (97%) children (Table 1). Thus, the A allele comprised 83% of all the 258 alleles. The respective figures were 10%, 0.4% and 6% for the B, C, and D alleles. The MBL genotype A/O was associated with the presence of two or more viral findings (Table 1). There were no other significant associations between different MBL genotypes and viral findings.

No significant associations were found between the MBL genotypes and disease severity, need of supplemental oxygen, need of feeding support or length of hospital stay (Table 2).
*MBL2* genotype A/O was associated with the need of treatment with corticosteroids because of post-bronchiolitis wheezing (Table 3). There were no other significant associations between MBL genetics and post-bronchiolitis outcomes.

## Discussion

There are two main results in the present study on *MBL2* gene polymorphisms in children hospitalized for bronchiolitis in early infancy. First, the distribution of the three MBL genotypes A/A (68%), A/O (29%) and O/O (3%) was nearly identical with that in the Finnish population, A/A in 65%, A/O in 30% and O/O in 5% [14–16]. Second, preliminary evidence was found that the variant genotype A/O in the present study may be associated with multiple viral findings and more severe post-bronchiolitis wheezing requiring treatment with corticosteroids. There were no

other significant associations between *MBL2* genotypes or frequencies of single alleles and viral findings, clinical characteristics or subsequent wheezing.

In line with our results, MBL deficiency and *MBL2* genotypes were not associated with the need of hospitalizations for RSV infections in a case-control study in 168 infants from South Africa [17]. In a study from Brazil, however, 81 children less than 5 years old with RSV infections had lower MBL levels than were observed in 40 healthy controls, and in particular, patients with severe disease requiring hospitalization had the lowest levels [18]. In a prospective population-based study from Greenland, 250 children aged less than 2 years were followed-up weekly for 2 years, and those who were heterozygous or homozygous for variant alleles had an increased risk for acute respiratory infections, but not earlier than from the age of 6 months onwards [3]. Several factors may explain the differences between these studies, including disease severity, infections by different viral strains or different serotypes of the same virus, other concomitant infections and differences in MBL haplotypes which vary worldwide [19]. Our results offer preliminary evidence, that variant MBL genotypes may be associated with viral co-infections in infants hospitalized for bronchiolitis at age less than 6 months.

Some studies have documented an association between *MBL2* gene polymorphisms and repeated respiratory infections in children [20,21]. *MBL2* gene mutations were more common in 335 Polish children with recurrent respiratory infections, aged 1-16 years, than in 78 age- and sex-matched healthy controls [20]. Though coexisting immune defects were common, presenting in 47% of the cases, *MBL2* gene mutations and low serum MBL levels were independent risk factors for recurrent infections. In a large population-based study in non-selected, less than 6 years old Chinese children, low serum MBL level and the presence of the B allele in the *MBL2* gene were associated with recurrent respiratory infections [21]. In the present post-bronchiolitis study, no association was found between *MBL2* gene polymorphisms and the presence of otitis media and

the use of antibiotics until 18 months of age; both parameters reflect the susceptibility to respiratory infections.

Data on the association between serums MBL level or MBL gene polymorphisms and allergy or asthma in children are conflicting [5-7]. To our knowledge, there are no previous studies focusing on bronchiolitis, post-bronchiolitis wheezing or other early childhood wheezing. The expectation is that high serum MBL levels rather than low levels would be associated with allergy, wheezing and asthma, as was seen in 72 Turkish schoolchildren with asthma compared with 30 healthy age- and sex-matched controls [5]. One *MBL2* gene polymorphism associating with increased serum MBL level, eosinophilia and asthma with allergic rhinitis and bronchopulmonary aspergillosis has been discovered [22]. However, large population-based studies made in blood donors, school children and adults have failed to confirm any association between *MBL2* gene polymorphisms and asthma or allergy [6,7,16,23,24].

As part of a birth cohort study from UK, serum MBL levels were measured in 124 Caucasian and 30 Asian children with a mean age of 10 years, and MBL levels did not differ between atopic and non-atopic children or between wheezing and non-wheezing children [25]. In wheezing children, however, MBL levels were associated with the severity of wheezing assessed by the required treatment; serum MBL was higher in children treated with inhaled corticosteroids compared with non-treated wheezing children and with healthy children [25]. Though there was no association between *MBL2* gene polymorphisms and clinical characteristics or disease severity of bronchiolitis in young infants, we found some evidence that the variant non-AA MBL genotype may be associated with severe post-bronchiolitis wheezing requiring treatment with corticosteroids. The post-bronchiolitis use of corticosteroids offers also indirect evidence for the

presence of repeated wheezing. These observations may reflect long-term or chronic airway inflammation or only vulnerability to repeated infections as well.

The main strengths of the present study are the prospective design, an extensive virological testing panel including RSV, rhinoviruses, metapneumovirus and bocavirus, and the homogeneity of the patients consisting of young Finnish infants hospitalized for bronchiolitis at less than 6 months of age. A shortcoming of the study is the lack of data on serum MBL concentrations. However, serum MBL concentrations have been associated rather constantly with MBL genotypes; when compared to subjects with the A/A genotype, those with the variant A/O genotype have presented with lowered concentrations and those with the variant O/O genotype with very low concentrations [8]. Another shortcoming is the number of cases which is small for genetic studies. In addition, no healthy controls were recruited. However, the distribution of MBL genotypes and different alleles is well studied in the Finnish population, which is, as is well-known, still rather homogenous.

In conclusion, preliminary evidence was found that in young infants hospitalized for bronchiolitis, variant non-A/A MBL genotypes may be associated with susceptibility to multiple viral infections and repeated or severe post-bronchiolitis wheezing.

