PETRI KOPONEN

Genetic and Viral Factors Predicting Preschool Asthma After Early-Life Bronchiolitis

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ACADEMIC DISSERTATION
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UNIVERSITY OF TAMPERE

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CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis consists of the following papers, which are referred to in the text by their Roman number.

I. Koponen, P., Helminen, M., Paassilta, M., Luukkaala, T., & Korppi, M. (2012). Preschool asthma after bronchiolitis in infancy. European Respiratory Journal, 39(1), 76-80.

II. Koponen, P., Nuolivirta, K., Virta, M., Helminen, M., Hurme, M., & Korppi, M. (2014). Polymorphism of the rs1800896 IL10 promoter gene protects children from post-bronchiolitis asthma. Pediatric Pulmonology, 49(8), 800-806.

III. Koponen, P., He, Q., Helminen, M., Nuolivirta, K., & Korppi, M. (2012). Association of MBL2 polymorphism with asthma after bronchiolitis in infancy. Pediatrics International, 54(5), 619-622.

IV. Koponen, P., Vuononvirta, J., Nuolivirta, K., Helminen, M., He, Q., & Korppi, M. (2014). The association of genetic variants in toll-like receptor 2 subfamily with allergy and asthma after hospitalization for bronchiolitis in infancy. Pediatric Infectious Disease Journal, 33(5), 463-466.

ABBREVIATIONS

aOR	Adjusted odds ratio
BHR	Bronchial hyper-responsiveness
DNA	Deoxyribonucleic acid
ECT	Exercise challenge test
hMPV	Human metapneumovirus
HRV	Human rhinovirus
ICAM	Intercellular adhesion molecule
IF	Immunofluorescence
IFN-ϒ	Interferon-gamma, protein
IFN-γ	Interferon-gamma, gene
Ig	Immunoglobulin
IL	Interleukin
IOS	Impulse oscillometry
LRTI	Lower respiratory tract infection
MBL	Mannose-binding lectin
NPA	Nasopharyngeal aspirate
OR	Odds Ratio
PCR	Polymerase chain reaction
PAMP	Pathogen-associated molecular pattern
PRR	Pattern recognition receptor
mRNA	messenger ribonucleic acid
RSV	Respiratory syncytial virus
RRS	Respiratory resistance
SD	Standard deviation
SPT	Skin prick test
SNP	Single nucleotide polymorphism
Th	T helper cell
TLR	Toll-like receptor
Treg	Regulatory T-cell

ABSTRACT

BACKGROUND: Bronchiolitis is a common cause for hospitalisation among young children and young infants present with the most difficult form of the disease. Respiratory syncytial virus (RSV) is the common cause, but other viruses are also known to cause bronchiolitis. Respiratory viruses and host-dependent factors, for example mutations in genes regulating innate immunity, may influence the long-term outcome of bronchiolitis. However, it is not clear whether bronchiolitis causes the increased asthma risk, or whether bronchiolitis is merely a sign of early stage asthma inflammation.

AIMS: The aim of this prospective cohort study was to evaluate associations between microbial, environmental and genetic factors and thus, identify predictive factors for preschool asthma after infant bronchiolitis. The more specific questions to address were: (1) the long-term prognosis until 5-7 years of age in a paediatric cohort hospitalised due to bronchiolitis at less than 6 months of age; (2) The link between viral etiology of early life bronchiolitis and subsequent asthma in childhood; (3) The role of early-life risk factors, like asthma or atopy in parents, or parental smoking or atopic dermatitis in infancy, as potential predictive factors for increased asthma or allergy prevalence; (4) the *IL10*, *IL18* and *IFN-γ* genes as potential candidate genes for increased asthma susceptibility in childhood; and (5) the connection between the *MBL2* and *TLR2* subfamily gene polymorphisms and childhood asthma prevalence after early life bronchiolitis.

SUBJECTS AND METHODS: The study was conducted at the Department of Pediatrics, Tampere University Hospital. Originally, 187 children who were less than six-months-of-age at hospital admission due to bronchiolitis, were enrolled during three different infection seasons between 2001 and 2004. Follow-up visits were arranged at preschool age between 2008 and 2010 and clinical data from 166 children were available for further analyses. Data on viral etiology of bronchiolitis were studied on admission by polymerase chain reaction (PCR). Background information regarding asthma and allergies was collected by comprehensive questionnaires which parents were asked to complete before the follow-up visit. During the follow-up visit, a doctor examined all participants and any allergic or asthmatic status findings were registered. Impulse oscillometry was used to diagnose bronchial hyper-responsiveness and skin-prick tests were conducted to diagnose atopy. For genetic analyses, polymorphisms of the selected genes were studied by PCR from DNA extracted from whole blood samples.

RESULTS: Asthma at preschool age was present in 21 (12.7%) children. In RSV infected children asthma was diagnosed in 8.2%, whereas in non-RSV infected children, this was present in 24% (p=0.01). Thirty-three percent of children with asthmatic mothers had asthma at the control visit (vs. 9.2% in those without maternal asthma; p=0.001). The prevalence of preschool asthma among children with atopic dermatitis in infancy was 30% (vs. 5% in those without infantile atopic dermatitis; p=0.01). In genetic analyses, the *IL10* polymorphism -1082 G/A was associated with asthma; only 1/32 (3.1%) of children with the A/A genotype had asthma during preschool-aged follow-up. The variant genotype of *MBL2* was associated with higher asthma risk, as 19.6% of non-A/A children had asthma vs. 7.4% of those with the A/A genotype (p=0.03). In *TLR2* subfamily polymorphisms, children who were homozygous for the major allele G of *TLR1* rs5743618 had a significantly smaller risk of asthma after bronchiolitis, since the asthma risk during childhood was 1.5-fold among non-G/G children (24% vs. 38%, p=0.04). In haplotype analyses, children with major alleles in all of the investigated *TLR2* subfamily single nucleotide polymorphisms (SNPs) were

less often asthmatics during first six years of life when compared with children who had variant alleles in *TLR2* subfamily SNPs (7.7% vs. 30%, p=0.02).

CONCLUSIONS: In our cohort, 12.7% of children hospitalised for bronchiolitis in the early months of life had asthma at preschool age. Furthermore, the risk of later asthma development seems to be dependent on the causative virus of bronchiolitis as children with non-RSV as the primary infection had a nearly 3-fold asthma risk when compared with those infected by RSV. Other statistically significant predictors for preschool asthma were atopic dermatitis in infancy and a history of asthma in mothers. Furthermore, polymorphisms in genes regulating innate immunity, and host defense in general, seem to play important role in asthma pathogenesis. Current work presents several preliminary associations between polymorphisms in genes coding key cytokines and pattern recognition receptors (PRRs) and asthma development after infantile bronchiolitis.

TIIVISTELMÄ

TAUSTAA: Bronkioliitti on yleinen sairaalahoidon syy imeväisikäisillä, ja pienillä imeväisillä on myös usein vaikein taudin kuva. Respiratory syncytial virus (RSV) on yleisin bronkioliitin aiheuttaja, mutta on olemassa myös useita muita bronkioliitin aiheuttajaviruksia. Hengitystietulehduksen aiheuttavat virukset sekä isännästä riippuvat tekijät, kuten mutaatiot synnynnäiseen immuniteettiin vaikuttavissa geeneissä, voivat vaikuttaa bronkioliitin pitkäaikaisennusteeseen ja astmariskiin. Ei ole kuitenkaan selvää, onko lisääntynyt astmariski bronkioliitin aiheuttamaa vai onko bronkioliitti pikemminkin merkki jo olemassa olevasta varhaisesta astmatulehduksesta.

TUTKIMUKSEN TARKOITUS: Tämän prospektiivisen kohorttitutkimuksen tarkoitus on tarkastella mikrobien, ympäristötekijöiden ja geneettisten tekijöiden yhteisvaikutuksia ja siten tunnistaa riskitekijät esikouluiän astman kehittymiselle varhaisvaiheen bronkioliitin jälkeen. Tarkemmat päätavoitteet tutkimukselle ovat: (1) selvittää pitkäaikaisennuste 5-7 vuoden ikäisiltä lapsilta, jotka joutuivat sairaalahoitoon bronkioliitin johdosta alle puolen vuoden iässä; (2) määrittää bronkioliitin aiheuttajaviruksen ja myöhemmän lapsuusiän astman puhkeamisen yhteyttä; (3) analysoida varhaislapsuuden riskitekijöiden, kuten vanhempien astman tai atopian, tai vanhempien tupakoinnin ja imeväisiän atopian yhteyttä esikouluiän astmaan tai allergiaan; (4) tutkia IL-10, IL-18 ja IFN-γ geenien yhteys esikouluiän astmariskiin; (5) selvittää MBL2- ja TLR1-, TLR2- ja TLR6-geenien monimuotoisuuden ja varhaisvaiheen bronkioliitiin yhteyttä lapsuusiän astmaan.

AINEISTO JA MENETELMÄT: Tutkimus tehtiin Tampereen yliopistossa, lastentautien yksikössä. Tutkimukseen otettiin 187 alle puolivuotiasta lasta, jotka olivat sairaalahoidossa Tampereen yliopistollisessa sairaalassa bronkioliitin johdosta kolmen eri infektiokauden aikana vuosina 2001-2004. Kontrollikäynnit järjestettiin vuosina 2008-2010 ja tutkimustiedot saatiin 166 lapsesta. Bronkioliitin aiheuttajavirus selvitettiin polymeraasiketjureaktiolla (PCR). Ennen kontrollikäyntiä vanhempien täyttämällä haastattelulomakkeella selvitettiin astman ja allergioiden esiintyvyys lapsuudessa. Kontrollikäynnin yhteydessä lääkäri suoritti kliinisen tutkimuksen ja kaikki allergiaan tai astmaan viittaavat löydökset rekisteröitiin. Impulssioskillometrilla selvitettiin keuhkojen hyperreaktiviteettia, ja lisäksi Prick-testillä tutkittiin lapsilla esiintynyttä atopiaa. Genetiikan analyyseissä valittujen geenien monimuotoisuutta tutkittiin PCR:llä kokoverinäytteistä valmistetuilla DNA-näytteillä.

TULOKSET: Esikouluiän astma todettiin 21 (12,7 %) lapsella. RSV:n sairastaneilla lapsilla astma todettiin 8,2 %:lla, kun taas ei-RSV:n sairastaneilla lapsilla astma todettiin 24 %:lla (p=0,01). 33 % lapsilla, joiden äidillä oli astmadiagnoosi, todettiin astma kontrollikäynnillä (verrattuna 9,2 % lapsilla, joiden äidillä ei ollut astmaa, p=0,001). Lapsilla, joilla oli todettu imeväisiän atooppinen ihottuma, esikouluiän astmaa esiintyi 30 %:lla (verrattuna 5 % lapsilla, joilla ei esiintynyt imeväisiän atooppista ihottumaa, p=0,01). Geneettisissä analyyseissä IL10 -1082 G/A-geenin monimuotoisuus oli yhteydessä astmaan; vain 1/32 (3,1 %) lapsista, joilla oli A/A-genotyyppi, todettiin astma esikouluiässä. Lisäksi MBL2-geenin monimuotoisuus liittyi astmariskiin, sillä 19,6 % joko A/O- tai O/O-genotyypin lapsista oli astmaatikkoja, verrattuna 7,4 %:iin A/A genotyypin tutkituista (p=0,03). Lapset, jotka olivat homotsygootteja TLR1-alleeli G:n suhteen olivat pienemmässä astmariskissä ensimmäisen kuuden ikävuoden aikana (24 %) muihin TLR1-genotyypin (38 %) lapsiin verrattuna (p=0,04). Haplotyyppianalyyseissä havaittiin, että lapset joilla oli TLR1-G/G, TLR2-G/G ja TLR6-C/C-genotyyppi, olivat harvoin astmaatikkoja (7,7 % astmaesiintyvyys

ensimmäisen kuuden vuoden aikana verrattuna 30 % esiintyvyyteen muilla TLR1-, TLR2- ja TLR6-genotyypeillä, p=0,02).

JOHTOPÄÄTÖKSET: Tuloksemme osoittavat, että 12,7 %:lla varhaisvaiheen bronkioliitin sairastaneista lapsista oli astma esikouluiässä. Erityisesti astmariskiin vaikutti bronkioliitin aiheuttanut virus, sillä tutkimuksessa ne lapset, joiden varhaisvaiheen bronkioliitin aiheuttaja oli jokin muu virus kuin RSV, olivat miltei kolminkertaisessa astmariskissä RSV-lapsiin verrattuna. Muina esikouluiän astmariskiin vaikuttavina tekijöinä ilmeni imeväisiän atooppinen ihottuma ja äidin astmadiagnoosi. Myös synnynnäisen immuniteetin säätelyyn osallistuvien geenien monimuotoisuus on yhteydessä astman puhkeamiseen. Tutkimuksessa löytyi myös useita alustavia yhteyksiä sytokiineja ja hahmontunnistus reseptoreita (PRR) koodaavien geenien monimuotoisuuden ja astmariskin välillä.

1. INTRODUCTION

Bronchiolitis is a viral lower respiratory tract infection and it is the most common cause of hospitalisation in infants under the age of six months (AAP. 2006, Panitch. 2007). Also, the severity of the infection is correlated with age, as bronchiolitis occurring in early infancy is usually more severe than infection during the second or third year of life (Damore et al. 2008; Singh et al. 2007). However, more than a third of all children will have expiratory breathing difficulties, i.e. wheezing, by the age of three years (Taussig et al. 2003). Both individual and environmental factors have been linked with the susceptibility for bronchiolitis (Houben et al. 2011, Simoes. 1999). In addition, the host immunity and especially the innate immunity responses play important role in defence against pathogens encountered in early life (Godfrey. 1996).

Many earlier studies have reported an increased risk of asthma after early-life bronchiolitis, having found a prevalence of asthma of up to 50% preschool age after hospitalisation for bronchiolitis (Castro et al. 2008, Kotaniemi-Syrjänen et al. 2002; Kuikka et al. 1994, Sigurs et al. 2000, Wennergren et al. 2004). However, the exact mechanism between bronchiolitis and asthma susceptibility has not yet been determined. There is preliminary evidence that children who are infected with severe bronchiolitis in infancy differ from healthy children regarding their immune responses before the bronchiolitis infection (Juntti et al. 2009). This suggests that a certain predisposition to bronchiolitis and possibly to asthma already exists already at birth, at least in some subgroup of bronchiolitis patients. Further, it has also been reported that early-life infection can cause alterations in the immune system, which may lead to increased asthma susceptibility (Copenhaver et al. 2004). Based on these findings, it may be that bronchiolitis is an early marker of already existing asthma susceptibility, and that bronchiolitis infection acts as a trigger that initiates asthma development. Further, many different individual and environmental predictive factors for asthma

susceptibility after bronchiolitis have been revealed (Goksör et al. 2006, Hyvärinen et al. 2005, Piippo-Savolainen et al. 2008, Sigurs et al. 2005). The strongest predictive factors for increased asthma risk after the bronchiolitis are a parental history of asthma (Hyvärinen et al. 2005, Piippo-Savolainen et al. 2006, Sigurs et al. 2005), early atopy (Hyvärinen et al. 2005; Piippo-Savolainen et al. 2007) and parental smoking (Goksör et al. 2006).

Today, with the help of improved technology, it is possible to detect a specific virus in almost 100% of bronchiolitis cases. RSV and human rhinovirus (HRV) are the most common causative agents for bronchiolitis (Greenough et al., 2001; Jartti et al. 2005, Korppi et al. 2004); RSV has been detected in up to 70% and HRV in up to 40% of bronchiolitis patients (Jartti et al. 2004). The prognosis for later asthma prevalence is different between RSV and non-RSV patients. Non-RSV infections, and HRV infections in particular, are associated with a higher risk of asthma than RSV infections (Jackson et al. 2008, Kotaniemi-Syrjänen et al. 2003). The clinical characteristics between RSV and non-RSV patients have varied, which may partly explain the difference in asthma risk (Korppi et al. 2004). However, post-bronchiolitis follow-up studies at preschool age, which include HRV as an etiological factor of bronchiolitis, are limited in number (Jackson et al. 2008, Kusel et al. 2007, Lemanske et al. 2005, Reijonen et al. 2000).

The immune system is immature and still developing during the first two years of life; therefore, early-life viral infection may cause permanent changes in immune responses leading to an increased risk of asthma (Jartti et al. 2005). Furthermore, early-life viral infection has resulted in a dominance of T helper cell 2 (Th2)-type immunoresponses (Pala et al. 2002, Strachan 1989, von Mutius 2007), which are typically associated with asthma development. Also, insufficient defence mechanisms against pathogens in early-life have resulted in a higher asthma prevalence (Hoffjan et al. 2005, Kormann et al. 2008, Tantisira et al. 2004). Also, alterations in genes regulating innate immunity, the main line of defence in first months of life, have also been associated with increased risk of chronic diseases, like asthma (Akdis et al. 2011, Jartti et al. 2005, Tesse et al.