**Acknowledgments**

The authors thank Elisa Rehnberg for excellent technical assistance for MBL genotyping.

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**Table 1.** MBL genetics in 129 children with bronchiolitis in relation to viral findings

| Genotypes and alleles | Total (N=129) | Single RSV infections (N=81) | Other single infections[&] (N=17) | Two or more viruses[#] detected (N=17) | No viruses detected (N=14) |
|---|---|---|---|---|---|
| Genotypes | | | | | |
| AA | 88 (68%) | 60 (74%) | 10 (59%) | 8(47%) | 10 (71%) |
| AO | 37 (29%) | 17 (21%) | 7 (41%) | 9 (53%)* | 4 (29%) |
| OO | 4 (3%) | 4 (5%) | 0 | 0 | 0 |
| Alleles | | | | | |
| A | 125 (97%) | 77 (95%) | 17 (100%) | 17 (100%) | 14 (100%) |
| B | 27 (21%) | 13 (16%) | 6 (35%) | 4 (24%) | 4(29%) |
| C | 1 (0.8%) | 0 | 1 (6%) | 0 | 0 |
| D | 15 (12%) | 10 (13%) | 0 | 5 (29%) ** | 0 |

[&] Rhinovirus (RV) in 9 cases, human metapneumovirus (hMPV) in 2 cases, influenza A virus (IVA) in 6 cases

[#] RSV and RV in 6 cases, RSV and adenovirus in 3 cases, RSV and human bocavirus (hBoV) in 4 cases, RV and hBoV in 3 cases ,IVA and adenovirus in 1 case.

* p=0.047 vs. single infections; p=0.034 vs. single RSV infections

** p=0.046 vs. single infections

**Table 2.** The association between MBL genetics and basic and clinical characteristics of bronchiolitis patients

| Patients (N=129) | Genotype AA (N=88) (%) | Genotype AO or OO (N=41) (%) | p | All (N=129)(%) |
|---|---|---|---|---|
| Age, weeks, median (range) | 9.0 (1-25) | 10.0 (2-25) | 0.55 | 9.0 (1-25) |
| Boys | 41 (47) | 22 (54) | 0.57 | 63 (49) |
| Oxygen required | 18(21) | 5(12) | 0.33 | 23 (18) |
| Feeding support | 27(31) | 17(42) | 0.24 | 44 (34) |
| Length of hospital stay, days, mean (SD, range) | 4.6 (2.7, 0-12) | 4.7 (3.9, 0-22) | 0.22 | 4.6 (3.1, 0-22) |

**Table 3.** The association between MBL genetics and post-bronchiolitis outcomes by age 18 months

| Patients (N=129) | Genotype AA (N=88) (%) | Genotype AO or OO (N=41) (%) | p |
|---|---|---|---|
| History of antibiotic use | 76 (86) | 31 (76) | 0.14 |
| History of otitis media | 72 (84) | 30 (78) | 0.46 |
| Post-bronchiolitis wheezing | 28 (32) | 19 (46) | 0.24 |
| Repeated post-bronchiolitis wheezing | 15 (17) | 10 (24) | 0.35 |
| Use of corticosteroids | 12 (14) | 13 (32)[#] | 0.016 |

[#] The genotype AO in 13 cases (the B allele in 7 and D allele in 6 cases).

# *Bordetella pertussis* Infection Is Common in Nonvaccinated Infants Admitted for Bronchiolitis

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**Background:** Preliminary evidence suggests that viral-pertussis coinfections are common in nonvaccinated infants.

**Subjects and Methods:** *Bordetella pertussis* infection was studied by polymerase chain reaction in nasopharyngeal aspirates in 142 infants <6 months of age, who were admitted for bronchiolitis. Viral etiology, documented by antigen detection or polymerase chain reaction in nasopharyngeal aspirate, was respiratory syncytial virus (RSV) in 105, rhinovirus in 8, influenza A virus in 8, and other viruses in 10 cases. Only 11 samples were negative.

**Results:** *B. pertussis* infection was found in 12 (8.5%) cases, being coinfection with RSV in 8 (67%) cases (7.6% of all RSV infections). In a retrospective analysis, RSV-pertussis coinfections and sole RSV infections did not differ for the presence of cough. Preliminary evidence was found that a history of coughing spells was associated with *B. pertussis* identification.

**Conclusions:** Coinfection with *B. pertussis* was present in 8.5% of <6-month-old infants, who were hospitalized for viral bronchiolitis. To avoid underdiagnosis, pertussis should be considered in all nonvaccinated infants admitted for lower respiratory tract infection.

**Key Words:** *Bordetella pertussis*, bronchiolitis, infant, mixed infection, respiratory syncytial virus

(*Pediatr Infect Dis J* 2010;29: 1013–1015)

The simultaneous occurrence of respiratory syncytial virus (RSV) and *Bordetella pertussis* infection was first reported 20 years ago.[1] Currently, the development of polymerase chain reaction (PCR) tests for RSV and for *B. pertussis,* in particular, has allowed confirmation of this observation.[2–5] *B. pertussis* coinfection has been documented in from <1%[6,7] to 16%[5] of RSV infections in nonvaccinated infants. *B. pertussis* was identified in 23% of <5-month-old infants hospitalized for severe respiratory disorders like apnea and respiratory failure.[2]

Until the early, 2000, children developed pertussis in our country (National Institute for Health and Welfare, Finland) despite approximately 95% coverage with whole cell pertussis vaccine given at 3, 4, 5, and 24 months of age. In 2003, the vaccination program was modified and booster vaccination by acellular pertussis vaccine was given at 6 and 11 to 13 years of age. Since 2005, acellular pertussis vaccine has been given at 3, 5, and 12 months and at 4 and 14 to 15 years of age, resulting in a decrease of pertussis incidence in children of all ages.

Between 2001 and 2004, 205 infants aged <6 months were enrolled in a study of the viral etiology of bronchiolitis. Nasopharyngeal aspirates (NPAs) were obtained for antigen detection by immunofluorescence for 7 respiratory viruses and for genome detection by PCR for 9 respiratory viruses; both tests were available for RSV.[8] In 2009, 142 good-quality frozen NPA samples were available for *B. pertussis* PCR. To supplement prospectively collected data (A. Halkosalo et al, unpublished data),[8] data on cough symptoms were collected retrospectively from the patients' records. The aim of the study was to evaluate how often respiratory viral infections, RSV infections in particular, are mixed infections with *B. pertussis* in infants with bronchiolitis.

## MATERIALS AND METHODS

A total of 205 healthy full-term infants younger than 6 months of age, who were hospitalized for bronchiolitis in the Department of Pediatrics, Tampere University Hospital (Finland) between December 1, 2001 and May 31, 2002 and between October 28, 2002 and May 31, 2004, were enrolled in the study. The Ethics Committee of the Tampere University Hospital District approved the study. An informed consent was obtained from parents before enrolling the children.

Bronchiolitis was defined as acute lower respiratory illness characterized by rhinorrhea, cough, and diffuse wheezes or crackles. NPA were obtained from all infants for antigen detection by indirect immunofluorescence for RSV, influenza A and B virus, adenovirus and parainfluenza virus 1, 2, and 3, according to routine practice at the hospital laboratory.[8] In addition, reverse transcription (RT) PCR was used for detection of the same viruses and also of rhinovirus[8] and human metapneumovirus[8] and human bocavirus (A. Halkosalo et al, unpublished data). For the present analysis, combined results of immunofluorescence and RT-PCR were used.

One year after bronchiolitis, 129 children (63%) attended the follow-up visit in 2003 to 2005, at an average of 18 months of age (range: 13–24). The parents had recorded prospectively the illness history of the child in diaries during the follow-up period. At the time of the follow-up visit, a questionnaire was used to register the data on postbronchiolitis episodes of wheezing, ear infections, and inserted tympanostomy tubes, as well as on the use of antibiotics, bronchodilators, and corticosteroids.[9]

In 2009, good-quality frozen NPA samples were available for *B. pertussis* PCR from 142 (69%) children hospitalized for bronchiolitis 5 to 8 years earlier. *B. pertussis* was studied by an in-house PCR at National Institute for Health and Welfare, Turku (Finland), as described earlier in more detail.[10] One of the authors (P.K.) reviewed retrospectively the patient cards of the hospital and classified, being not aware of the viral or pertussis findings, the cough symptoms present on admission into 3 categories: cough with spells, cough without spells, and no cough. In addition, the use of antibiotics, with special focus on macrolides effective for pertussis, was registered. All infants were <6 months old; 96

Accepted for publication April 12, 2010.

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Supported by the University Hospital of Tampere, Finland (to P.K.) and by the Central Hospital of Seinäjoki (to K.N.).

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ISSN: 0891-3668/10/2911-1013
DOI: 10.1097/INF.0b013e3181f537c6

(67.6%) infants were entirely nonvaccinated for <3-month olds and 46 were 3- to 5-month olds who were vaccinated once or twice. Thus, no 5- to 6-month-old children had completed the primary pertussis vaccination series.

Student *t* test and Fisher exact text were used in the statistical analyze of the data.

## RESULTS

*B. pertussis* involvement was found in 12 of 142 (8.5%) infants hospitalized for bronchiolitis. Their mean age was 10.4 weeks (SD: 5.1) compared with 9.9 weeks (SD: 6.6) in pertussis-negative infants, and 8 of 12 (67%) were girls. Pertussis-positive infants were not more likely to have older siblings than infants with viral bronchiolitis alone. All infants with *B. pertussis* involvement were <20 weeks old (Table 1).

Pertussis was associated with RSV in 6 cases, with RSV and another virus in 2 cases, and with other viruses than RSV in additional 3 cases (Table 1). Therefore, *B. pertussis* was identified in 8 (7.6%) of all 105 RSV infections. Only 1 case was solely associated *B. pertussis* infection.

Overall, 135 (95%) infants presented with cough; 24 had coughing spells and 111 had cough with no spells (Table 2). Coughing spells were present in 5 (41.7%) pertussis-positive infants compared with 19 (14.6%) pertussis-negative infants ($P = 0.038$). The 1 infant with *B. pertussis* infection alone had coughing spells.

There were no differences in the clinical findings for those with only viral infection compared with *B. pertussis* coinfection. The need or duration of supplementary oxygen, feeding support, and presence of fever, need, and duration of inhalation therapy or duration of intensive care or hospital treatment were similar (Table 3). Only 2 pertussis-positive infants (17%) were treated with macrolides and 5 (42%) were treated with other antibiotics (vs. 51% of pertussis-negative ones, $P = 0.163$).

Twelve months after bronchiolitis, 114 of 142 (80%) children attended the control visit, at which time the parents reported doctor-diagnosed postbronchiolitis wheezing in 39 (34%) cases and use of steroids for wheezing in 22 (19%) cases. There was no significant difference between the pertussis-positive and pertussis-negative cases (data not shown). Likewise, there were no significant differences between the groups in the use of antibiotics, ear infections, or inserted tympanostomy tubes after bronchiolitis (data not shown).