2011). Overall, there is a lot of evidence that interaction between early-life immunity and certain environmental factors, like viruses, play a pivotal role in future development of asthma.

The main purpose of the present study was to identify the predictive factors for preschool asthma after infant bronchiolitis. The focus was on viral etiology of early-life infection, as well as on hereditary and environmental factors associated with asthma susceptibility in previous studies. Further, and of equal importance, the associations between genes regulating innate immunity and asthma susceptibility were analysed.

2. REVIEW OF THE LITERATURE

2.1 Bronchiolitis in early infancy

2.1.1 Definition of bronchiolitis

Bronchiolitis is a lower respiratory tract infection in infants caused by viral pathogens. The pathogenesis of bronchiolitis includes acute inflammation, edema and necrosis of epithelial cells in small airways, increased mucus production and bronchospasm. Typical clinical symptoms and signs associated with bronchiolitis are tachypnoea, rhinitis, cough, wheezing, crackles and the use of accessory muscles and/or nasal flaring (AAP. 2006). The diagnosis of bronchiolitis is mostly clinical and in current UK and US guidelines the upper age limit of bronchiolitis is 2 years of age (AAP.2006, SIGN. 2006). However, in Europe the upper age limit of bronchiolitis is commonly 12 months (Smyth & Openshaw. 2006) and only children with their first episode of wheezing should be diagnosed as bronchiolitis patients (Jartti et al. 2009). In children aged >12 months, repeated wheezing episodes associated with respiratory infections should be considered as own entity and be separated from bronchiolitis patients (AAP. 2006; Jartti et al. 2005). These wheezing episodes are often labeled as virus-associated wheezy bronchitis and are not always easily separable from bronchiolitis (see Chapter 2.3.2).

The most severe disease is usually the primary infection and, accordingly, the most difficult symptoms are commonly among the very youngest patients (Damore et al. 2008, Singh et al. 2007). Also, high birth weight (Houben et al. 2011) as well as environmental factors, such as tobacco smoke and crowded living conditions have been associated with more severe bronchiolitis (Simoes & Carbonell-Estrany. 2003).

Nevertheless, the susceptibility to severe bronchiolitis is dependent on multiple factors, e.g. genetics, environment and the aetiology of infection (Godfrey. 1996). Previous post-bronchiolitis studies with preschool age data available have included children <36 months (Jackson et al. 2008, Jartti et al. 2009, Martinez et al. 1995), <24 months (Kuikka et al. 1994, Wennergren et al. 1992), or < 12 months (Sigurs et al. 2000, Sznajder et al. 2005) of age on admission. No previous follow-up studies enrolling only children aged 6 months or less have been conducted.

2.1.2 Prevalence of early-life bronchiolitis

The prevalence of bronchiolitis is approximately 18-32% in the first year of life and 9-17% in the second (Jartti & Korppi. 2011). In addition, reinfections are common, as infection with RSV, the most common virus causing bronchiolitis, does not grant permanent or long-term immunity (AAP. 2006). The hospitalisation rate due to bronchiolitis is 1-3 % in previously healthy infants (Shay et al. 1999). Furthermore, 10-20% of children suffer from repeated lower respiratory tract infections during the first years of life (Taussig et al. 2003). Despite of several studies, it is not clear why some children are more vulnerable to severe disease, whereas others have only mild flu-like symptoms.

2.1.3 Viral aetiology of early life bronchiolitis

With the latest technologies available, today it is possible to reach a detection rate of 100% for bronchiolitis virus infection (Jartti et al. 2005). The most common etiology for bronchiolitis is RSV, as 90% of children are infected with RSV during their first two years of life (Greenough et al. 2001). In recent decades, the prevalence of HRV, and non-RSV infections in general, have largely been underestimated. It was commonly believed that HRV causes mostly upper respiratory tract infections and is not an important causative agent for bronchiolitis. However, due to advances in viral

diagnostic methods, mostly PCR, the important role of HRV as cause for a lower respiratory tract has been recognised (Korppi et al. 2004). The causative virus of bronchiolitis varies by age, as RSV is the most common virus infecting children under 6 months of age and HRV is predominant among 12 month-old children and older (Jartti et al. 2009). Other bronchiolitis-causing viruses are human metapneumovirus (hMPV), human bocavirus (HBoV), adenovirus, influenza virus and parainfluenza viruses, especially type 3. However, due to the smaller number of patients infected by these viruses in previous studies, the correlation between hMPV, HBoV, influenza and parainfluenza virus and clinical characteristics of bronchiolitis patients is not clearly defined. In a recent multicentre bronchiolitis cohort study, with a total of 408 less than 2-years-of-age participants, RSV was the most common finding (43%). The second most common causative virus was HRV (32%), then hMPV (7%), parainfluenzavirus (7%), coronavirus (6%), adenovirus (3%), enterovirus (3%) and influenza A(2%) (Jartti et al., 2014).

2.1.4 RSV and non-RSV viruses causing bronchiolitis

RSV is single-strand RNA virus of the *Paramyxoviridae* family. It consists of a single serotype and two antigenic subtypes, A or B. RSV epidemics follow a seasonal pattern. Typically, most RSV infections occur between winter and early spring. HRV is a single-stranded RNA virus of the *Picornaviridae* family. Varying by age, the prevalence of HRV bronchiolitis in hospital setting is 20-40%, making rhinovirus infection second most common lower respiratory tract infection (LRTI) among children (Jartti et al. 2009, Kotaniemi-Syrjänen et al. 2003, Midulla et al. 2012) There are three genetically distinct HRV groups, A, B and C, with over 100 different serotypes in total. HRV provoke serotype-specific antibody responses, which means that repeated infections to HRV are common (Jartti & Korppi. 2011). There is accumulating evidence from recent years that HRV is a lower respiratory tract pathogen as well (Jacobs et al. 2013). Unlike RSV, HRV can only be detected by PCR as it is difficult to culture and serology is not feasible (Jartti & Korppi. 2011). In addition to the age difference among infected

children, HRV infected infants more often have characteristics of atopic predisposition and eosinophilic reaction pattern (Korppi et al. 2004) and different secreted cytokine profiles (Gern. 2010, Kusel et al. 2007) than RSV patients.

2.2 Immunology and genetics of early-life bronchiolitis

2.2.1 Innate immunity and the pattern recognition receptors

During the first months of life, the innate immune system is mainly responsible for defence against viral pathogens. Innate immunity consists of both soluble and cellular pattern recognition receptors (PRR) for microbial products (Rämet et al. 2011). The recognition of pathogen-associated molecular patterns (PAMPs) led to the activation of signaling pathways, which was followed by the increased expression of cytokines, chemokines and other anti-inflammatory molecules (Hallman et al. 2001, Ramet et al. 2011). The host-defence can be compromised if there is a deficiency of any of these inflammatory agents against foreign microbes.

Toll-like receptors (TLR) are evolutionary conserved receptor complexes that are able to recognise molecular patterns from micro-organisms but not in the host (Ramet et al. 2011, Takeda et al. 2003). TLRs are pivotal in activating and controlling immune signaling and monitoring adaptive immune responses (Akira et al. 2006). The TLR2 subfamily is comprised of TLR1, TLR2, TLR6 and TLR10. TLR1, TLR2 and TLR6 are regarded as co-receptors, but TLR10 has a separate signaling pathway (Guan et al. 2010, Tapping et al. 2007). TLRs are expressed in immune cells such as antigen-presenting cells, and in epithelial cells. Genetic variations in the components of innate immunity, e.g. TLRs, may decrease the ability to defend against pathogens (Takeda et al. 2003).

Mannose-binding lectin (MBL) is an endocytic pattern-recognition receptor that recognises pathogen-associated molecular patterns. The identification process is followed by phagocytosis of pathogens by antigen-presenting macrophages and dendritic cells, and finally, the lectin complement pathway is activated (Medzhitov & Janeway. 2000). Polymorphisms in MBL2 coding genes have led to the deficient secretion of MBL protein. One third of the population have reduced levels of MBL secretion and, further, very low levels of MBL secretion have been measured in about 10% of study participants in clinical trials (Turner. 1996)

2.2.2 Innate immunity cytokines IL-10, IFN-γ and IL-18

Cytokines responsible for controlling the innate immunity responses are pivotal in early infancy. Interleukin-10 (IL-10) is a key regulator in the inflammatory and anti-inflammatory processes and thus controls the development of immune tolerance in early childhood (Akdis et al. 2011). Due to the ability of IL-10 to control both humoral and cellular immunity, the *IL10* gene has been selected as a candidate gene in the regulation of anti-inflammatory diseases, like asthma (Akdis et al. 2011). Interferon–gamma (IFN-γ) is a pro-inflammatory cytokine and, thus is associated with Th1- type response when defending against viral pathogens. IL-18 similarly enhances innate immunity inflammatory processes and has an important immunoregulatory role in allergic and autoimmune diseases. It is also involved in controlling immunoglobulin E (IgE) synthesis; a decline in IL-18 concentrations may lead to disturbances in the Th1/Th2 balance (Cebeci et al. 2006).

2.2.3 Genetics of bronchiolitis

The great diversity of the clinical presentation of infantile bronchiolitis largely results from genetic variety of the host. Single nucleotide polymorphisms (SNPs) are sequence

variations in DNA, which occur when a single nucleotide in the genome differs between members of a biological species or paired chromosomes in an individual. SNPs are highly abundant and are estimated to occur at an average rate of 1 per 1000 bases in the common genome (Sachidanandam et al. 2001). In many chronic conditions, e.g. asthma, researchers are trying to identify the key SNPs which correlate with disease susceptibility or severity. Previously, numerous genes conferring susceptibility to asthma (Vercelli. 2008) and also to bronchiolitis occurrence and severity have been found (Gentile et al. 2003, Helminen et al. 2008, Janssen et al. 2007). In addition, viral aetiology of bronchiolitis has also been linked with with *IL10* (Helminen et al. 2008) and *MBL2* (Nuolivirta et al. 2012) SNPs. As adaptive immunity has not yet been fully developed, innate immune responses are pivotal against pathogens encountered in early life. Hence, genes regulating the adaptive immunity are of particular interest in post-bronchiolitis follow-up studies.

2.3 Preschool asthma after early life bronchiolitis

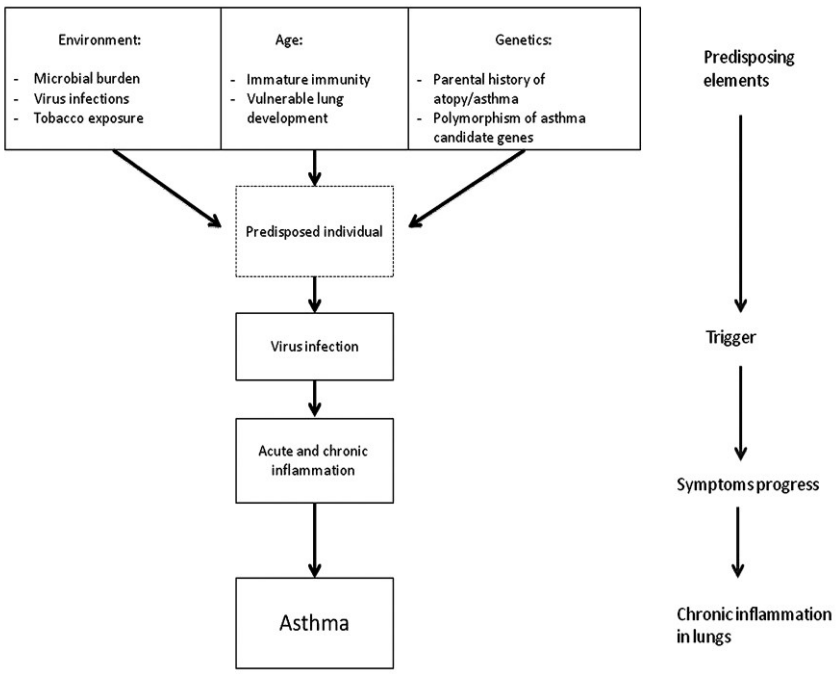
Bronchiolitis in early infancy increases the susceptibility to asthma in later childhood (Piippo-Savolainen et al. 2008, Taussig et al. 2003, Wennergren et al. 2004). Despite the many years of active research, the exact link between asthma and bronchiolitis has not yet been identified. It has been debated whether primary lower respiratory tract infection is the direct cause of post-bronchiolitis lung function decline, or if infected children are more susceptible to lower respiratory infections due to their pre-existing atopic characteristics or inflammation in their airways. Further, there is evidence from previous studies that at least a certain subgroup of children have a pre-existing decline in lung function prior to any LRTI (Taussig et al. 2003).

2.3.1 The link between bronchiolitis and asthma

According to the hygiene hypothesis theory, exposure to house dust or farm animals in early life leads to dominance in Th1-type immunity and, thus, decreases Th2 immunity and lowers the secretion of IgE (Lynch et al. 2014, von Mutius. 2007). In the pathogenesis of asthma, immunity is balanced towards Th2-type responses, which leads to Th2-cell activation and Th2-type cytokine release, as well as to allergy-related immunoglobulin secretion by B cells (Kormann et al. 2008). Chronic inflammation in the airways and chronic respiratory symptoms develop on the basis of this process. In accordance, the increased asthma susceptibility after bronchiolitis in infancy derives from the effect of early-life infection on child´s immunoregulatory mechanisms and a disturbance of Th1/Th2-type cytokine secretion. In the case of an early-life respiratory infection, such as bronchiolitis, the index infection could serve as a "first hit" agent that causes immunity to balance more towards Th2-type skewed responses. Evidence for this theory comes from animal models, where murine parainfluenza LRTI caused

the up-regulation of Th2 inflammatory cells and inflammatory mediators (Cheung et al. 2010). In accordance, children infected by RSV in early life more often had IL-4 secretion responding to RSV and cat antigens suggesting Th2 polarisation after early-life infection (Pala et al. 2002). However, it seems likely that certain environmental, genetic or age-related predisposition is required in order for chronic respiratory problems to develop after being infected by bronchiolitis (Figure 1).

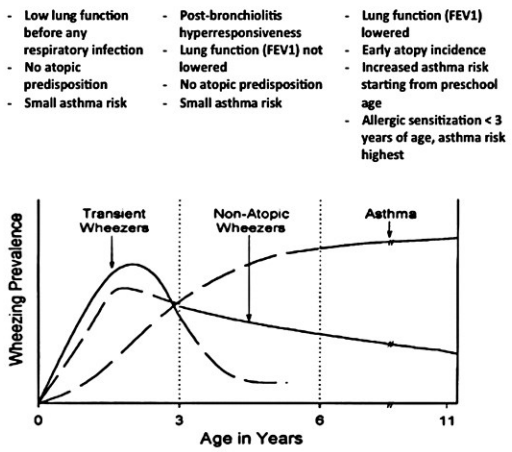
Figure 1. Multifactorial influences on the development of asthma after early-life bronchiolitis. Modified from Openshaw et al. (2004) and Jartti et al. (2005).



2.3.2 Different wheezing phenotypes in childhood

Different wheezing phenotypes among children have been identified in earlier clinical studies (Figure 2). Sixty percent of early-life wheezers have respiratory symptoms only during respiratory tract infections and they gradually become symptom-free after the age of three years. An interesting finding has been that many of these individuals have subnormal lung functions before any respiratory infection (Stein et al. 1999). In addition, 40% of early wheezers have continuous respiratory symptoms from the age of three until six years of age. Half of them become sensitised to an allergen and are therefore called sensitised wheezers. Out of the three subgroups of early life wheezers, the sensitised wheezers are at the highest risk of developing chronic lung disease, like asthma. Their subnormal lung function only develops after the first LRTI (Taussig et al. 2003).

Figure 2: Different wheezing phenotypes and asthma predicting factors between different wheezing subgroups. Modified from Taussig et al. (2003).



2.3.3 Prospective follow-up studies at preschool age after hospitalisation for bronchiolitis

Earlier post-bronchiolitis follow-up studies have reported prevalence figures of up to 50% for preschool asthma (Castro et al. 2008, Kotaniemi-Syrjänen et al. 2002, Kuikka et al. 1994, Sigurs et al. 2000, Wennergren et al. 2004). Also, the severity of primary infection, as well as the criteria for asthma diagnosis, has varied, which also explains differences in the study outcomes. Furthermore, the positive correlation between the severity of early bronchiolitis and later asthma risk has been reported (Carroll et al. 2009), which may also explain the variations in study results. In post-bronchiolitis follow-up studies from Sweden and Finland, the preschool asthma prevalence rate was 30% when participants were under 12 months at admission, and up to 50% when under 24 months (Kotaniemi-Syrjänen et al. 2002, Kuikka et al. 1994, Sigurs et al. 2000, Wennergren et al. 1992). Previous post-bronchiolitis studies are listed in Table 1.

Table 1. Follow-up studies evaluating preschool age asthma after hospitalisation for bronchiolitis.