During the study years 2001 to 2004, the incidence of pertussis, based on laboratory reports was constantly increasing in Finnish children of all ages (Table 4). The respective incidence rates and their increases in the primary area of our hospital were similar (data not shown). In 2006, 3 years after the first modification of the pertussis vaccination program and 1 year after the final change of the vaccination program, the figures were lower than in 2001 (Table 4).

**TABLE 1.** Age, Gender, and Viral Findings in Infants With Bronchiolitis, in Relation to the Presence or Absence of *Bordetella pertussis* by Polymerase Chain Reaction

| Findings | *B. pertussis* Positive (N = 12) | *B. pertussis* Negative (N = 130) |
|---|---|---|
| Age in weeks, mean (SD) | 10.4 (5.1) | 9.9 (6.5) |
| Boys/girls | 4/8 | 67/63 |
| Older siblings | 8 (67%) | 91 (70%) |
| Breastfeeding | 6 (50%) | 91 (70%) |
| Age <4 weeks | 1 (8%) | 31 (24%) |
| Age 5–8 weeks | 4 (33%) | 33 (25%) |
| Age 9–12 weeks | 1 (8%) | 26 (20%) |
| Age 13–16 weeks | 4 (33%) | 16 (12%) |
| Age 17–20 weeks | 2 (17%) | 11 (9%) |
| Age 21–24 weeks | 0 (0%) | 13 (10%) |
| Viral infection | 11 (92%) | 120 (92%) |
| RSV | 8 (67%)* | 97 (81%) |
| Rhinovirus | 1 (8%) | 7 (6%) |
| Influenza A | 1 (8%) | 7 (6%) |
| Other viruses | 1 (8%)† | 9 (7%) |

*RSV with bocavirus in 1 case and RSV with adenovirus in 1 case.
†One case with parainfluenza 3 virus.
Statistical significance: no significant differences between the groups.
RSV indicates respiratory syncytial virus.

**TABLE 2.** Respiratory Syncytial Virus (RSV) and *Bordetella pertussis* Findings in Infants in Relation to the Presence of Cough

| Microbe Findings | Group 1 (Coughing Spells) (N = 24) | Group 2 (Cough, No Spells) (N = 111) | Group 3 (No Cough) (N = 7) |
|---|---|---|---|
| *B. pertussis*+ RSV− | 2 | 2 | 0 |
| *B. pertussis*+ RSV+ | 3 | 5 | 0 |
| *B. pertussis*− RSV+ | 15 | 77 | 5 |
| *B. pertussis*− RSV− | 4 | 27 | 2 |
| All *B. pertussis*+ | 5 (21%)* | 7 (6%)† | 0 (0%) |

*$P = 0.038$ versus group 2; †$P = 0.644$ versus group 3.

**TABLE 3.** Treatment During Hospitalization in the Infants With *Bordetella pertussis* Infection

| | *B. pertussis* Positive (N = 12) | *B. pertussis* Negative (N = 130) |
|---|---|---|
| Supplementary oxygen | 4 (29%) | 24 (19%) |
| Duration of oxygen therapy* | 4 (1–10) | 3.5 (1–14) |
| Feeding support | 5 (36%) | 49 (38%) |
| Duration of feeding support* | 7.8 (3–13) | 4.5 (1–12) |
| Duration of hospital stay* | 6.8 (3–22) | 4.7 (1–15) |
| Intensive care treatment | 1 (8%) | 0 |
| Duration of intensive care* | 5 (5) | 0 |
| Antibiotics | 7 (58%) | 51 (39%) |
| Macrolide | 2 (29%) | 1 (2%) |
| Amoxicillin | 4 (68%) | 39 (76%) |
| Penicillin | 1 (14%) | 7 (14%) |
| Cephalosporin | 0 | 3 (6%) |

*Duration in days, mean (range).
Statistical significance: no significant differences between the groups.

**TABLE 4.** Incidence of Pertussis in Children (Cases/100,000) in the Study Years 2001–2004, in the Year 2006 and in the Year 2009 in Finland

| Year | Age 0–4 yr | Age 5–9 yr | Age 10–14 yr |
|---|---|---|---|
| 2001 | 7.9 | 16.2 | 27.3 |
| 2002 | 24.5 | 40.7 | 41.3 |
| 2003 | 53.7 | 100.2 | 109.0 |
| 2004 | 82.3 | 128.5 | 179.0 |
| 2006 | 10.4 | 7.9 | 6.5 |
| 2009 | 9.8 | 7.0 | 7.1 |

Data obtained from the National Infections Register of Finland, maintained by the Institute of Health and Welfare, Helsinki, Finland. Both physicians and laboratories are obligated to report microbiologically verified pertussis cases in the register.

## DISCUSSION

There are 3 main results in the present study. First, pertussis diagnosed by PCR was surprisingly common, nearly 10%, in

nonvaccinated infants hospitalized for bronchiolitis. Second, two-thirds of the cases had mixed infections with RSV. The findings are similar to another study from Finland,[4] in which 8% of <6-month-old bronchiolitis patients had pertussis by PCR during an RSV epidemic. Similarly, more than 20% of infants requiring intensive care during an RSV epidemic had pertussis in a study from the United Kingdom.[2] Third, our preliminary evidence suggests that the coughing spells were associated with *B. pertussis* coinfection. However, RSV and pertussis cases could not be separated by the presence of cough, in agreement with the 20-year-old observation from the United States[1] and with newer studies from the United Kingdom and Finland.[2,4] Therefore, the typical features of RSV bronchiolitis, or even the detection of RSV etiology, do not exclude pertussis in nonvaccinated infants.

In the 1980s, Nelson et al studied upper respiratory secretions by culture and direct fluorescent antibody assay, for RSV and *B. pertussis* in 180 infants hospitalized for respiratory tract infection.[1] Pertussis was identified in 29 (15%) cases; 15 were mixed infections with RSV and 14 were single *B. pertussis* infections. Pertussis was associated with prematurity, but there were no other significant differences between infants with pertussis alone, RSV infection alone, or mixed pertussis-RSV infection. About 20 years later, Crowcroft et al[2] studied *B. pertussis* culture and PCR in 126 infants aged <6 months requiring intensive care for lower respiratory tract infection; 25 (20%) had pertussis and 9 were mixed infections with RSV. Infants with pertussis suffered from cough, apnea, and whooping cough more often than infants without pertussis.[2] Korppi and Hiltunen[4] studied viral etiology of infection by antigen detection in 117 infants hospitalized for bronchiolitis at <6 months age and *B. pertussis* etiology by PCR in those 88 in whom parents or nurses reported cough. *B. pertussis* was identified in 9 (8%) infants, and 7 were mixed infections with RSV. In a recent study from France, PCR to *B. pertussis* was positive in 16% of <4-month-old children hospitalized for RSV infection.[5] In the present study, pertussis was not clinically suspected in any bronchiolitis case, but nearly 10% of them proved to be *B. pertussis* positive by PCR. Thus, all these studies suggest that pertussis is underdiagnosed in infants with respiratory infection, including infants with presumably viral bronchiolitis and with positive RSV identification.

There are 2 recent studies from the United States with opposite results. Siberry et al found only 1 *B. pertussis* positive case by PCR in 166 infants admitted to hospital during RSV season,[6] and Walsh et al found no *B. pertussis* positive cases by PCR in 204 infants <18 months of age presenting with bronchiolitis.[7] The results from the United States suggest that screening of *B. pertussis* is not useful if the prevalence of pertussis is low, as was the case in those study populations. Our results from 2001 to 2004 represent an era of a higher pertussis incidence than currently the case in Finland. The incidence decreased substantially in children at all ages after the 2 alterations in the national vaccination program in 2003 and 2005. Currently, the last booster vaccination is given at 14 to 15 years of age in our country. The occurrence of pertussis needs continuous monitoring in different age groups, including young adults also with no regular booster vaccinations against pertussis.

The in-house PCR used in the present study has been sensitive and specific in diagnosing pertussis. The test was positive in 57 (48%) of 117 nasopharyngeal swaps obtained from elementary school children during an outbreak, including all 6 culture-positive cases.[10] During another outbreak, PCR was positive in 18 (45%) of 40 NPA samples obtained from children with susceptible pertussis, including all 3 culture-positive cases and 5 (35%) of the 14 seropositive cases.[10] In contrast, the high sensitivity of PCR carries a risk of positive findings that are not clinically significant. However, this risk is less in infants than in older children. In addition, the percentage increase in laboratory confirmed pertussis cases diagnosed by PCR compared with culture was moderate, ranging from 9% to 26%, in a multicenter 5-year study in <6-month-old infants from the United Kingdom.[11]

Preliminary evidence was found that coughing spells as such are not diagnostic for pertussis. In addition, there were no significant differences between *B. pertussis* positive and negative cases in the severity of the disease, although estimated by many objective measures. In a recent retrospective study from Israel, *B. pertussis* was identified by PCR in 11 (15%) of 74 <12-month-old infants treated in the pediatric intensive care unit.[12] The pertussis-positive and -negative cases differed similar to our results, only for presence of paroxysmal and prolonged cough. Our pertussis-positive group, like the respective groups in many other studies,[4,5,12] was quite small, being underpowered to reveal small albeit real differences between the groups. In the present study, all infants improved despite the fact that antibiotics effective for pertussis were not used. The patients of the present study were collected prospectively, but data on cough history and antibiotic treatment were retrospective. In future, prospective studies powered sufficiently to allow multivariate, and subgroup-specific stratified analyses are needed to answer to these 2 important questions: the clinical value of coughing history and the effectiveness of antibiotics.

## ACKNOWLEDGMENTS

*The authors thank Päivi Haaranen and Tiina Haarala for excellent technical assistance for pertussis PCR.*

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ACTA PÆDIATRICA
NURTURING THE CHILD

Acta Pædiatrica ISSN 0803–5253

REGULAR ARTICLE

# Weight gain in infancy and post-bronchiolitis wheezing

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**Keywords**
Birth weight, Bronchiolitis, Infancy, Overweight, Wheezing

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**Received**
19 April 2011; revised 1 July 2011; accepted 8 July 2011.

DOI:10.1111/j.1651-2227.2011.02414.x

## ABSTRACT

**Aim:** Low birth weight, high birth weight and excessive weight gain after birth may be risk factors for asthma in childhood, but their associations with wheezing in early childhood are poorly studied. The aim of the study was to evaluate birth weight, weight gain in early infancy and overweight in infancy assessed by weight for length (WFL) as risk factors for wheezing after hospitalization for bronchiolitis in early infancy.

**Methods:** In all, 127 full-term infants hospitalized for bronchiolitis at age <6 months have been followed up until the mean age of 1.5 years. The weights and lengths of the infants were measured on admission to hospital and at the control visit. Birth weights were obtained from the hospital records.

**Results:** Both occurrence and recurrence of post-bronchiolitis wheezing were associated with birth weight >4000 g and the recurrence of post-bronchiolitis wheezing with WFL >110% at age 1.5 years. The associations were robust to adjustments with gender and allergy. Higher weight gain from birth to hospitalization at age <6 months was associated with wheezing in the subgroup of children with birth weight >4000 g.