Author	Study participants	Age on admission (Md, months)	Age at the follow-up (Md, years)	Virus etiology / main virus	Asthma prevalence	Asthma criteria *	Virus predictive for asthma	Study type	Control group
Wennergren (1992)	101	10	5.2	50%/ RSV 65%	15%	≥ 3 doses of reliever medication/week	No **	Prospective	No
Reijonen (2000)	81	10.6	4.0	70%/ RSV 67%	51%	≥ 2 doctor-diagnosed wheezing episodes	HRV (OR 4.14 95% Cl 1.02-16-77)	Prospective	Yes
Sigurs (2000)	47	3.9	7.5	100%/ RSV †	23%	≥ 3 doctor-diagnosed wheezing episodes	RSV (OR 10.8 95% Cl 2.51-47.11)	Prospective	Yes
Henderson (2005)	41	<12	7.5	100%/ RSV †	38%	Parent-reported asthma	RSV (aOR 2.5 95% Cl 1.4-4.3)	Retrospective	Yes

* Asthma was registered if the patient was on continuous asthma maintenance medication ** HRV not included in viral aetiology analyses

† RSV infection was inclusion criterion in the study group

2.4 The predictive factors for preschool asthma after early-life bronchiolitis

2.4.1 Viral aetiology of early life bronchiolitis and subsequent preschool asthma risk

The association between asthma susceptibility and RSV bronchiolitis has been known since the 1980s. Furthermore, as novel PCR methodologies have been developed, the link between asthma and non-RSV viruses, mainly rhinovirus, has also been reported (Hyvärinen et al. 2005, Kotaniemi-Syrjänen et al. 2003). As the viral aetiology of bronchiolitis is dependent on the age at admission (Jartti et al. 2009), comparisons between cohort studies should include only those children who were admitted to hospital at the same age.

2.4.2 RSV infection in infancy and subsequent asthma risk

Children wheezing during RSV infection in early life have lowered lung functions and airway hyper-responsiveness long after the primary infection, sometimes lasting until early adulthood (Sigurs et al. 2010, Strope et al. 1991) In the longest prospective follow-up study from Sweden with participants being under 12 months when hospitalised, the asthma prevalence after early life RSV was up to 8-fold higher compared with healthy controls (Sigurs et al. 2000). In earlier prospective studies from Finland and Sweden, the childhood asthma rate after RSV bronchiolitis (Reijonen et al. 2000, Wennergren et al. 1997) was 15-29%. In a recent prospective follow-up study from the US, among 206 children diagnosed with severe RSV during first 12 months, approximately one third were physician-diagnosed asthmatics at preschool age (Bacharier et al. 2012). Finally, in birth cohort studies with less severe primary infection,

the preschool asthma risk after RSV bronchiolitis was up to 4-fold when compared with population controls without early-life LRTI (Stein et al. 1999).

2.4.3 Rhinovirus and non-RSV bronchiolitis and subsequent asthma risk

HRV infections are common causes of upper respiratory tract infections; however, in the last few decades, it has become evident that rhinovirus infections are also important causes of LRTIs (Kellner et al. 1989). Although it is not possible to clinically differentiate HRV from RSV infection (Korppi et al. 2004), improved methods to detect non-RSV lower respiratory pathogens have made it possible to evaluate their association with asthma as well. When compared with other respiratory viruses, children infected with rhinovirus were older and more often had signs of atopic eczema and increased blood eosinophilia (Korppi et al. 2004). In addition, those children who were hospitalised due to HRV, more often had maternal asthma as a clinical characteristic when compared to RSV-infected children (Carroll et al. 2012). Based on these findings, it has been suggested that HRV infected children are more likely to have atopic predisposition which leads to asthma development during early infections (Jartti & Korppi. 2011).

Of all of the children who were hospitalised due to HRV bronchiolitis, 52% were doctor-diagnosed preschool asthmatics in a Finnish study. The risk of asthma was up to four-fold when compared with other respiratory viruses (Kotaniemi-Syrjanen et al. 2003). In a recent Norwegian study (Mikalsen et al. 2012) children hospitalised due to non-RSV bronchiolitis more often demonstrated an obstructive lung function pattern and the asthma rate in the teenage years was significantly more common among non-RSV vs. RSV-infected study participants. In a high asthma risk cohort, all participants of which had a family predisposition to asthma, children with a HRV infection in the first 36 months-of-life had a 9.8-fold preschool asthma risk compared with a 2.6-fold risk among RSV-infected children (Jackson et al. 2008). Finally, in an Italian 12-month

follow-up study of 313 <12-month old participants, HRV bronchiolitis had a strong correlation with repeated wheezing episodes and the association was even stronger than the traditional risk factors, like, for example, a family history of asthma. Also, eosinophilia was strongly associated with asthma risk (Midulla et al. 2012).

2.4.4 Other risk factors for preschool asthma after bronchiolitis in infancy

Atopic dermatitis, family history of atopy, asthma in mothers and passive smoking have been linked with an increased risk of later asthma in earlier post-bronchiolitis studies (Goksör et al. 2007, Hyvärinen et al. 2005; Kotaniemi-Syrjänen et al. 2002, Sigurs et al. 2000). In addition, maternal smoking has been associated with increased bronchial hyper-responsiveness (BHR) and reduced lung function, whereas paternal smoking increased the active smoking at teen age (Goksör et al. 2007). It has also been postulated that early viral infection, especially HRV, and atopic inflammation may enhance synergistically the risk of later asthma (Kusel et al. 2007). However, there have been some negative results concerning family atopy and later asthma or repeated wheeze risk after early life bronchiolitis as well (Jackson et al. 2008; Lemanske et al. 2005). Conflicting results can be explained by differences in study participants; for example, severity of index infection, and age on admission has varied greatly between different studies. Also, not all post-bronchiolitis studies have included control group, and when included, clinical characteristics of controls may have been different between the studies.

2.5 Genetics of preschool asthma after early life bronchiolitis

2.5.1 Genetic factors associated with preschool asthma and atopy after early-life bronchiolitis

The understanding of asthma genetics has increased greatly in the last two decades. It has become more evident, that there are genes that act either alone or combined with other genes to influence asthma risk (Xu et al. 2001). However, the simultaneous effects of genetic heterogeneity and heterogeneous phenotypes of asthma, as well as genotype- environment and gene-gene interactions, can cause confounding in results (Heaton et al. 2005). The genetic predisposition to either strong pro-inflammatory or weak anti-inflammatory responses have been linked to susceptibility to an atopic disease (Jartti et al. 2005). Furthermore, early-life contact with microbes can be protective from allergy, but severe early-life infection can also lead to prolonged Th2-type responses (Strachan. 1989, von Mutius. 2007). Thus, lowered or increased immune responses against foreign pathogens in infancy have been associated with asthma and allergies (Hoffjan et al. 2005, Kormann et al. 2008, Tantisira et al. 2004). Accordingly, increasing evidence has been reported that alterations in innate immunity regulating genes, not adaptive immunity, have been associated with chronic diseases, such as allergies and asthma (Tesse et al., 2011).

2.5.2 Polymorphism of *IL10* and preschool asthma risk after bronchiolitis

Polymorphisms of the *IL10* gene has been linked to asthma and atopy in previous studies (Chatterjee et al. 2005; Hussein et al. 2011, Raedler et al. 2013). However, association between *IL10* polymorphism and post-bronchiolitis asthma has not been previously studied. *IL10* SNPs have shown association with IL-10 production (Turner et al. 1997); further, lower levels of secreted IL-10 have increased the risk of later wheezing (Hoffmann et al. 2002; Lim et al. 1998). In accordance, in two recent meta-analyses with 4478 and 4716 study participants from Europe and Asia, the association between *IL10* polymorphism and asthma risk was well demonstrated (Nie et al. 2012; Zheng et al. 2014). Also in a pediatric setting, *IL10* SNPs have resulted in a higher asthma and atopy risk, as well as in milder wheezing symptoms in early childhood (Hussein et al. 2011, Raedler et al. 2013). Interestingly, in a recent Danish study children who were asthmatics at preschool age had an abnormal immune response, including IL-10 response, against pathogenic bacteria already in their infancy (Larsen et al. 2014).

2.5.3 Polymorphism of *IFN-γ* gene and preschool asthma risk after bronchiolitis

IFN-γ is a pro-inflammatory cytokine that is associated with Th1- type response when defending against viral pathogens. The polymorphism of *IFN-γ* is associated with deficiency of secreted proteins (Pravica et al. 2000). Earlier, there have been reports that a deficiency of IFN-γ leads to the increased prevalence of atopy and asthma (Guerra et al. 2004, Stern et al. 2007). Further, studies with adult and paediatric participants evaluating the *IFN-γ* polymorphism and asthma risk have reported conflicting results (Huang et al. 2012, Kumar & Ghosh. 2008, Nakao et al. 2001, Shao et al. 2004). Finally, in an earlier report from this cohort, we demonstrated that *IFN-γ* SNPs were associated with the frequency of post-bronchiolitis viral respiratory tract

infections at 1.5 years as well as with post-hospitalisation corticosteroid usage suggesting more severe disease (Nuolivirta et al. 2009).

2.5.4 Polymorphism of *IL18* gene and preschool asthma risk after bronchiolitis

IL-18 is a pro-inflammatory cytokine that has an immunoregulatory role in allergic and autoimmune diseases. It stimulates Th1-mediated immune responses and inhibits IgE synthesis. Thus, the decreased expression of IL-18 may lead to disturbances in Th1/Th2 balance (Akdis et al. 2011; Cebeci et al. 2006). In one study, IL-18 serum levels were negatively correlated with peak expiratory flow during asthma exacerbations, suggesting a disease modifying role for IL-18 among asthmatics (Chen et al. 2003). However, evidence of a link between the *IL-18* polymorphism and asthma or allergy susceptibility has not been convincing so far (Birbian et al. 2013, Heinzmann et al. 2004). In the 1.5-year follow-up, there were no significant associations regarding bronchiolitis and *IL18* polymorphisms (Nuolivirta et al. 2009).

2.5.5 Polymorphism of Toll-like receptor 2 subfamily and preschool asthma risk after bronchiolitis

Even though the association between early life bronchiolitis and childhood asthma regarding TLR polymorphism has not been previously studied, there are earlier reports showing an association between childhood asthma and polymorphism in TLR 1, 2,6 and 10 (TLR2 subfamily) (Bottema et al. 2010, Daley et al. 2012, Eder et al. 2004, Kormann et al. 2008). Polymorphism in TLR coding genes can lead to declined immune responses against lower respiratory pathogens, which have been linked with asthma and atopy (Akira et al. 2006). The impaired function of innate immunity due to TLR polymorphism has been shown to delay the shift from infantile Th2 type

immunity towards "non-atopic" Th1-type immune responses (Tesse et al. 2011). In addition, there is evidence that more severe early-life respiratory infection is associated with higher asthma risk in later life (Singh et al. 2007).

Only a few previous reports about an association between childhood asthma and TLR2 subfamily exist (Hoffjan et al. 2005, Kormann et al. 2008, Tantisira et al. 2004), and there are no earlier studies investigating associations between the prognosis of wheezing after bronchiolitis and *TLR* polymorphism. Some evidence about lowered immune responses regarding *TLR 1, 2* and *6* polymorphism and increased atopy risk have been reported (Hoffjan et al. 2005, Kormann et al. 2008). Kormann *et al.* found that two different polymorphisms of the *TLR1* gene were associated with asthma at 9-11 years of age and, further, two different SNPs were associated with the increased mRNA expression of TLR1 (Kormann et al. 2008). Also, TLR1 was significantly associated with SPT positive atopy among Canadian and Australian children, whereas no correlation between TLR6 and atopy was found (Daley et al. 2012). In a Dutch study, it was shown that TLR1 was positively associated with IgE secretion at 6-8 years of age (Reijmerink et al. 2011). Earlier, from this same cohort, there were no associations between the severity of initial bronchiolitis, or with the risk of post-bronchiolitis wheezing at 1.5 years of age and *TLR2* subfamily SNPs (Nuolivirta et al. 2013).

2.5.6 Polymorphism of Mannose-binding lectin (*MBL2) gene* and preschool asthma risk after bronchiolitis

The genetically determined deficient secretion of MBL has been associated with increased susceptibility to recurrent respiratory tract infections (Koch et al. 2001, Rantala et al. 2008). Further, wheezing episodes are usually caused by viral LRTIs and a history of repeated LRTIs usually precede asthma development in childhood (AAP. 2006, Piippo-Savolainen & Korppi. 2008, Taussig et al. 2003). Regardless of these findings, earlier findings of asthma susceptibility and *MBL2* polymorphism have been conflicting (Aittoniemi et al. 2005, Leung et al. 2006, Muller et al. 2007, Nagy et al. 2003, Uguz et al. 2005). While in a Finnish study (Aittoniemi et al. 2005), a positive association between low MBL secretion and non-atopic asthma among adults was reported, no correlation between MBL polymorphism and asthma susceptibility among pediatric participants was found in German or Chinese study participants (Leung et al. 2006, Muller et al. 2007). Further, in a study from Turkey, higher serum levels of MBL were demonstrated among asthmatic children during asthma attacks and quiescence phase compared to healthy controls (Uguz et al. 2005). There are no earlier studies evaluating post-bronchiolitis wheezing and MBL genotypes, but, interestingly, those children that had been infected earlier by *Chlamydia pneumoniae* infection and had variant MBL genotype were more often asthmatic than those with the wild-type MBL genotype (Nagy et al. 2003).

Similarly, the findings regarding MBL polymorphism and atopy prevalence have been conflicting (Aittoniemi et al. 2005, Carrera et al. 2010, Richardson et al. 1983, Thorarinsdottir et al. 2005). Theoretically, MBL deficiency in early infancy may cause alterations in immune responses and hence lead to increased atopy susceptibility (Carrera et al. 2010). Earlier, no significant associations between atopy and MBL polymorphism among pediatric study participants were found (Muller et al. 2007, Thorarinsdottir et al. 2005). A Brazilian birth cohort study showed contradictory

results, the prevalence of atopic dermatitis associated significantly with MBL polymorphism (Carrera et al. 2010). Similarly, in a Chinese paediatric cohort, specific MBL haplotypes were less likely to be associated with current atopy, but the association disappeared after adjustment with potential confounding factors making this result rather weak (Leung et al. 2006). In an earlier 1.5-year follow-up from this same cohort, the susceptibility to more severe post-bronchiolitis wheezing was significantly associated with MBL2 polymorphism (Nuolivirta et al. 2012).

3. AIMS OF THE STUDY

The main goal for this thesis was to evaluate the association between early-life bronchiolitis and the subsequent prevalence of preschool age asthma. Further, the aim was to identify the most relevant individual characteristics, mainly hereditary or genetic, which are responsible for increased vulnerability for later asthma after severe early-life respiratory infection. In conclusion, the more specific questions to be addressed in this thesis are:

1. The long-term prognosis until 5-7-years of age in a pediatric cohort hospitalised due to bronchiolitis at less than 6 months of age.

2. The link between viral aetiology of early-life bronchiolitis and subsequent asthma in childhood.

3. The role of early-life risk factors, like asthma and atopy in parents, parental smoking or atopic dermatitis in infancy, as potential predictive factors for increased asthma or allergy prevalence.

4. The association of IL-10, IL-18 and IFN-γ gene polymorphisms with later asthma occurrence as potential candidate genes for increased asthma susceptibility in childhood.

5. The connection between MBL2 and TLR2 subfamily polymorphisms and childhood asthma prevalence after early life bronchiolitis.