**Conclusion:** High birth weight and the development of overweight may be associated with post-bronchiolitis wheezing in infancy.

## INTRODUCTION

Excessive weight gain is a risk factor for asthma in adults (1,2), and there is increasing evidence that overweight and obesity may increase the asthma risk also in children (3). Thus far, only two studies have been published on the connection between weight status and wheezing in infants (4,5). In a study from South Korea, overweight assessed by WFL increased the risk of wheezing in 3–12 months old infants (4). In a study from the US, higher WFL at 6 months of age increased the risk of wheezing by age 3 years, but not at age 1 or 2 years (5).

Low birth weight, especially when caused by poor intrauterine growth, has been associated with rapid post-natal weight gain and later obesity and asthma (6). Lower birth weight increased asthma risk in Danish twin pairs at 3–9 years of age (7). In a nation-wide, register-based, nested case-control study from Finland (8), a low ponderal index at birth was an independently significant risk factor for asthma in children at <3 years of age. In a recent birth cohort from the UK (9), greater infant weight and adiposity gains at 0–6 and 6–12 months were associated with both atopic and non-atopic wheezing by 3 years of age.

We have prospectively followed up a group of children hospitalized for bronchiolitis at 0–6 months of age (10,11). The aim of the present paper was to evaluate birth weight, weight gain from birth to hospitalization and overweight at the control visit about 12 months after hospitalization assessed by WFL, as risk factors for post-bronchiolitis wheezing until 1.5 years of age.

## PATIENTS AND METHODS

Previously, healthy full-term infants hospitalized for bronchiolitis at age <6 months between December 1, 2001 and May 31, 2002 and between October 28, 2002 and May 31, 2004 were eligible to the study. The Ethics Committee of the Tampere University Hospital district had approved the study, and an informed consent was obtained from the parents.

The weights of the infants were measured and registered on admission to hospital, and birth weights were obtained from the hospital records. Birth weights were classified into

### Key notes

- High birth weight >4000 g was associated with both occurrence and recurrence of post-bronchiolitis wheezing.
- High weight for length (WFL) >110% at age 1.5 years was associated with recurrence of post-bronchiolitis wheezing.
- Low birth weight (<3000 g) and low WFL (<90%) were not associated with post-bronchiolitis wheezing.

three groups: <3000, 3000–4000 and >4000 g. The birth weight of 3000 g approximates the 5th percentile and that of 4000 the 95th percentile of birth weights in Finnish newborns (12). The rate of weight gain was calculated as weight on admission minus birth weight divided by age in weeks on admission and was expressed as gram per week. Weight gain was regarded as poor, if the mean weight gain was <70 g/week (<10 g/day).

In 2003–2005, about 12 months after hospitalization for bronchiolitis, 127 of 143 (89%) enrolled children attended the follow-up visit at on average 1.5 years of age (11). At the follow-up visit, the parents were interviewed and structured questionnaires were filled in by the doctor. The data registered for the post-bronchiolitis period consisted of episodes of wheezing and otitis media, and of the use of antibiotics, bronchodilators and corticosteroids. Parent-reported allergy was registered and consisted of atopic dermatitis or food allergy confirmed by the family doctors (10). The weights and lengths of the children were measured, and the WFLs were calculated by using the Finnish national population-based gender-specific growth charts as references (13,14). WFLs were categorized by the limits of 110% (overweight) and 120% (obesity). Underweight was defined by two ways, WFL <90% or WFL <80%.

### Statistical analyses

Data were analysed with the SPSS package version 19 (IBM SPSS Co., NY, USA). Chi-square and Fisher's exact tests were used for categorized and student's *t*-test for normally distributed and Mann–Whitney test for non-normally distributed continuous variables. The results are expressed as frequencies, proportional frequencies, means, standard deviations (SD), odds ratios (OR) and 95% confidence intervals (95% CI). As published recently (11), the causative virus, place of residence, length of breast feeding, parental smoking, keeping of pets in the family, form of day care, allergy in the family or number of siblings had no association with the number of wheezing episodes. Instead, children with doctor-diagnosed allergy had more wheezing episodes assessed as events per patient month (mean 0.1, SD 0.16, $p = 0.02$) than had non-allergic children (mean 0.04, SD 0.07) (11). Therefore, logistic regression with adjustments for gender and allergy was used to analyse the associations between weight parameters and post-bronchiolitis wheezing.

## RESULTS

The mean birth weight of the 127 children was 3525 g (SD 543, 95% CI 3430–3620), ranging from 1635 to 5455 g. The respective figures were 3475 g (SD 507, 95% CI 3355–3596) in the 66 girls and 3586 g (SD 582, 95% CI 3432–3741) in the 61 boys ($p = 0.25$). There were only four children with birth weight <2500 g, 19 children with birth weight <3000 g and 23 children with birth weight >4000 g. Weight gain per week from birth to hospitalization depended on birth weight; the mean (SD, 95% CI) was 202.2 g/week (86.3, 185.4–219.0) if birth weight was <4000, and 170.7 g (66.0, 142.2–199.2) if birth weight was >4000 g ($p = 0.06$).

Weight for length was assessed at the follow-up visit, when the children were on average 18 months old. The mean WFL was −1.0% (SD 9.8, 95% CI −2.8 to +0.7), ranging from −20% to +53%. WFL was >110% (overweight) in 11 children and >120% (obesity) in only three children. WFL was <90% in 13 children and <80% in only one child. Only four (17%) of the 23 children with birth weight >4000 g had WFL >110% 18 months later ($p = 0.11$), and correspondingly, only four (21%) of the 19 children with birth weight <3000 g had WFL <90% ($p = 0.68$).