4. MATERIALS AND METHODS

4.1 Cohort study

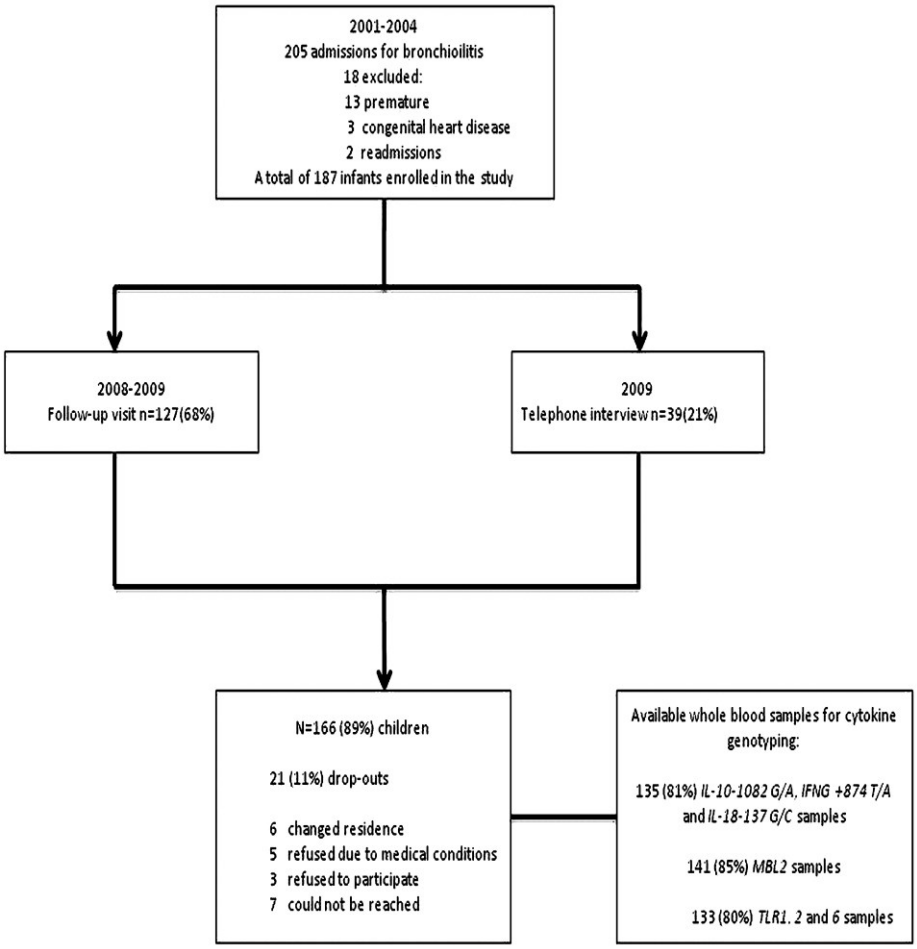
Healthy, full-term infants who were under six months of age when hospitalised at the Department of Pediatrics, Tampere University Hospital (Finland) due to bronchiolitis were enrolled in the study. Diagnosis of bronchiolitis was strictly based on clinical findings: rhinorrhea, cough, tachypnoea, and diffuse wheeze and/or inspiratory crackles, and/or apnea, and/or feeding problems (AAP. 2006). The enrolment periods were between December 1, 2001 and May 31, 2002 and between October 28, 2002 and May 31, 2004. The follow-up visits were arranged between 2008 and 2010, when children were 5-7 years-of-age. A total of three different follow-up periods outside of pollen and main infectious seasons were arranged, which allowed some of the children that had cancelled their first follow-up visit due to respiratory infection to participate in the study.

4.2 Patients

A total of 187 children aged less than 6-months were enrolled between 2001 and 2004. All patients were hospitalised due to LRTI and were diagnosed with J21.0 or J21.9 (WHO ICD-10, 1992). The etiology of bronchiolitis was analysed using PCR and immunofluorescence from nasopharyngeal aspirates with no remarkable differences in results. The children were invited to participate in the follow-up visit at the age of 5-7 years. In total, 127 (68%) children attended the follow-up visit at preschool age. In addition, parents of the 39 children who were not able to participate in an actual study visit were interviewed by telephone. Thus, preschool age follow-up data from 166 (89%) original study participants were available.

4.3 Study design

Flow chart of study participants:



4.4 Follow-up visit at preschool age

The follow-up visit was arranged at 5-7 years, since by that age early childhood transient wheezing had either finished, or if still present, changed to more permanent, asthma-like symptoms (Piippo-Savolainen & Korppi. 2008, Taussig et al. 2003). Thus, the selected time period enabled a first time opportunity to evaluate cases with potentially long-term lung function reduction after early-life bronchiolitis. A detailed questionnaire was sent to parents prior to the follow-up visit and children had to be free of respiratory infection symptoms for at least two weeks before the visit. The main investigator was available for telephone consultation in the case of questions related to the visit; for example, enquiries concerning discontinuations of medications due to lung function testing.

4.4.1 Questionnaire data

Parents were asked to complete a comprehensive questionnaire (Appendix 1.) before the follow-up visit to record cases of doctor-diagnosed asthma, the age when asthma was diagnosed and the continuous or intermittent use of inhaled corticosteroid for asthma treatment. All data on various wheezing episodes were collected, as well as episodes of other asthma-like symptoms, including prolonged cough lasting over 4 weeks or night cough apart from infection. Further, present cases, i.e. active within the last 12 months, of atopic dermatitis and allergic rhinitis were collected, as well other potential asthma or allergy risk factors, like doctor-diagnosed parental asthma or allergy, keeping of indoor furred pets and parental smoking during and after the pregnancy. All enquiries were made separately for mothers and fathers.

4.4.2 Clinical data

All participants of the follow-up visit went through a thorough clinical examination by the main investigator. All findings suggestive of present allergic status, like atopic dermatitis, allergic rhinitis or conjunctivitis were carefully registered. Skin-prick tests (SPTs) were performed to 124 children for eight allergens: birch, timothy grass and mugwort pollens, cat and dog dander, house dust mites (*Dermatophagoides pteronyssimus* and *D.farinae*) and spores of the mould Alternaria alternate. The positive reaction was registered if the wheal reached a diameter of $\geqslant$ 3mm vs. negative control. No antihistamine medications were allowed in 5 days before testing. If the child had experienced a respiratory infection in last two weeks, the testing was rescheduled. Finally, the pre-sent questionnaire was reviewed to confirm a mutual understanding of the answers.

Impulse oscillometry (IOS, Master Screen IOS; Jaeger, Höchberg, Germany) was performed to identify any findings of BHR before and after exercise challenge test (ECT). IOS is a technique, which enables lung function measurement during tidal breathing and no forced expiratory flows are required. Therefore, IOS is an ideal device with which to measure lung function in preschool-aged children, since group co-operation at this age has been a common problem with spirometry(Malmberg et al. 2002) IOS was repeated until three reliable post-exercise curves were available. Resistance values were measured at 5 Hz (Rrs5) and expressed as standard deviations from national height-related, sex-specific reference values (Malmberg et al. 2002). BHR was registered, if the best post-exercise Rrs5 value had increased $\geqslant$ 35% from the best pre-exercise value (Malmberg et al. 2008). ECT was carried out by running outdoors for 8 minutes and exercise was considered sufficient if heart rate reached $\geqslant$ 90% of the predicted maximum for $\geqslant$ 2 minutes. Heart rate was monitored using a heart rate monitor (Polar Ltd, Kempele, Finland).

Asthma was considered to be present if the child was on continuous maintenance medication for asthma, or if the child had suffered from doctor-diagnosed wheezing or prolonged cough (>4 weeks) or night cough apart from infection during the previous year, and BHR was positive. Earlier asthma during childhood was recorded if the child had been on inhaled corticosteroids for > 3 months.

4.5 Genetic methods of the study

4.5.1 Genotyping of cytokine polymorphisms *IL10* –1082 G/A (rs1800896), *IL18* –137 G/C (rs187238) and *IFN-γ* +874 T/A (rs2430561)

Overall, 135 frozen whole blood samples were available for *IL10, IL18* and *IFN-γ* genotyping. Blood samples for further DNA analysis were collected and frozen during hospitalisation for bronchiolitis. Missing DNA samples were collected during the follow-up visit. DNA was extracted from whole blood using a commercially available kit (QIAGEN Inc., USA) according to the manufacturer's instructions at the Department of Clinical Microbiology, Tampere University, Finland. The remaining genomic DNA was frozen at –70°C. Genotyping of the *IL10* –1082 G/A (rs1800896) and *IL18* –137 G/C (rs187238) SNPs was carried out by using the ABI PRISM® 7000 Sequence Detection System for both PCR and allelic discrimination (Applied Biosystems, CA, USA). For *IL18* –137 G/C analysis, 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 synthesised in conjunction with Applied Biosystems (Assay by Design). For *IL10* –1082 G/A, a commercial kit was used (Assay on Demand, C_1747360_10 IL10). The universal PCR thermal cycling conditions from ABI were as follows: 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 performed in a 25 μl volume containing the 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 *IFN-γ* +874 T/A (rs2430561) polymorphism was analysed by the amplification refractory mutational system-PCR method. Genomic DNA was amplified using ThermoprimePLUS 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 of reaction and the 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 analysed by the Agilent DNA 1000 Kit according to the manufacturer`s instructions.

4.5.2 Genotyping of the *MBL2* polymorphism

A total of 141 frozen whole blood samples were available for *MBL2* polymorphism evaluation. The genotyping of MBL2 structural gene mutations was performed by pyrosequencing for the simultaneous detection of three SNPs. The genomic DNA was purified from peripheral blood using the commercial kit (QiagenR); 200μl DNA was collected and 1 μl (20 ng) was used for amplification of the first exon of the MBL2 gene by PCR. The PCR product was used for pyrosequencing (Roos, Dieltjes, Vossen, Daha, & de Knijff, 2006), and dNTPs was sequentially added in the following order: CTCTGTGTCATCACAGC. Three functional SNPs in exon 1 of the MBL2 gene can

be detected in the same pyrosequencing reaction, resulting in pyrograms with unique patterns for each allele combination.

4.5.3 Genotyping of *TLR2* subfamily polymorphism

A total of 133 whole blood samples were available for *TLR2* subfamily genetics. The genotyping of SNPs *TLR1* T1805G (rs5743618), *TLR2* G2258A (rs5743708) and *TLR6* C745T (rs5743810) was performed by pyrosequencing. The genomic DNA was extracted from peripheral blood with the Qiagen QiAmp DNA blood Mini Kit 250 (Qiagen, Hilden, Germany) according to the instructions of the manufacturer. PCR reactions with selected primer pairs for the *TLR*s studied and the PCR amplifications were performed. The pyrosequencing assay was performed using an automated pyrosequencer (PSQTM 96MA Pyrosequencer, Biotage, Uppsala, Sweden). PCR products with potential SNPs were recognised as the template in the pyrosequencing reactions, using PSQTM 96 Pyro Gold Q96 reagent kit according to the manufacturer's protocol. To guarantee the specificity of pyrosequencing, three negative controls (water only, annealing buffer including sequencing primers together with water; PCR products together with water without annealing buffer including sequencing primer) were included in each run.

4.6 Statistics

The statistical analyses were performed using SPSS 17.0-19.0 (SPSS Inc., IL, USA and IBM SPSS Co., NY, USA). The statistical significances of differences between categorical variables were calculated with the Chi-square test and Fisher´s exact test was used with small sample sizes and when the expected frequency for any cell was <5. For continuous variables, the t-test was used for normally distributed variables and the Mann-Whitney U-test was used for non-normally distributed variables. Logistic regression was used to control the effect of potentially confounding factors for the main end-points, mainly asthma and allergy. Logistic regression analyses were first performed as univariate analyses and then as multivariate analyses adjusted for relevant clinical characteristics depending on the evaluated end-point (gender, age, allergy status, virus etiology of index infection and hereditary risk factors for asthma). Odds ratios (OR) with 95% confidence intervals (95%Cl) were reported from both the univariate and multivariate analyses. 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% Cls of ORs or aORs did not include the value 1.0. Hardy-Weinberg equilibrium (HWE) analysis was performed for all cytokine polymorphic alleles to evaluate the representativeness of the analysed cohort sample of SNPs.

4.7 Ethics

The Ethics Committee of the Tampere University Hospital District approved the study protocol. All study participants were thoroughly informed about the study program and a written consent from one parent was required before enrolment. The personal data of the study subjects were not given to the laboratory which performed the genetic studies.

5. RESULTS

5.1 Asthma at preschool age after early-life bronchiolitis (I)

Follow-up data regarding asthma and allergy at preschool age after bronchiolitis in infancy were available from 166 (89%) children at the mean age of 6.5 years (SD 0.57) and 86 (52%) of the study participants were male. Asthma was considered to be present in 21 (12.7%) children (Table 2). Further, there were an additional 24 children who had received continuous maintenance medication for asthma earlier than the preceding 12 months before the follow-up visit. In total, 45 (27%) children had received an asthma diagnosis during their childhood after hospitalisation for early-life bronchiolitis. Three out of 21 children did not have an asthma diagnosis prior to the follow-up visit, but if a child had symptoms consistent with asthma and was also hyper-responsive in ECT, he/she was counted as a "present asthmatic". Bronchial hyper-responsiveness was also documented in five other children, but they did not have any respiratory symptoms and they were not registered as current asthmatics.

5.2 Allergy at preschool age after early-life bronchiolitis (I)

Present allergic symptoms were rather common, as 48 (29%) of the study children had symptoms of allergic rhinitis and 30 (18%) children had doctor-diagnosed atopic dermatitis during the prior 12 months. Thirteen (27%) children with allergic rhinitis also had a present asthma diagnosis (vs. 6.8% in those 118 with no allergic rhinitis, $p<0.001$). A total of 124 SPTs were performed to study participants, and 8/15 (53.3%) of present asthmatics were SPT positive. Birch pollen (22.8%), timothy grass pollen (19.2%) and dog (12.7%) and cat (11.7%) were most common allergens.

Table 2. Baseline data in 166 children hospitalised for bronchiolitis at less than 6 months of age, presented in relation asthma at preschool age.

	Current asthma (n=21)		No asthma (n=145)		p-value
Age at admission, in days, Md (IQR)	113	(63-147)	77	(38-118)	0.027
Age at admission, n (%)					0.06
< 3 months	9	(42.9)	93	(64.1)	
≥ 3 months	12	(57.1)	52	(35.9)	
Gender (male), n (%)	14	(66.7)	72	(49.7)	0.145
RSV bronchiolitis, n (%)	9	(42.9)	108	(74.5)	0.015
Non-RSV bronchiolitis, n (%)	12	(57.1)	38	(26.2)	0.01
Rhinovirus bronchiolitis, n (%)	3	(14.3)	18	(12.4)	0.733
Atopic dermatitis <12 months, n (%)	15	(71.4)	34	(23.4)	<0.001

Student`s t-test was used for continuous variables and Pearson`s chi-square test or Fisher's exact test for categorised variables.

5.3 Risk factors for preschool asthma after early life bronchiolitis (I-IV)

5.3.1 Viral etiology of bronchiolitis (RSV vs. non-RSV virus) (I)

Out of all 166 bronchiolitis hospitalisations, RSV was the causative virus in 117 (70.5%) cases and HRV in 21 (12.7%) cases. Influenza accounted for 6 (3.6%) cases and hMPV for 2 (1.2%) cases. In only 5 (3.0%) bronchiolitis cases, the causative virus could not be detected. Current asthma at preschool age was found among 9 (8.2%) former RSV patients (vs. 12 (24%) of the former non-RSV patients, p=0.01). In further virological analyses, 3/21 (14.2%) of former HRV patients were present asthmatics (vs. 12% of those with other viruses, p=0.7). In further subgroup analyses, former RSV and non-RSV patients had similar figures (29%) for positive SPT reaction as a marker of atopic tendency. In multivariate analyses the findings between asthma at preschool age and virus aetiology of bronchiolitis (RSV vs. non-RSV) remained statistically significant (aOR 3.74 95% Cl 1.28-10.99). Adjustment was done to potentially confounding factors of age on admission, gender, atopic dermatitis in infancy and maternal asthma (Table 4).

5.3.2 Family history of asthma and environmental factors (I)

Eight out of 24 (33.3%) children with asthmatic mothers had asthma at control visit (vs. 9.2% of those 142 without maternal asthma, p=0.001) (Table 3). In contrast, paternal history of asthma had no association with preschool asthma. Atopy was common among asthmatic parents as 21(87.5%) of the 24 asthmatic mothers had both doctor-diagnosed asthma and doctor-diagnosed allergic rhinitis or atopic dermatitis.

Due to this finding, parental asthma, but not parental atopy was included in the multivariate analyses and the association remained statistically significant after adjustments (aOR 3.39 95% Cl 1.03-11.24) (Table 4).

Maternal smoking during pregnancy was reported in 29 (17.5%) cases and, of those, only one child (3.5%) was present asthmatic at the follow-up visit. Maternal smoking in infancy, either indoors or outdoors, was reported by 47 (28.3%) mothers. Paternal smoking during infancy was reported up to 70 (43.8%) fathers, but neither paternal nor maternal smoking during infancy had a significant association with asthma risk at preschool age.

Table 3. 166 children hospitalised for bronchiolitis at <6 months of age, in relation to asthma

	Current asthma (n=21)		No asthma (n=145)		p-value
Maternal smoking during pregnancy, n (%)	1	(4.8)	28	(19.3)	0.129
Maternal history of asthma, n (%)	8	(38.1)	16	(11.0)	0.001
Paternal history of asthma, n (%)	0	(0.0)	10	(6.8)	0.231
Maternal history of atopy, n (%)	13	(61.9)	60	(41.4)	0.077
Paternal history of atopy, n (%)	8	(38.1)	32	(22.1)	0.109
Maternal smoking in infancy, n (%)	5	(23.8)	42	(29.0)	0.624
Paternal smoking in infancy, n (%)	9	(42.9)	61	(42.1)	0.946
Furry pet at home in infancy, n (%)	5	(23.8)	46	(31.7)	0.462

Pearson`s chi-square test or Fisher's exact test was used for categorized variables.

Table 4. Logistic regression: Risk factors for asthma at the mean age of 6.5 years (n=166).