The use of antibiotics and the presence of otitis media between hospitalization for bronchiolitis and the control visit were not associated with birth weight (Table 1) or with overweight at control visit (Table 2). Both occurrence and recurrence of post-bronchiolitis wheezing were associated with birth weight >4000 g vs. birth weight <4000 g (Table 1). The recurrence of post-bronchiolitis wheezing was associated with WFL >110% at control visit vs. WFL <110% (Table 2). No corresponding associations were seen with birth weight <3000 g or WFL <90% (Tables 1 and 2).

There was no significant association between weight gain and post-bronchiolitis wheezing (Table 3). When the analyses were carried out separately in the three birth weight groups, there was a significant association between weight gain before bronchiolitis and wheezing after bronchiolitis if the birth weight was >4000 g (Table 3). Poor weight gain

**Table 1** Post-bronchiolitis use of antibiotics, presence of otitis media, wheezing episodes and use of corticosteroids in relation to birth weight

| Outcome | Birth weight <3000g (N = 19) (%) | Birth weight 3000–4000g (N = 85) (%) | Birth weight >4000g (N = 23) (%) |
|---|---|---|---|
| Antibiotics | 15 (79) p = 0.39* | 71 (84) | 20 (87) p = 0.44** |
| Otitis media | 14 (74) p = 0.34* | 69 (81) | 18 (78) p = 0.53** |
| One or more subsequent wheezing episodes | 6 (32) p = 0.46* | 25 (29) | 13 (57) p = 0.015** |
| Two or more subsequent wheezing episodes | 2 (11) p = 0.25* | 13 (15) | 8 (35) p = 0.028** |
| Corticosteroid use | 2 (11) p = 0.20* | 17 (20) | 6 (26) p = 0.28** |

*Compared to 108 children with birth weight >3000 g.
**Compared to 104 children with birth weight <4000 g.

**Table 2** Post-bronchiolitis use of antibiotics, presence of otitis media, wheezing episodes and use of corticosteroids in relation to weight for length (WFL) at the age of 18 months

| Outcome | WFL <90% (N = 13) (%) | WFL 90–110% (N = 103) (%) | WFL >110% (N = 11) (%) |
|---|---|---|---|
| Antibiotics | 9 (69) p = 0.14* | 87 (84) | 10 (91) p = 0.43** |
| Otitis media | 8 (62) p = 0.14* | 83 (81) | 10 (91) p = 0.30** |
| One or more subsequent wheezing episodes | 4 (31) p = 0.51* | 34 (33) | 6 (55) p = 0.13** |
| Two or more subsequent wheezing episodes | 0 (0) p = 0.06* | 18 (17) | 5 (45) p = 0.028** |
| Corticosteroid use | 0 (0) p = 0.05* | 22 (21) | 3 (27) p = 0.34** |

*Compared to 114 children with weight for height >90%.
**Compared to 116 children with weight for height <110%.

**Table 3** Weight gain from birth to hospitalization in relation to post-bronchiolitis wheezing until 18 months of age

| | One or more post-bronchiolitis wheezing episodes | Two or more post-bronchiolitis wheezing episodes | No post-bronchiolitis wheezing episodes |
|---|---|---|---|
| All children (N = 127) | (N = 44) | (N = 23) | (N = 83) |
| Weight gain, mean in g/week (SD, 95% CI) | 202.2 g (64.9, 184.4–223.9) p = 0.48 | 206.4 g (53.0, 183.6–229.2) p = 0.54 | 192.4 g (92.1, 172.3–212.5) |
| Children with birth weight >4000 g (N = 23) | (N = 13) | (N = 8) | (N = 10) |
| Weight gain, mean in g/week (SD, 95% CI) | 194.9 g (55.5, 161.4–228.4) p = 0.042* | 175.8 g (39.3, 142.9–208.6) p = 0.20 | 139.2 g (67.8, 90.7–187.7) |

*Compared to 10 children with birth weight >4000 g without post-bronchiolitis wheezing.

(<70 g/week) was present in only 10 (8%) children; two had suffered from wheezing but none from recurrent wheezing.

Post-bronchiolitis wheezing did not differ significantly between boys and girls: for ≥1 episode, the figures were 28/61 (46%) in boys vs. 19/66 (29%) in girls (p = 0.07), and for ≥2 episodes 25% (boys) vs. 12% (girls) (p = 0.11), respectively. All analyses were repeated separately for the 88 infants hospitalized for bronchiolitis at <3 months of age. The association between the occurrence and recurrence of post-bronchiolitis wheezing and birth weight >4000 g lost statistical significance, but the associations between WFL >110% and recurrent wheezing remained as significant (data not shown).

As doctor-diagnosed allergy in children (11), birth weight >4000 g and WFL >110% at age 1.5 years were associated with an increased risk of post-bronchiolitis wheezing, the analyses were continued by logistic regression. Allergy and high birth weight >4000 g were significant risk factors for both occurrence and recurrence of wheezing, WFL >110% was a significant risk factor for only recurrence of wheezing, but gender had no predictive value (Table 4).

**Table 4** Logistic regression: association of birth weight >4000 g and weight for length (WFL) >110% with post-bronchiolitis wheezing

| Variables | Adjusted odds ratio | 95% confidence interval |
|---|---|---|
| One or more post-bronchiolitis wheezing episodes | | |
| Birth weight >4000 g | 3.11 | 1.19–8.143 |
| WFL >110% | 2.19 | 0.59–8.20 |
| Allergy* | 2.76 | 1.15–6.67 |
| Gender (males) | 1.66 | 0.76–5.10 |
| Two or more post-bronchiolitis wheezing episodes | | |
| Birth weight >4000 g | 3.21 | 1.06–9.78 |
| WFL >110% | 4.63 | 1.14–18.89 |
| Allergy* | 4.39 | 1.53–12.66 |
| Gender (males) | 1.87 | 0.20–1.46 |

*Parent-reported doctor-diagnosed atopic dermatitis or food allergy.