		Crude		Adjusted	
	n	OR	(95% CI)	OR	(95% CI)
Age > 3 months at admission	64	2.31	(0.91-5.85)	2.04	(0.69-6.04)
Gender (male)	86	2.03	(0.77-5.32)	2.01	(0.64-6.29)
Atopic dermatitis at <12 months of age	49	8.16	(2.94-22.7)	7.45	(2.45-22.89)
Non-RSV bronchiolitis	50	4.04	(1.57-10.36)	3.74	(1.28-10.99)
Maternal history of asthma	24	4.96	(1.78-13.79)	3.39	(1.03-11.24)
SPT, positive reaction	124	3.6	(1.19-10.9)	2.81	(0.72-10.9)

Multivariate analyses were performed as adjusted for age on admission, gender, atopic dermatitis in infancy, viral etiology of bronchiolitis and maternal asthma. SPT positivity was significantly associated with asthma risk in univariate analyses (OR 3.6, 95% Cl 1.19-10.9), but not in multivariate analyses (aOR 2.81, 95% Cl 0.72-10.9, n=124).

5.5.3 Polymorphism in genes regulating cytokines, MBL and receptors of toll-like-2 receptors

5.3.3.1 *IL10* –1082 G/A, *IL18* –137 G/C and *IFN-γ* +874 T/A

In all, 135 samples were available for genotyping, and 69 (51%) children had the G/A, 34 (25%) A/A and 32 (24%) G/G genotype at *IL10* -1082 G/A (rs1800896). In all, 68 (50.4%) children had the G/C, 60 (44.4%) C/C and 7 (5.2%) had the G/G genotype at *IL18* –137 G/C (rs187238). Further, 68 (50.4%) children had the T/A, 49 (36.3%) the T/T and 18 (13.3%) the A/A genotypes at *IFN-γ* +874 T/A (rs2430561).

Homozygosity of the allele G of *IL10* -1082 G/A was associated with low asthma prevalence at preschool age, as 1/32 (3.1%) individuals had present asthma (vs. 15.5% of 103 allele A carriers, p=0.04) (Table 5). In further multivariate analyses, the presence of allele A at *IL10* -1082 G/A was predictive for asthma after early life bronchiolitis (aOR 1.84 95% Cl 1.2-3.87) even after adjusting for potential confounding factors of age on admission, gender and current atopy.

The prevalence of current atopy did not have statistically significant association with *IL10* SNP. Similarly, the *IL18* –137 G/C or *IFN-γ* +874 T/A polymorphisms did not associate significantly with present asthma or atopy at preschool age or with asthma diagnose during first six years of life. However, interestingly, the presence of the allele A at *IFN-γ* +874 T/A, especially the A/A genotype, was more common among those children that were non-asthmatics during first six years of life. None out of eighteen children with A/A at *IFN-γ* +874 T/A had present asthma at preschool age, and a further three (16.7%) children had been diagnosed with asthma during childhood (vs. 36 (31%) allele T carriers, p=0.09).

Table 5. Clinical characteristics of 135 study children in relation to *IL10* -1082 G/A, *IL18* -137 G/C or *IFN-γ* +874 T/A genotype, and asthma or atopic findings during follow-up visit

Cytokine genotype	Asthma at follow -up n=17	Asthma between 1-6 years n=39	Atopic eczema at follow-up n=47	Allergic rhinitis at follow-up n=36	Atopy at follow-up n=57
***IL10*-1082G/A**					
A/A n=34	7(41.2%)	11(28.2%)	15(31.9%)	11(30.6%)	19(33.3%)
G/A n=69	9(52.9%)	22(56.4%)	20(42.6%)	16(44.4%)	25(43.9%)
G/G n=32	1(5.9%)*	6(15.3%)	12(25.5%)	9(25%)	13(22.8%)
G allele n=101	10(58.8%)	28(71.8%)	32(68.1%)	25(69.4%)	38(66.7%)
A allele n=103	16(94.1%)*	33(84.6%)	35(74.5%)	27(75%)	44(77.2%)
***IL18*-137 G/C**					
G/G n=7	1(5.9%)	3(7.7%)	3(6.4%)	2(5.6%)	3(5.3%)
G/C n=68	11(64.7%)	18(46.2%)	27(57.4%)	21(58.3%)	32(56.1%)
C/C n=60	5(29.4%)	18(46.2%)	17(36.2%)	13(36.1%)	22(38.6%)
G allele n=75	12(70.6%)	21(53.8%)	30(63.8%)	23(63.9%)	35(61.4%)
C allele n=128	16(94.1%)	35(89.7%)	42(89.4%)	33(91.7%)	52(91.2%)
***IFN-γ*+874 T/A**					
T/T n=49	7(41.1%)	15(38.5%)	15(32.6%)	14(38.9%)	22(38.6%)
T/A n=68	10(58.8%)	21(53.8%)	25(54.3%)	18(50%)	29(50.9%)
A/A n=18	0	3(7.7%)	6(13%)	4(11.1%)	6(10.5%)
A allele n=86	12(71%)	24(61.5%)	31(67.4%)	22(61.1%)	35(61.4%)
T allele n=116	17(100%)	36(92.3%)	40(87%)	32(88.9%)	50(89.3%)

Pearson's Chi-squared test or Fisher´s exact test were used for categorised variables

* p=0.04, other results are not statistically significant (p>0.05).

5.3.3.2 *MBL2* gene

Out of 141 samples genotyped for the *MBL2* gene, 95 (67.4%) children had the wild-type *MBL* genotype A/A, 43 (30.5%) had variant A/O and 3(2.1%) had variant O/O genotypes. *MBL2* had a positive association with asthma at the control visit, as nine (19.6%) children with the non-A/A genotype had asthma (vs. 7.4% of A/A genotype children, p=0.03). This finding remained significant after further adjustments for gender, age at control and atopy (aOR 3.15 95% Cl 1.04-9.56). On the contrary, no significant associations between atopy status or asthma during the first 5 years of life could be demonstrated. All three study participants who had asthma at the follow-up visit and who had not any signs of simultaneous atopy were of the non-A/A *MBL2* genotype (p=0.03).

5.3.3.3 *TLR2* subfamily genes

Overall, 133 blood samples were available for analysis. The genotype distribution for *TLR1* rs5743618 was G/G in 99 (74%), G/T in 29 (22%) and T/T in 5 (4%) cases. Distribution of *TLR2* rs5743708 was G/G in 123 (93%) and G/A in 10 (5%) cases. Similarly, genotypic distribution for *TLR6* rs5743810 was C/C in 42 (31%), C/T in 60 (45%) and T/T in 31 (24%) cases. All of the observed genotype frequencies of the *TLR2* subfamily were in HW equilibrium.

Twenty-four (24%) children who were homozygous for the major allele G at *TLR1* had asthma as diagnosed between the ages of 1 and 5 years (vs. 13 (38%) of those with G/T or T/T genotypes, p=0.04) (Table 6). This result was robust to further adjustment for age and sex (aOR 0.48, 95% Cl 0.2-0.9). *TLR2* polymorphism had no association with present or previous asthma status or with present atopy. Eleven of 60 children (18%) with *TLR6* C/T genotype vs. 49% of other genotypes had current atopy at the control visit (aOR 0.25 (0.11-0.56), adjusted for gender and age) (Table 7). In haplotype analyses combining all three *TLR2* subfamily genotypes, only two of the 26 (7.7%) children with wild-type genotypes for all SNPs had an asthma diagnosis

during the first six years of life (vs. 30% in those children with variant genotypes in one or all *TLR2* genes, aOR adjusted for sex and age 0.66, 95% Cl 0.3-0.89). The significant associations after multivariate analyses between *IL10*, *MBL2* and *TLR2* subfamily genotype SNPs and preschool asthma risk are listed in Table 8.

Table 6. Clinical characteristics of 133 study children in relation to *TLR1* rs5743618 genotype and asthma or atopic findings during follow-up visit.

TLR1 **rs5743618**	**G/G n=99**	**G/T n=29**	**T/T n=5**	**G allele n=126**	**T allele n=34**
Asthma between 1- 6 years n=39	24(24%) *, †	11(38%)	2(40%)	35(28%)	13(38%) *
Present asthma n=17	12(12%)	3(10%)	2(40%)	15(112%)	5(15%)
Present non-atopic asthma n=3	1(1%)	2(7%)	0	3(24%)	2(6%)
Present atopic eczema n=47	38(38%)	6(21%)	3(60%) *, **	44(35%) *	9(26%)
Present allergic rhinitis n=57	43(43%)	11(38%)	3(60%)	54(43%)	14(41%)

* p=0.04; ** aOR: 0.19 (0.14-1.2); † aOR(sex,age): 0.49(0.2-0.9)

Table 7. Clinical characteristics of 133 study children in relation to *TLR6* rs5743810 genotype and asthma or atopic findings during follow-up visit.

TLR6 **rs5743810**	**C/C** **n=42**	**C/T** **n=60**	**T/T** **n=31**	**C allele** **n=102**	**T allele** **n=91**
Asthma between 1- 6 years (n=39)	10(24%)	21(35%)	8(26%)	31(30%)	29(32%)
Present asthma (n=17)	5(12%)	8(13%)	4(13%)	13(13%)	12(13%)
Present non-atopic asthma (n=3)	1(2%)	2(3%)	0	3(3%)	2(2%)
Present atopic eczema (n=47)	16(38%)	11(18%) *, †	20(65%) *, ‡	27(26%) *	31(34%)
Present allergic rhinitis (n=57)	16(38%)	22(37%)	19(61%) **, §	38(37%) **, ¥	41(45%)

* p<0.001; ** p=0.01; † aOR: 0.25 (0.11-0.56); ‡ aOR: 2.78 (1.2-6.4); § aOR: 4.5(1.87-10.3); ¥ aOR: 0.36 (0.16-0.83)

Table 8. Univariate and multivariate analyses for childhood asthma risk in relation to *IL10*, *MBL2* and *TLR2* subfamily genotype.

		Crude		Adjusted	
	n	**OR**	**(95% CI)**	**OR**	**(95% CI)**
***IL10* -1082 allele A presence ***	103	1.96	(1.5-3.52)	1.84	(1.2-3.87)**
***MBL2* non A/A genotype ***	46	3.6	(1.3-8.9)	3.15	(1.04-9.56)**
***TLR1* rs5743618 G/G genotype †**	99	0.4	(0.22-0.85)	0.48	(0.2-0.9)‡
***TLR1* G/G and *TLR2* G/G and *TLR6* C/C genotype (haplotype) †**	26	0.52	(0.37-0.78)	0.66	(0.3-0.89)‡

* association between preschool age asthma and SNP; ** multivariate adjustment with age, gender and present atopy; † association between asthma during 1-6 years and SNP/haplotype; ‡ multivariate adjustment with age and gender

6. DISCUSSION

6.1 Preschool asthma prevalence after early-life bronchiolitis

In the cohort of 166 children, who were hospitalised due to bronchiolitis under the age of six months, the preschool asthma rate was 12.7%. This figure is higher than the preschool asthma rate of 6 % in the Finnish healthy child population (Pekkanen et al. 1997, Remes et al. 1996), but lower than the previously reported post-bronchiolitis preschool asthma rates of 15-48% (Castro et al. 2008, Kotaniemi-Syrjänen et al. 2002, Kuikka et al. 1994, Sigurs et al. 2000, Wennergren et al. 2004). The variance in results is likely to be derived from differences in the clinical characteristics between previous post-bronchiolitis cohorts. There are no other earlier post-bronchiolitis follow-up studies that enrolled only children <6 months of age upon hospitalisation; the earlier studies have enrolled children aged 12 months up to 3 years. Also, the severity of index infection has varied greatly.

Studies from Finland and Sweden have reported a preschool asthma rate of up to 30% when participants were hospitalised for bronchiolitis at less than 12 months of age (Sigurs et al. 2000) and 25-47% when children were younger than 24 months upon hospitalization (Kotaniemi-Syrjänen et al. 2002, Kuikka et al. 1994, Wennergren et al. 1992). Further, in birth cohort studies that included mild, parent-reported wheezing symptoms, the later asthma rate has been remarkably higher, with up to 60% of former wheezers having asthma-like symptoms during preschool age (Martinez et al. 1995). In a high-risk birth cohort that enrolled only children with parental asthma or allergy, the preschool asthma rate was 28% after early-life wheezing (Jackson et al. 2008). However, contrary to the basics of their diagnostics, we included only doctor-diagnosed cases of

asthma in our study.We were also able to perform objective testing of BHR with IOS, which increases the reliability of our results.

In order to report reliable associations between early-life infections and following asthma susceptibility it is vital that the index infection under research is the first LRTI encountered by the child in their life. In patient cohorts that included up to two or even three year-old children, this might not be the case, as nearly all children have been infected by RSV by their second birthday (Greenough et al. 2001). Also, other LRTI-causing viruses are common, the prevalence of HRV bronchiolitis, when recurring, was up to 80% in first year of life in children from atopic families (Jartti et al. 2009). In our cohort, this potential bias of repeated infections is very unlikely, as most of the children were under 3 months of age when hospitalised.

One of the theories behind the present study was that LRTI occurring during the very first months of life might be linked with later asthma development, as the neonatal immunology is still immature and probably sensitive to environmental factors, like viral infections. To support this view, it has been shown that first LRTI in early-life was significantly associated with elevated IgE levels among children who went on to become persistent wheezers (Martinez et al. 1998). However, the result of the present study is quite the opposite. In fact, we found that children under three months of age when hospitalized had an asthma rate at preschool age of only 8.9%, which shows that particularly young age upon hospitalisation is not a prognostic factor for later asthma. In line with this, RSV infections in infants without any predisposition to allergy, and hence smaller asthma risk, are more common among the youngest bronchiolitis patients. Further, in large birth cohort study from the US, later wheezing was more common among those who wheezed only during the second or third year than among those who wheezed only during the first year of life (Taussig et al. 2003).

6.2 Prognostic value of viruses in preschool asthma after early-life bronchiolitis

Children infected with non-RSV bronchiolitis had a significant 3-fold increase in preschool asthma risk when compared with solely RSV-infected children (24% vs. 8%). Also, 14% of former HRV patients had present asthma at the follow-up visit, but the number of HRV cases was too small for definite conclusions. There are only a few earlier studies evaluating HRV infection and asthma prognosis, because PCR testing for HRV has only recently become available and no antigen test is available and serology for HRV is not feasible. However, our results are in line with the previous findings, where bronchiolitis in general has been associated with an increased asthma risk and children with rhinovirus or non-RSV infections have had an up to 2- to 4-fold asthma risk vs. RSV-infected children (Jackson et al. 2008, Kotaniemi-Syrjänen et al. 2003, Kusel et al. 2007).

Clinical characteristics between children hospitalised due to RSV and non-RSV have differed with age, atopic predisposition and hereditary characteristics of the child (Jartti et al. 2009; Jartti & Korppi. 2011, Papadopoulos et al. 2002). The dividing age between the prevalence of RSV and HRV has been 12 months in previous studies (Jartti et al. 2009), meaning that older children are more likely to be infected by HRV. Also, atopic inflammation has been linked with susceptibility to rhinovirus infection due to the increased expression of major HRV receptor Intercellular adhesion molecule-1 (ICAM-1) among atopic individuals (Jartti & Korppi. 2011). In the present study, there were no statistically significant differences between RSV and HRV-infected children and their hereditary characteristics, i.e. parental asthma and/or atopy. Parental atopy was slightly more common among children infected with HRV (59% vs. 55% of children infected with non-HRV), but this finding did not reach statistical significance. Further, atopic dermatitis and/or food allergies were slightly more common among

non-RSV (33%) vs. RSV-(24%) infected children. Also, HRV-infected children were slightly older upon index infection vs. RSV-infected study participants, which is in accordance with the previous results (Korppi et al. 2004).

The associations between the viral aetiology and the clinical characteristics of the present study are consistent with reports where RSV and non-RSV bronchiolitis infections are actually seen as two separate entities (Jartti et al. 2009; Korppi et al. 2004, Papadopoulos et al. 2002). Clinically these two conditions cannot be separated, but the history and risk factors of the patients differ. Acute HRV infection more closely resembles asthma exacerbation, whereas RSV bronchiolitis is an independent viral infection, occurring regardless of predisposing factors of a child (Papadopoulos et al. 2002). Our results support the significance of the predisposing factors among HRV bronchiolitis patients, as atopy and asthma at preschool age were more common among them compared to patients with the history of RSV bronchiolitis.

6.3 Early-life predictive factors for preschool asthma

The association between early life respiratory infection and increased asthma susceptibility in later childhood has been clear for years (Martinez et al. 1995, Piippo-Savolainen & Korppi. 2008). Still, even after two decades of active researching, the outstanding question is: what comes first, lower respiratory tract infection, which causes increased asthma risk, or pre-existing asthma predisposition, which is revealed by bronchiolitis, as a marker of this tendency? Studies collecting data prior to any LRTIs have shown, that cytokine profile at birth was different among those who had severe RSV infection when compared among healthy controls (Juntti et al. 2009). Similarly, the RSV-bronchiolitis group demonstrated decreased lung function (Taussig

et al. 2003) and increased BHR (Chawes et al. 2012) in premorbid lung function measurements. Based on these findings, it seems that certain predisposition to RSV-bronchiolitis exists, which might also reflect early susceptibility to repeated wheezing episodes and even to asthma.

Based on our recent findings, there are at least three subgroups of post-bronchiolitis patients with different background mechanisms for asthma susceptibility. The aetiology of primary LRTI and individual clinical characteristics of a child will play a part in determining the future asthma risk (Koponen et al. 2012). Further, and equally importantly, polymorphisms in genes regulating immune defence against early-life pathogens (Koponen et al. 2012, Koponen et al. 2013) seem to be associated with an increased asthma risk as well. In conclusion, the combined effect of various predictive factors and the causative virus will ultimately dictate the following asthma risk in childhood.

6.3.1 Early-life atopy and family history of asthma

Over two-thirds of the present asthmatics had presented with doctor-diagnosed atopic dermatitis in the first year of life; most of them had signs of allergic rhinitis later during childhood as well. Although not as striking as in our study, the association between early-life atopy and later asthma has been similarly reported in previous post-bronchiolitis studies after the hospitalisation (Heymann et al. 2004, Kotaniemi-Syrjänen et al. 2002, Wennergren et al. 1992). However, in some birth cohort studies, the association between early atopy and asthma has either been weak (Lemanske et al. 2005), or not found (Martinez et al. 1995), which probably results from different patient characteristics in dissimilar study settings. In several previous post-bronchiolitis follow-up studies maternal asthma has been predictive for childhood asthma (Goksör et al. 2006, Piippo-Savolainen & Korppi 2009, Sigurs et al. 2005). In accordance, one-

third of children with asthmatic mothers were present asthmatics in our study. However, paternal asthma was not significantly associated with preschool asthma, which probably demonstrates the fetal immunity modifying effect of maternal immune responses (Platts-Mills et al. 2003). In our cohort, parental allergy was not significantly associated with preschool asthma, whereas biparental allergy in particular has been linked with increased allergy and wheezing risk in previous studies (Cantani 1999, Clough et al. 1999).

6.3.2 Exposure to environmental tobacco smoke

Scandinavian post-bronchiolitis follow-up studies after the hospitalisation, which have evaluated the link between the exposure of environmental tobacco smoke and asthma risk, have reported inconsistent results. Two studies found a positive association (Goksör et al. 2007, Piippo-Savolainen et al. 2006), but negative results have also been reported (Kotaniemi-Syrjänen et al. 2002, Reijonen et al. 2000, Sigurs et al. 2000). In a Swedish study, maternal smoking led to bronchial hyper-responsiveness and reduced lung function, whereas paternal smoking increased the risk of own smoking as a teenager. Their study stressed that maternal smoking during pregnancy is a particular risk factor for lung function impairment (Goksor et al. 2007). In our study, both the maternal smoking during pregnancy (18%) and maternal smoking during infancy (28%) were rather common, but no association with preschool asthma could be shown. Mixed results regarding the association between tobacco exposure and asthma may also derive from selection bias, since exposure to tobacco smoke is a well-documented risk factor for early-life bronchiolitis (Carroll et al. 2007).

6.4 Genetic risk factors for preschool asthma after bronchiolitis

We found several preliminary results regarding genes linked to the pathophysiology of asthma and asthma prevalence at preschool age after infantile bronchiolitis. As previous post-bronchiolitis follow-up studies evaluating the association between genetics and increased asthma risk in childhood do not exist, the comparisons of our results to previous studies examining the link between genetics and susceptibility for asthma or infections was performed. Most of the available studies have evaluated RSV-associated disease and are based on the candidate gene approach (Singh et al. 2007). Candidate genes are selected on the basis of their role in immunity, and particularly innate immunity responses. More specifically, genes involved in direct pathogen control or genes that are postulated to later modify immunopathology are the key targets when investigating the link between early-life respiratory infections, genetics and disease outcome (Miyairi & DeVincenzo. 2008).

6.4.1 *IL10* polymorphism and asthma at preschool age

We found two noteworthy associations between *IL10*-1082 G/A SNP and asthma at preschool age. First, those children who are non-carriers of allele G were at increased risk of preschool asthma after infantile bronchiolitis and the carriage of allele A was significantly associated with elevated asthma risk even after adjustment for gender, age and present atopy. Second, only 3.1% of the children with the G/G genotype at *IL10*-1082 G/A were present asthmatics, compared to 15% of other genotypes at the same *loci*. These findings are well in line with other recent results, where the meta-analysis of 18 case-control studies found a correlation between the *IL10*-1082 G/A SNP and asthma risk. Further, an association between *IL10* SNP and wheezing at 3 years of age was found in a study from Germany (Raedler et al. 2013). In line with this, in a study of Egyptian asthmatic children, it was noted that children with the G/G genotype at -

1082 G/A were nearly 50% less likely to have atopic asthma than other children (Hussein et al. 2011).

Genetic predisposition to either strong or weak anti-inflammatory responses against pathogens may increase the risk of atopic diseases like asthma (Jartti et al. 2005). Previously, it was found that lower levels of secreted IL-10 led to more Th2-type responsiveness and to less immunological tolerance, which may also lead to an increased risk of asthma (Jartti et al. 2009). This is in accordance with our results, where children with the low-producing genotype of *IL10* were more commonly asthmatic at preschool age. IL-10 has been found to have an effect on suppressing early-life inflammatory processes, and hence, Th1-type cytokine responses. However, simultaneously there is growing evidence that IL-10 has a regulatory, and even up-regulatory role on some Th1 cytokines, like IFN-γ, as well (Akdis et al. 2011, Raedler et al. 2013). Recently the role of Treg cells, a vital source of IL-10 production, has been emphasized, as the number of Treg cells is lower with *IL10* SNPs, and they could play a more pivotal role in early phase of immune maturation than just Th1/Th2 cytokine lineages (Raedler et al. 2013).

Importantly, IL-10 has a pivotal role in host defence as well. In addition to having a regulatory role in cytokine secretion, IL-10 inhibits the maturation of dendritic cells and, also, macrophages from killing intracellular organisms. Thus, IL-10 is a key factor in regulating the immunity responses against microbes (Hawrylowicz & O'Garra. 2005). It is vital that a sufficient amount of IL-10 is produced during inflammatory responses, especially when the necessary levels of IL-10 will even increase during respiratory virus infection (Jartti et al. 2009). In our study, 80% of HRV-infected children in infancy with the low-producing *IL10* genotype had an asthma diagnosis during childhood. In accordance, in a Japanese study, secreted IL-10 levels from mononuclear cells were lower if they were obtained from asthmatic children during rhinovirus infection. More interestingly, children who did not outgrow their wheezing symptoms during childhood had lowered IL-10 production when compared to those who outgrew their

symptoms by 6 years of age (Iikura et al. 2011). These findings provide evidence that a low capacity to control early-life HRV-induced inflammation in airways due to *IL10* SNPs seems to be associated with longer term changes in lung function, even with asthma.

6.4.2 *IFN-γ* and *IL18* polymorphisms and asthma at preschool age

There were no statistically significant associations between *IFN- γ* +874 T/A or *IL18* –137 G/C SNPs and asthma symptoms during childhood in our study. However, the presence of the allele A at *IFN- γ* +874 T/A, especially the low producing A/A genotype (Pravica et al. 2000), was numerically more common among children who did not have any asthmatic symptoms during childhood. In line with this, in earlier follow-up from this cohort, it was found that those children with allele A of *IFN- γ* +874 T/A were at a lower risk of repeated viral infections after the hospitalisation due to bronchiolitis (Nuolivirta et al. 2009).

The earlier studies evaluating *IFN-γ* SNPs and the development of asthma have reported conflicting results. An adult cohort from India (Kumar & Ghosh- 2008), and similarly, a paediatric cohort from Japan (Nakao et al. 2001) reported that *IFN-γ* SNPs had an association with asthma, whereas studies from Japan or Taiwan did not find any such associations (Huang et al. 2012, Shao et al. 2004)among paediatric study participants. Further, the low production of IFN-γ has been linked with susceptibility to HRV infection and also to atopic predisposition of study participants (Gern. 2010, Kusel et al. 2007). Nonetheless, none of these association studies had severe lower respiratory infection in early-life as an inclusion criterion, so the results are not totally comparable with each other.

IL-18 is an important factor in host defence, as it enhances both the innate and acquired immunity. It stimulates the development of Th1 cells and it also stimulates the production of IFN-γ, while inhibiting IgE synthesis (Akdis et al. 2011). Despite these interesting characteristics potentially being linked to asthma development, we could not find any associations with asthma risk and the *IL18* –137 G/C SNP. This is in accordance with previous conflicting results, where only associations with the severity of asthma and *IL18* SNPs have been previously published (Harada et al. 2009; Tanaka et al. 2001).

6.4.3 *MBL2* polymorphism and asthma at preschool age

The main finding regarding *MBL* polymorphism and asthma risk was that carriers of the variant genotypes of *MBL2* gene had a 3-fold risk of preschool asthma when compared with wild-type carriers. There are no earlier post-bronchiolitis follow-up studies investigating *MBL2* polymorphisms, but MBL deficiency, associated with variant genotypes, has been linked with increased susceptibility to repeated respiratory tract infections (Koch et al. 2001, Rantala et al. 2008). Association studies between *MBL2* SNPs and asthma in children have provided mainly negative results (Aittoniemi et al. 2005, Leung et al. 2006, Muller et al. 2007, Nagy et al. 2003, Uguz et al. 2005). Positive associations have only been found in two studies, where asthmatics had higher serum levels of MBL during acute attacks of symptoms when compared to healthy controls (Kaur et al. 2006, Uguz et al. 2005). Further, in contrast to our results, a cohort study from India found a significant association between the *MBL2* wild-type genotype and asthma risk (Birbian et al. 2012).

In a previous paediatric study, the variant genotype of *MBL2* gene and past *C. pneumoniae* infection were linked with asthma risk (Nagy et al. 2003). The study design had similar elements to our present study. The results offer an interesting viewpoint to a setting of suboptimal immune defence due to MBL polymorphism, and the study detected previous microbe infection and the subsequent increased asthma susceptibility.

Moreover, *C. pneumonia* infection is inhibited by MBL similar to the respiratory viruses investigated in our study. It may be, that respiratory infection among individuals with compromised immunity, due to polymorphism of MBL, leads to chronic respiratory symptoms, and further to asthma. This might also be the rationale behind the 3-fold increase in current asthma prevalence among *MBL2* variants after the infantile severe respiratory infection observed in our study.

6.4.4 *TLR2* subfamily polymorphism and asthma at preschool age

In the present study there were two interesting associations between *TLR2* subfamily genetics and asthma occurrence during childhood. First, the *TLR1* gene polymorphism was associated with asthma prevalence between 1 and 5 years of age, after being hospitalised due to bronchiolitis in early infancy. Second, in further haplotype analyses, it was found that children with wild-type genotypes for all three oh the investigated *TLR* SNPs were rarely asthmatics. In line with this, the risk of asthma was up to 4-fold if the child had one or more variant genotypes for *TL1, TLR2* or *TLR6* SNP. Combining these two results brings preliminary evidence that variant *TLR* genotypes are linked with increased asthma risk after severe early-life respiratory infection.

Due to the lowered recognition of microbes, *TLR* polymorphisms have been associated with impaired immune responses and, further, with pronounced asthma risk (Akira et al. 2001). The explanation for increased asthma risk could be domination in Th2-type responses, which have been associated with *TLR* SNPs in children with early-life respiratory infections (Message & Johnston. 2001). In addition, the severity of early-life respiratory tract infection has been associated with later asthma risk, which emphasises the importance of proper immune responses, e.g. TLR function. This is particularly important in the first months of life when the adaptive immunity has not yet been fully developed (Singh et al. 2007).

In previous paediatric studies, it has been demonstrated that polymorphism in the *TLR* genes, including *TLR1* and *TLR6*, were associated with the development and different phenotypes of asthma (Kormann et al. 2008). However, no association between *TLR2* subfamily SNPs and asthma was found in another study (Hoffjan et al. 2005). Moreover, there are earlier results suggesting that *TLR1, TLR2* and *TLR6* SNPs are predictive of atopy (Hoffjan et al. 2005, Kormann et al. 2008, Tantisira et al. 2004), which could also result in an increased asthma risk. In accordance with this, we found that *TLR1* and *TLR6* were associated with present allergic symptoms also in our cohort. The sustained Th2-response after immunology modulating severe early-life infection is the most likely explanation for our finding.

6.5 Methodological aspects

6.5.1 Strengths of the study

The main strength of the study is the prospective design. Overall, 166 children participated in the follow-up at the preschool age, so the clinical follow-up data were received from 89% of the original study participants, who were followed-up over 5 years. All clinical data were verified upon follow-up visit and only doctor-diagnosed asthma and allergy cases were registered. We were able to measure BHR using IOS, and also to perform SPTs during follow-up visits. By using IOS, we were able to detect cases of lowered lung function that would have not otherwise been detected.

The present study included a unique cohort of children, as all study participants were under six- months-of-age when hospitalised due to bronchiolitis. This enables us to analyse the associations between infantile severe respiratory infection and various predictive factors for preschool-age asthma. Moreover, as most children were only two months of age upon hospitalisation, the analysed infection is most likely the first primary infection that the child has encountered in his/her life. Further, all children were from a rather small geographical area and were of Finnish origin, which is a definitive strength for a genetic study. Finally, we were able to reach a viral detection rate of over 90% due to extensive virological testing. RSV, HRV, adenovirus, influenza, PIV type 3 and hMPV were included in the testing panel. PCR and antigen detection were both performed with no remarkable differences in results.

6.5.2 Shortcomings of the study

There are three main shortcomings of the present study. First, the lack of a population-based control group to some extent hampers asthma risk comparisons between healthy controls and the present cohort with severe infection in early life. Second, the number of HRV infections was rather low, 21 cases, which made it impossible to analyse the correlation between sole HRV infection and later asthma and/or atopy risk. Third, the number of participants is quite low for a genetic study.

Our study was designed to evaluate the asthma risk, specifically among those who suffered severe bronchiolitis in infancy, and to find the most important asthma predictive factors, such as various viruses, genetic factors and hereditary components within this post-bronchiolitis study group. In this sense, the comparisons between different cohort subgroups need to be performed inside the study group in any case, and thus, a population-based control-group does not provide much additional information. Moreover, the asthma prevalence at preschool age in a healthy Finnish population has been well documented in earlier studies (Pekkanen et al. 1997, Remes et al. 1996).

The study was primarily designed to assess various virologic and genetic factors and their role in preschool asthma as an outcome after bronchiolitis in early infancy. Due to that decision, the upper age limit of study participants was only 6 months. There are no previous post-bronchiolitis cohorts with all participants having severe infection in the first months of life. On the other hand, by selecting only the youngest bronchiolitis patients, RSV dominated greatly, and in particular, the number of HRV infection cases was not sufficient for statistical analyses. By dividing children on the basis of their index infection to RSV and non-RSV study groups, we were able to perform statistical calculations between the groups.

Finally, the sample size is quite small for a genetic study. However, the small sample size is more likely to lead to false-negative, rather than to false-positive results. Furthermore, all studied SNPs were in HWE, which was determined by the exact HWE test.

7. Conclusions

In the present follow-up study, after hospitalisation for early-life bronchiolitis at less than six months of age, the prevalence for preschool asthma was 13%. This rate is approximately two-fold when compared with the healthy Finnish population without severe infection in infancy. In earlier post-bronchiolitis studies, asthma risk has been even greater, but all previous bronchiolitis follow-up studies enrolled children under 12 or even under 24-months-of-age, which offers feasible explanation for the different results. Our cohort consists of "pure bronchiolitis" patients, who were only two months of age, on average, upon hospitalization. Older study participants in earlier follow-up studies have most likely already demonstrated asthma-like symptoms, which may explain the higher asthma prevalence figures observed in those studies.

Moreover, children infected with a respiratory virus other than RSV had even higher asthma prevalence at preschool age, as one-quarter of the previous bronchiolitis patients were present asthmatics at preschool age. The main driver for this finding is HRV, which was the second most common virus after RSV. In contrast, RSV, as a causative virus, was not heavily predictive for asthma, as only 8% of former RSV patients had asthma at preschool age. As we enrolled only children aged six-months or less, RSV was the highly dominant virus in our cohort; therefore, the study was not powered to analyse other lower respiratory tract viruses separately. Finally, we found that children with non-RSV aetiology more often had atopy in infancy and, further, children with HRV were slightly older than RSV-infected children in our study.

Atopic eczema in the first 12 months of life and maternal asthma were associated with preschool asthma in our present study. Instead, paternal asthma was not associated with present asthma prevalence. Atopic tendency has been linked with lowered immune responses against different pathogens. This results in a certain level of

predisposition to respiratory infections among the atopic individuals, and therefore, early-life respiratory infection may also serve as a marker of later atopic characteristics. On the contrary, we did not find any associations between smoking during pregnancy or exposure to passive smoking during infancy. However, as exposure to tobacco smoke is a proven risk factor for bronchiolitis, our results regarding smoking effects might have been biased due to this selectness of our cohort.