## DISCUSSION

There are three main results in the present secondary analysis of the 1-year follow-up data in children hospitalized for bronchiolitis at <6 months of age. First, high birth weight of >4000 g (>95th percentile in population) was associated with post-bronchiolitis wheezing, and second, overweight (WFL >110%) at 1.5 years of age was associated with recurrent post-bronchiolitis wheezing. And third, the rate of weight gain during the first months of life was associated with post-bronchiolitis wheezing if birth weight was >4000 g.

On average, birth weights of the study children were rather similar to those in non-selected Finnish newborns: in boys 3586 vs. 3582 g in the population and in girls 3475 vs. 3461 g in the population (National Institute for Health and Welfare, Finland). Surprisingly, there were more girls than boys, and as expected, more hospitalizations at <3 months than at >3 months of age. We have no explanation for this female preponderance. In a recent retrospective study in 738 Korean infants hospitalized for lower respiratory infection at 3–12 months of age (4), male gender, younger age and higher WFL were associated with wheezing.

Earlier register-based studies (15,16) and a recent birth cohort study (17) have evidenced that high birth weight (>4500 g) may be associated with an increased risk of


 

childhood asthma. On the other hand, some other studies have found an association between low birth weight and childhood asthma (7,8,18). In a register-based study including over 20 000 Finnish children, low gestational age and low ponderal index at birth were independently significant risk factors for doctor-diagnosed asthma at <3 years of age (8). In a recent birth cohort study in nearly 5000 Danish twins, the lower birth-weight twin had a 1.3-fold risk for asthma compared to the counterpart at 3–9 years of age (7). In an American birth cohort of 1372 children, male sex, smoking exposure and parental asthma were associated with asthma-related outcomes at age 2 years, but birth weight, intrauterine growth and length of gestation (those born before 34 gestational weeks excluded) were not (18). Thus, the association between birth weight or intrauterine growth and later asthma may be non-linear, and both low and high birth weights may be risk factors for wheezing and asthma.

The association between weight and early childhood wheezing is less studied than the association between weight and childhood asthma (19). Children with low birth weight and rapid post-natal weight gain have an increased risk of having an impaired lung function in infancy (20) and overweight and obesity in adolescence (6). In a birth cohort study including 3627 Dutch children born at term, lower birth weight was associated with wheezing at 2–6 years of age, but not with wheezing at 1 year of age (21). In a prospective cohort of 932 children from the US, infants with higher WFL at 6 months of age had a greater risk of recurrent wheezing by age 3 years, but not with wheezing at 1 or 2 years of age (5). In a recent birth cohort from the UK (9), greater length-related weight gain and adiposity gain assessed by subscapular skinfold thickness at 0–6 and 6–12 months were associated with both atopic and non-atopic wheezing by 3 years of age. In addition, greater foetal abdominal circumference at 11–19 weeks was associated with atopy and with wheezing in atopic children but was not associated with wheezing in non-atopic children (9). In the present study, birth weight >4000 g (95th percentile in the population) was associated with post-bronchiolitis wheezing at 0.5–1.5 years of age, but birth weight <3000 g (5th percentile in the population) was not. We excluded infants born at pre-term before 37 gestational weeks. The number of low birth weight (<2500 g) full-term infants reflecting decreased intrauterine growth was too small for any statistical analyses.

There is increasing evidence that overweight and obesity may increase asthma risk in children at school age (3,22). Excessive weight gain seems to be associated with asthma symptoms, treatments and diagnoses (22), and even with decreased lung function (23), but not with bronchial hyperresponsiveness (22). We found some evidence that an excessive weight gain assessed by WFL, in line with three other studies applying length-related weight gains for infants (4,5,9), increases the risk of wheezing in early years. The mechanisms are obscure, but genetic polymorphisms, hormonal reasons, mechanical effects on lung function, altered immune responses and increased susceptibility to gastric reflux have been speculated as potential links. An association between post-natal adiposity, weight gain and wheezing may even reflect prenatal influences (9).

From the age of 2 years onwards, overweight and obesity have usually been assessed by calculating body mass index (BMI) adjusted for age (24), but BMI is not necessarily the best method in infants. In addition, Finnish age- and sex-specific BMI references are not available for children <24 months of age (25). Like the three other thus far published studies on weight status and wheezing in infancy (4,5,9), we used WFL calculated on the basis of national gender-specific age-related growth references.

The results should be interpreted with caution, as the study evidently was underpowered to prove or disprove many potential associations. The study was a secondary analysis of the data collected prospectively for other purposes. On the other hand, the main results of the study, that is the association between high birth weight >4000 g with both occurrence and recurrence of post-bronchiolitis wheezing, and the association between overweight by WFL >110% with recurrence of post-bronchiolitis wheezing, were confirmed by multivariate analyses adjusted for gender and allergy. Doctor-diagnosed allergy in the child was, in the earlier study published from this material, the only factor among many which significantly predicted post-bronchiolitis wheezing (11).

In conclusion, we found some evidence that high birth weight may be associated with post-bronchiolitis wheezing at age 0.5–1.5 years and that overweight at age 1.5 years was associated with recurrent post-bronchiolitis wheezing by age 1.5 years.

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