In genetic studies, we found some interesting associations between *IL10* –1082 G/A, *MBL2* and *TLR2* subfamily SNPs and asthma susceptibility after infantile bronchiolitis. The main result was that only 3.1% of the children with the G/G genotype at *IL10* -1082 G/A had asthma at the follow-up, compared to 15% of other genotypes at the same *loci*. Moreover, the children with low-producing genotype of IL10 were more susceptible to asthma, which demonstrates the effect of insufficient host defence against virus infection in infancy. In addition, immunoresponses tend to balance more towards the Th2 direction, leading to asthma susceptibility, when IL-10 is not produced adequately.

Children with variant genotypes of *MBL2* were three times more likely to have asthma at preschool age. This is a preliminary finding and needs to be verified in future studies, but it shows the effect of compromised innate immunity, which is the key factor in host defence in the first months of life. Furthermore, *TLR1* polymorphism and *TLR2* subfamily haplotype analyses had associated with asthma prevalence during first six years of life. Insufficient responses against pathogens seem to lead to chronic respiratory inflammation, and further, these findings stress the role of properly functioning innate immunity during viral infections in early infancy.

7.1 Clinical implications of the present study

The main message for a clinician from the present study is that children hospitalised due to non-RSV infection in the first of months of life are clearly at elevated asthma risk in later life. We found that almost one-quarter of the study participants with non-RSV infection were asthmatic at preschool age. Further, if the child has some additional hereditary (maternal asthma) or individual atopic predispositions (atopic eczema), the asthma risk may be even higher. Thus, all families with a child hospitalised due to bronchiolitis, and with a high risk of allergy, should be informed about the symptoms suggestive of asthma.

Further, our genetic results provide preliminary data from a perspective that has not been previously studied. By clarifying the roles of genetic (host) and environmental (virus) factors regarding the prognosis after early-life infection, we are able to get more into why some children will be future asthmatics while some will never develop any asthma-like symptoms. Accordingly, the present findings regarding an association between the *TLR2* subfamily polymorphisms and asthma development may help to provide clinicians with a useful tool for asthma risk identification in the future.

Finally, present results bring new insights into interactions between cytokine regulating genes, like *IL10*, and the development of pediatric asthma. Our results show that individual alterations in immune mechanisms lead to increased risk of asthma development. Thus, comprehensive understanding of the immunity mechanisms is pivotal when planning new studies or developing new effective medicines.

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Appendix 1.

POST-BRONCHIOLITIS OUTCOME STUDY

University of Tampere, Paediatric Department

Questionnaire

Name: _____

Personal ID number : _____-_____

1. OBSTRUCTIVE RESPIRATORY SYMPTOMS

	No	**Yes**
Has your child experienced any respiratory wheezing symptoms during last 12 months?		
Has your child experienced wheezing symptoms more than once during last 12 months?	0	1
Has your child experienced any wheezing symptoms during flu-like illnesses?	0	1
Has your child experienced any wheezing symptoms during pollen season and/ or during contact with furry animals?	0	1
Has your child experienced wheezing during physical activities?	0	1
Have there been any wheezing symptoms during any other occasion? **If YES, please, describe the situation:**	0	1

2. COUGH SYMPTOMS

	No	Yes
Has your child suffered from prolonged coughing symptoms (>4 weeks) during last 12 months? (no respiratory infection present)	0	1
Has your child suffered from repeated night cough during last 12 months? (no respiratory infection present)	0	1

3. ASTHMA DIAGNOSIS

	No	Yes
Has your child been diagnosed with asthma during last 12 months? **If YES, please write the name of the health centre/ hospital/ private health care organisation:**	0	1
Has your child been ever hospitalized due to asthma or obstructive bronchitis? **If YES, please report the age when hospitalization occurred:** 1, 2, 3, 4, 5, 6 years **Please report the name of the hospital:**	0	1
Has your child ever been diagnosed with asthma: **If YES, please report the age when your child had asthma diagnosis:** 1, 2, 3, 4, 5, 6 years	0	1

3. ASTHMA MEDICATION:

	No	Yes
Has your child received inhaled asthma medication during last 12 months? (Pulmicort, Flixotide, Beclomet , Seretide, Symbicort, Singulair)	0	1

Has your child ever received inhaled asthma medication?: **If Yes, please report the age when your child received asthma medication:** 1, 2, 3, 4, 5, 6 years	0	1

	No	**Yes**
Has your child received inhaled symptom reliever medication during last 12 months (Bricanyl, Serevent, Ventoline, Airomir)**?** **daily / weekly / monthly / less than monthly**	0	1
Has your child ever received inhaled symptom reliever medication?: **If Yes, please report the age when your child received inhaled symptom reliever medication:** 1, 2, 3, 4, 5, 6 years	0	1

5. SYMPTOMS OF RHINITIS

	No	**Yes**
Has your child suffered from runny nose, sneezing or stuffy nose outside the infection during last 12 months?:	0	1

If NO, please proceed to question 6.

	No	**Yes**
Have the symptoms of rhinitis (see above) been linked with certain time of the year?: **If YES, when:** January, February, March, April, May, June, July, August, September, October, November, December	0	1
Have the symptoms of rhinitis been linked with animal contacts?:	0	1
Has your child been doctor-diagnosed with allergic rhinitis?: **If YES, please report the name of health center/ hospital / private health care organisation:**	0	1

6. OCULAR SYMPTOMS

	No	Yes
Has your child suffered from ocular itching, tearing or eyelid irritation?:	0	1

If NO, please proceed to question 7.

	No	Yes
Have the symptoms of ocular irritation been linked with certain time of the year?: **If YES, when:** January, February, March, April, May, June, July, August, September, October, November, December	0	1
Have the symptoms of ocular irritation been linked with animal contact ?:	0	1
Has your child been doctor-diagnosed with allergic conjunctivitis?:	0	1

7. SKIN ECZEMA

	No	Yes
Has your child experienced itching eczema during last 12 months?:	0	1

If NO, please proceed to question number 8.

	No	Yes
Has your child been doctor-diagnosed with allergic eczema?: **If YES, please describe where eczema developed:** Face, trunk, legs, hands, neck	0	1

8. FOOD ALLERGY

	No	Yes
Has your child experienced eczema or bowel symptoms that are caused by food allergy during last 12 months?:	0	1

If NO, please proceed to question number 9.

	No	Yes
Has your child been doctor-diagnosed with food allergy? **If YES, please describe what allergies have been found?:** Milk, egg, grain, other:	0	1

9. ECZEMA DURING FIRST 12 MONTHS OF LIFE

	No	Yes
Did your child receive diagnosis of allergic eczema during first 12 months of life?	0	1

If NO, please proceed to question number 10.

	No	Yes
If YES, please describe where eczema developed: Face, trunk, legs, hands, neck	0	1

10. FOOD ALLERGY DURING FIRST 12 MONTHS OF LIFE

	No	Yes
Did your child receive a diagnosis of food allergy during first 12 months of life?	0	1

If NO, please proceed to question 11.

	No	Yes
If YES, please describe the symptoms: Diarhhea, vomiting, eczema **If YES, please describe what allergies were found:** Milk, grain, egg, other:	0	1

11. ASTHMA IN FAMILY

	No	Yes
Has mother ever been diagnosed with asthma?	0	1
Has father ever been diagnosed with asthma?	0	1
Has any of the siblings ever been diagnosed with asthma?	0	1
Has any of the siblings ever been hospitalized due to repisratory infection?	0	1

12. ALLERGY IN FAMILY

	No	Yes
Has mother ever been diagnosed with allergic rhinitis or allergic conjunctivitis?:	0	1

Has father ever been diagnosed with allergic rhinitis or allergic conjunctivitis?:	0	1
Has any of the siblings ever been diagnosed with allergic rhinitis or allergic conjunctivitis?:	0	1

13. PETS

	No	**Yes**
Was there a cat in the family when child was under 12 months of age?	0	1
Was there a cat in the family when child was over 12 months of age?	0	1
Was there a dog in the family when child was under 12 months of age?	0	1
Was there a dog in the family when child was over 12 months of age?	0	1

14. EXPOSURE TO TOBACCO SMOKE

	No	**Yes**
Did father smoke when child was under 12 months of age?: **If YES, which is correct:** indoors only outdoors	0	1
Did father smoke when child was over 12 months of age?: **If YES, which is correct:** indoors only outdoors	0	1
Did mother smoke when child was under 12 months of age?: **If YES, which is correct:** indoors only outdoors Smoking during pregnancy No smoking during pregnancy	0	1
Did mother smoke when child was over 12 months of age?: **If YES, which is correct:** indoors only outdoors	0	1

15. HEIGHT AND WEIGHT

DATE: ___/____ 20xx

Weight	
Height	

THANK YOU!

LIST OF ORIGINAL PUBLICATIONS

I. Koponen, P., Helminen, M., Paassilta, M., Luukkaala, T., & Korppi, M. (2012). Preschool asthma after bronchiolitis in infancy. European Respiratory Journal, 39(1), 76-80.

II. Koponen, P., Nuolivirta, K., Virta, M., Helminen, M., Hurme, M., & Korppi, M. (2014). Polymorphism of the rs1800896 IL10 promoter gene protects children from post-bronchiolitis asthma. Pediatric Pulmonology, 49(8), 800-806.

III. Koponen, P., He, Q., Helminen, M., Nuolivirta, K., & Korppi, M. (2012). Association of MBL2 polymorphism with asthma after bronchiolitis in infancy. Pediatrics International, 54(5), 619-622.

IV. Koponen, P., Vuononvirta, J., Nuolivirta, K., Helminen, M., He, Q., & Korppi, M. (2014). The association of genetic variants in toll-like receptor 2 subfamily with allergy and asthma after hospitalization for bronchiolitis in infancy. Pediatric Infectious Disease Journal, 33(5), 463-466.

Eur Respir J 2012; 39: 76–80
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Preschool asthma after bronchiolitis in infancy

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ABSTRACT: Asthma risk is lower after wheezing associated with respiratory syncytial virus (RSV) than with non-RSV infection in infancy. RSV is the main wheezing-associated virus in infants aged <6 months. We evaluated the outcome of children hospitalised for bronchiolitis at <6 months of age, with special focus on viral aetiology and early risk factors.

Out of 205 infants hospitalised for bronchiolitis at <6 months of age, 127 (62%) attended a control visit at a mean age of 6.5 yrs and the parents of an additional 39 children were interviewed by telephone. Thus, follow-up data collected by identical structured questionnaires were available from 166 (81%) children. Viral aetiology of bronchiolitis, studied on admission by antigen detection or PCR, was demonstrable in 97% of cases.

Current asthma was present in 21 (12.7%) children: 8.2% in the 110 former RSV patients *versus* 24% in non-RSV patients (p=0.01). 45 (27%) children had ever had asthma. In adjusted analyses, atopic dermatitis, non-RSV bronchiolitis and maternal asthma were independently significant early-life risk factors for asthma.

The risk of asthma was lower after RSV bronchiolitis than after bronchiolitis caused by other viruses in children hospitalised at <6 months of age.

KEYWORDS: Asthma, atopy, bronchiolitis, respiratory syncytial virus, rhinovirus

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Bronchiolitis is the most common lower respiratory infection (LRI) in infancy [1]. The American Academy of Paediatrics has defined bronchiolitis as a disorder in children aged <24 months caused by viral LRI and characterised by acute inflammation, mucus production and bronchospasm of small airways [2]. In most European countries, the upper age limit used, at least in clinical practice, has been 12 months [3]. The severity of bronchiolitis and need for hospital care decrease with increasing age [4]. The viral aetiology of bronchiolitis is age-dependent; respiratory syncytial virus (RSV) is the predominant virus at <6 months and rhinovirus at >12 months of age [5]. Bronchiolitis in infancy increases the asthma risk in later life [6–8]. Studies with outcome data available beyond age 5 yrs have been performed in children aged <36 months [5, 9, 10], <24 months [11, 12] or <12 months [13, 14] on admission; however, there are no age-specific data available for children hospitalised at <6 months of age.

We prospectively followed up a group of children hospitalised for bronchiolitis at <6 months of age in 2001–2002 and 2002–2004 [15, 16]. RSV was the causative agent in 70%, rhinovirus in 7% and other viruses in 7% of the cases [15]. When the children were 5–6 yrs of age, they were invited to a clinical follow-up study in 2008–2009. The hypotheses of the study were that asthma is more common after non-RSV bronchiolitis (especially after rhinovirus bronchiolitis) than after RSV bronchiolitis, and more common after bronchiolitis at <3 months than at 3–6 months of age.

The aim of the present study was to evaluate the outcome with special focus on asthma at preschool age after hospitalisation for RSV, non-RSV and rhinovirus bronchiolitis at <6 months of age. In addition, the age at admission for bronchiolitis and other early risk factors, such as asthma and atopy in parents and atopic dermatitis in children, were analysed as predictors of childhood asthma.

MATERIALS AND METHODS

205 healthy, full-term infants aged <6 months and hospitalised for bronchiolitis at the Dept of Paediatrics, Tampere University Hospital (Tampere, Finland) were enrolled in the study between December 1, 2001 and May 31, 2002 and between October 28, 2002 and May 31, 2004. The Ethics Committee of the Tampere University Hospital District approved the study. Informed consent was obtained from parents before enrolling the children.

Bronchiolitis was characterised by LRI with rhinitis, cough and diffuse wheezes or crackles [15]. The aetiology of bronchiolitis was assessed in nasopharyngeal aspirates by immunofluorescence for

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seven viruses, including RSV, by PCR for nine viruses, including RSV and rhinoviruses, and by PCR for *Bordetella pertussis* [17].

From 2008 to 2009, 127 (62%) children attended the study visit at 5–7 yrs of age. In addition, parents of the 39 children who did not attend the study visit were contacted and interviewed by telephone. Thus, follow-up data collected by identical structured questionnaires were available from 166 (81%) children (fig. 1). Doctor-diagnosed asthma, the age when asthma was diagnosed and the continuous or intermittent use of inhaled corticosteroids (ICS) as maintenance medication for asthma were recorded by year. Intermittent ICS medication means a pre-set, regular use during infections or respiratory symptoms. In addition, data were recorded on parent-reported wheezing episodes and episodes of other asthma-like symptoms, such as prolonged (>4 weeks) cough and night cough apart from infection. The presence of doctor-diagnosed atopic dermatitis and allergic rhinitis was recorded; only cases who were symptomatic during the preceding 12 months were included. In addition, parental doctor-diagnosed asthma and atopy (allergic rhinitis or atopic dermatitis), keeping of indoor furred pets, and parental smoking during and after pregnancy were surveyed. All data were collected separately for mothers and fathers.

Skin-prick tests (SPTs) were performed in 124 children for eight allergens: birch, timothy grass and mugwort pollens, cat and dog dander, house dust mites (*Dermatophagoides pteronyssimus* and *D. farinae*) and spores of the mould *Alternaria alternata*. Wheals with a mean diameter of $\geqslant$3 mm were regarded as positive. Children were not allowed to take any antihistamine medication for 5 days before testing.

Bronchial hyperresponsiveness (BHR) was studied by exercise challenge test (ECT), which consisted of free running outdoors for 8 min and measurements of pre- and post-exercise airway resistance by impulse oscillometry (IOS) (Master Screen IOS; Jaeger, Höchberg, Germany). Exercise was considered sufficient when heart rate, monitored using a heart rate monitor (Polar Ltd, Kempele, Finland), was $\geqslant$90% of the predicted maximum for $\geqslant$2 min. IOS was repeated until three acceptable pre-exercise and two acceptable post-exercise curves were obtained. The resistance curves had to be graphically appropriate and free from artefacts for the whole 30-s measurement time. Resistance values were measured at the 5-Hz level (total respiratory resistance at 5 Hz (R_{rs5})) and expressed as standard deviations from national height-related, sex-specific references [18]. BHR was considered to be present if the best post-exercise R_{rs5} value had increased $\geqslant$35% from the best pre-exercise value [19]. If the child had suffered from an infection during the two preceding weeks, IOS was rescheduled.

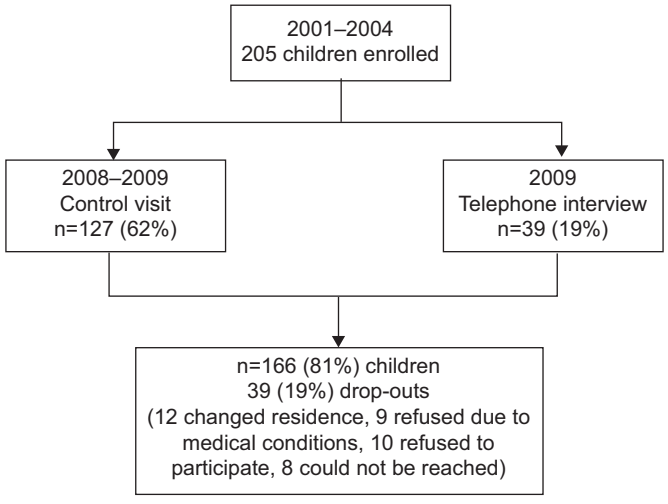

FIGURE 1. Flow chart of the study population.

Current asthma was considered to be present if the child was on continuous maintenance medication for asthma, or if the child had suffered from doctor-diagnosed wheezing or prolonged (>4 weeks) cough or night cough, apart from infection, during the preceding 12 months, and BHR was documented in ECT. Previous asthma before the control visit was defined by the use of ICS as continuous or intermittent maintenance medication for asthma. If the child had either previous or current asthma, the term ''asthma ever in life'' was used.

Statistics

The data were analysed using SPSS 18.0 (IBM, Helsinki, Finland). The statistical significances of differences between the groups were calculated with the unpaired t-test, Chi-squared test and Fisher's exact test. Logistic regression was used to analyse the associations between risk factors and asthma, first by univariate analyses and then by multivariate analyses adjusted for age on admission (<3 *versus* >3 months), sex and characteristics which were significant in univariate analyses. Odds ratios with 95% confidence intervals are reported from both univariate (OR) and multivariate adjusted (aOR) analyses.

RESULTS

The mean$\pm$SD age of the 166 children attending the study was 6.5$\pm$0.57 yrs and 86 (52%) were male. Current asthma was present in 21 (12.7%) children: in 14 males (16.3% of males; p=0.05 *versus* females) and in seven females (8.8% of females). In addition, there were 24 children with no current asthma who had been previously, but not during the preceding 12 months, taking ICS as maintenance medication for asthma. Thus, the number of children with asthma ever in life before or during the study was 45 (27%). The age-specific prevalence and cumulative incidence of asthma, defined by the use of continuous or intermittent ICS, are presented in figure 2. The highest prevalence, 26.9%, was seen at 2–3 yrs of age.

18 children with current asthma had used ICS during the preceding 12 months. 12 children were on continuous and six on intermittent ICS, and two of them also used leukotriene antagonists. Five (24%) children were symptomatic and six (29%) hyperresponsive in ECT despite maintenance medication. Three additional children had symptoms consistent with asthma and were hyperresponsive in ECT, and they were defined to have asthma. BHR was documented in five other children, but none of them reported doctor-diagnosed wheezing or prolonged or night cough. Six (4%) of the nonasthmatic children had suffered from repeated parent-reported wheezing, but none of them reported doctor-diagnosed wheezing, prolonged or night cough, or had BHR in ECT.

RSV had caused 117 (70.5%) and rhinovirus 21 (12.7%) of the 166 bronchiolitis cases (table 1). *B. pertussis* was involved in 10 (6%)

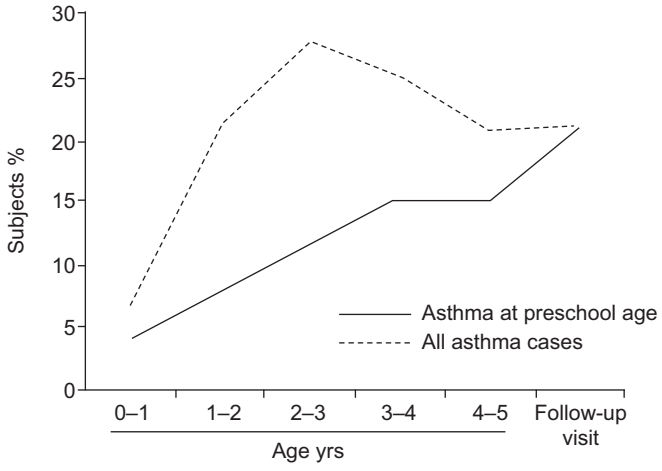

FIGURE 2. The age-specific prevalence and cumulative incidence of asthma, defined by the use of inhaled corticosteroids, in the 166 study subjects.

cases, but all were mixed infections with viruses. Current asthma at 6.5 yrs of age was present in nine (7.7%) of former RSV bronchiolitis patients (*versus* 24.4% of former non-RSV patients; p=0.01), in three (14.3%) former rhinovirus bronchiolitis patients and in one (10%) former *B. pertussis*-positive patients.

Age on admission as a continuous variable (but not categorised into <3 and >3 months), atopic dermatitis at <12 months of age (71.4% *versus* 23.4%; p<0.001) and asthma in mothers (38.1% *versus* 11.0%; p=0.001) but not in fathers were significantly associated with current asthma (table 1). Conversely, maternal smoking, paternal smoking and keeping furred pets at home during infancy had no association with later asthma (table 1).

48 (29%) study children had suffered from symptoms presumptive for allergic rhinitis during the preceding 12 months and 13 (27%) of them had current asthma (*versus* 6.8% in those 118 with no allergic rhinitis; p<0.001). Correspondingly, 61.9% of the 21 children with and 24.1% of those 145 without asthma had allergic rhinitis. SPTs were performed in 124 children; eight (53.3%) out of 15 children with asthma were SPT-positive (*versus* 6.4% of those 109 with no asthma; p=0.07). Birch pollen (22.8%), timothy grass pollen (19.2%), dog dander (12.7%) and cat dander (11.7%) were common, and mugwort pollen (1.0%), house dust mites (1.0%) and spores of moulds (0%) were rare allergens.

Maternal history of asthma was a significant risk factor for asthma in children (table 1). However, 21 (87.5%) out of the 24 mothers with asthma (p=<0.001 *versus* 52 mothers with no asthma) and six (60%) out of the 10 fathers with asthma (p=0.06 *versus* 34 fathers with no asthma) also had doctor-diagnosed allergic rhinitis or atopic dermatitis. The association between parental asthma and atopy was so strong that their independent associations with asthma in children could not be studied, and we included only maternal asthma in the multivariate analyses.

As seen in table 2, non-RSV bronchiolitis was an independent risk factor for preschool asthma in multivariate analyses adjusted for age on admission, sex, atopic dermatitis in infancy and maternal asthma (aOR 3.74, 95% CI 1.28–10.99). Atopic dermatitis in infancy and maternal asthma were other significant risk factors for current asthma in adjusted analyses (table 2).

TABLE 1 Baseline data in 166 children hospitalised for bronchiolitis at <6 months of age, presented in relation to asthma at preschool age

	Current asthma	No asthma	p-value
Subjects n	21	145	
Age at admission days	113 (63–147)	77 (38–118)	0.027
Age at admission months			0.06
<3	9 (42.9)	93 (64.1)	
>3	12 (57.1)	52 (35.9)	
Males	14 (66.7)	72 (49.7)	0.145
RSV bronchiolitis	9 (42.9)	108 (74.5)	0.015
Non-RSV bronchiolitis#	12 (57.1)	37 (25.5)	0.01
Atopic dermatitis at <12 months of age	15 (71.4)	34 (23.4)	<0.001
Maternal smoking during pregnancy	1 (4.8)	28 (19.3)	0.129¶
Maternal history of asthma	8 (38.1)	16 (11.0)	0.001
Paternal history of asthma	0 (0.0)	10 (6.8)	0.231¶
Maternal history of atopy	13 (61.9)	60 (41.4)	0.077
Paternal history of atopy	8 (38.1)	32 (22.1)	0.109
Maternal smoking in infancy	5 (23.8)	42 (29.0)	0.624
Paternal smoking in infancy	9 (42.9)	61 (42.1)	0.946
Furred pet at home in infancy	5 (23.8)	46 (31.7)	0.462

Data are presented as median (interquartile range) or n (%), unless otherwise stated. The t-test was used for continuous variables and Pearson's Chi-squared test was used for categorised variables, unless otherwise stated. RSV: respiratory syncytial virus. #: rhinovirus in three cases, influenza A virus in three cases, parainfluenza type 3 virus in three cases, adenovirus in one case and human metapneumovirus in one case, and two cases with no viral aetiology; ¶: Fisher's exact test.

The analyses were repeated in the subgroup of 124 children with SPT results available by including SPT positivity in the model. SPT positivity was associated with an increased asthma risk in univariate analyses (OR 3.60, 95% CI 1.19–10.9) but not in multivariate analyses (aOR 2.81, 95% CI 0.72–10.9). In these analyses, atopic dermatitis in infancy, non-RSV bronchiolitis and maternal asthma lost statistical significance as risk factors of current asthma (data not shown).

There were no significant differences in baseline characteristics, such as sex, age on admission and viral aetiology of bronchiolitis, between the 166 attendees and the 39 drop-outs (data not shown). Likewise, there were no significant differences in baseline or questionnaire-based characteristics, such as atopy, asthma and smoking in parents, or atopic dermatitis in infancy and allergic rhinitis at preschool age in study children, between those 39 interviewed by telephone and those 127 attending the study visit (data not shown).

DISCUSSION

There are four main results in the present prospective follow-up study at preschool age after hospitalisation for bronchiolitis at <6 months of age. First, asthma prevalence was only 12.7% at a mean age of 6.5 yrs. This figure is lower than the previously reported prevalence figures up to 48% after bronchiolitis in infancy [11–14, 20]. Secondly, atopic dermatitis in infancy was a

TABLE 2 Logistic regression: risk factors for asthma at a mean age of 6.5 yrs

	Subjects	Crude	Multivariate
Age ⩾3 months at admission	64	2.31 (0.91–5.85)	2.04 (0.69–6.04)
Male sex	86	2.03 (0.77–5.32)	2.01 (0.64–6.29)
Atopic dermatitis at <12 months of age	49	8.16 (2.94–22.7)	7.45 (2.45–22.89)
Non-RSV bronchiolitis	50	4.04 (1.57–10.36)	3.74 (1.28–10.99)
Maternal history of asthma	24	4.96 (1.78–13.79)	3.39 (1.03–11.24)

Data are presented as n or odds ratio (95% confidence interval). n=166. Multivariate analyses were performed and adjusted for age on admission, sex, atopic dermatitis in infancy, viral aetiology of bronchiolitis and maternal asthma. RSV: respiratory syncytial virus.

significant risk factor for asthma, in line with earlier post-bronchiolitis studies [12, 21, 22]. Thirdly, asthma in mothers, but not in fathers, was a significant risk factor for asthma. Asthma in mothers was associated more than asthma in other family members with asthma risk in children in birth cohorts [10]. Finally, confirming the study hypothesis, asthma at preschool age was more common after non-RSV bronchiolitis (24%) than after RSV bronchiolitis (8%) in infancy. This observation is in line with previous studies after early-life wheezing from Finland and Wisconsin, USA [9, 20, 23], but we were not able to confirm the specific role of rhinovirus aetiology of bronchiolitis as an asthma predictive factor.

The prevalence of preschool asthma has varied from 15% to 48% in previous post-bronchiolitis studies [11–14, 20], which means a four- to 10-fold increase in asthma prevalence compared with nonselected populations [24]. In earlier post-bronchiolitis studies from Finland and Sweden, the prevalence of asthma was 30% when the infants were hospitalised at <12 months of age [13] and 25–47% when hospitalised at <24 months of age [11, 12, 20]. In birth cohort studies, the prevalence figures have been higher (30–60%) after wheezing in early life, reflecting the inclusion of mild, parent-reported wheezing cases treated at home [9, 10]. In the present study, after hospitalisation for bronchiolitis at <6 months of age, asthma prevalence at preschool age was low (12.7%), and even lower (only 8.9%) after hospitalisation at <3 months of age. In a recent study from Missouri, USA, the cumulative prevalence of parent-reported, doctor-diagnosed asthma by age 6 yrs was as high as 48% after RSV bronchiolitis at age <12 months [14]. The cumulative prevalence in the present study, called asthma ever in life, was not higher than 27% when only cases treated with ICS were included.

Atopic dermatitis in infancy, parental atopy, parental asthma (especially asthma in mothers) and passive smoking (especially smoking mothers) have been linked with an increased risk for later asthma [13, 20, 21, 25]. In this study, one-third of children with atopic dermatitis presenting during the first year of life had asthma at preschool age. The figure is higher than in earlier post-bronchiolitis studies after hospitalisation at <24 months of age, which evidently included children with less severe atopy not presenting in early infancy [12, 20, 22]. Thus, atopy in infancy is an important risk factor for asthma in later life, and invasive RSV infections in infancy may increase, in addition to the risk of asthma, the risk of allergy at early school age [13, 14].

A recent post-bronchiolitis study from Sweden stressed the differences in the harmful effects of maternal and paternal smoking [25]. Maternal smoking led to BHR and reduced lung function, whereas paternal smoking increased the risk of active smoking at teen age. Many studies, like the present study, have not been able to confirm the increased asthma risk after *in utero* or early-life tobacco smoke exposure [13, 21, 23, 26]. A selection bias might have occurred since passive smoking in infancy is a risk factor of bronchiolitis [27]. In the present study, ~30% of mothers and ~40% of fathers smoked, which is more than reported in young Finnish females (20%) and males (30%) [28].

RSV has been shown to be the predominant virus in bronchiolitis in infants aged <6 months and rhinovirus in infants aged >12 months [5]. Asthma risk at preschool age after rhinovirus bronchiolitis has been two- to four-fold compared with RSV bronchiolitis [9, 29]. Consistent with this, only 8.2% of the former RSV bronchiolitis patients in our study had asthma at preschool age; in fact, the figure was close to 4–6% asthma prevalence in a nonselected, age-specific population in Finland [24]. Accordingly, non-RSV bronchiolitis was a significant risk factor for preschool asthma, even after adjustment with potential confounding factors. However, no single virus was predominant in the former non-RSV group with current asthma. The mechanisms behind the link from bronchiolitis in infancy to asthma in childhood are not known. Non-RSV bronchiolitis most probably reveals susceptible infants rather than directly causes later asthma [9, 21, 29]. The role of rhinoviruses as an asthma-predicting factor may be age dependent (not seen in bronchiolitis patients aged <6 months).

The study of SIGURS *et al*. [13] is the only post-bronchiolitis follow-up comparable with the present study. In that study, 47 former RSV bronchiolitis patients hospitalised at <12 months of age attended the control visit at the median age of 7.5 yrs, and 23% of them had asthma, compared with 3% in controls. In the present study, asthma prevalence at the mean age of 6.5 yrs was substantially lower, only 8.2% among 117 children hospitalised for RSV bronchiolitis at <6 months of age. In addition, SIGURS *et al*. [13] reported that 20% of former RSV bronchiolitis patients were sensitised to inhaled allergens documented by SPTs, compared with 6% in controls. In our study, former RSV and non-RSV patients had the same SPT positivity rate (29%). In the Swedish study, parental asthma was present in 45% of the infants with bronchiolitis, compared with 20% in our patients, which may partly explain the differences in outcome at preschool age.

The main strengths of the present post-bronchiolitis study are the prospective design, and large number and homogeneity of the enrolled patients; all were <6 months of age, all needed hospital care and 166 children were followed up over 5 yrs. When bronchiolitis is defined as viral LRI with wheezing at <24 months of age, which has been the practice in most earlier studies, the study population is more heterogeneous, consisting of patients with bronchiolitis, reactive airway disease and early-onset asthma.

The main shortcoming of the present study is that population-based controls were not enrolled. However, the age-specific prevalence of asthma in Finnish children is well known, being 4–6% at preschool age [24]. In addition, many subgroups were rather small and thus, the study was underpowered to find many obvious associations. Data on atopic dermatitis and family history of asthma and atopy were carefully collected, but no tests were available for allergen-specific immunoglobulin E, eosinophils or eosinophilic markers, which are well-known risk factors of childhood asthma after bronchiolitis [21, 30]. However, later asthma was so rare and atopic dermatitis in infancy was such a strong predictive factor that any additional data on risk factors would not have changed the main conclusions of the study.

In conclusion, asthma prevalence was low (only 12.7%) at the mean age of 6.5 yrs after hospitalisation for bronchiolitis at <6 months of age. In agreement with the study hypothesis, non-RSV aetiology of bronchiolitis was an independently significant risk factor of asthma in adjusted analyses, but in disagreement with the study hypothesis, age >3 months compared with age <3 months was not.

SUPPORT STATEMENT

This study was supported by the University Hospital of Tampere and Tampere Tuberculosis Foundation (Tampere, Finland).

STATEMENT OF INTEREST

None declared.

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Polymorphism of the rs1800896 *IL10* Promoter Gene Protects Children From Post-Bronchiolitis Asthma

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Summary. Viral bronchiolitis is a major cause of hospitalization in infancy, with increased asthma risk in later childhood. However, the principal mechanisms behind post-bronchiolitic asthma have remained unclear. Previously, different cytokine polymorphisms have been associated with asthma occurrence, but no previous follow-up study has investigated cytokine polymorphisms in relation to post-bronchiolitic asthma. We hypothesized that former bronchiolitis patients with cytokine gene variants associating with Th2 cell up-regulation are at asthma risk at preschool age. Our emphasis was in *IL10* rs1800896, since IL-10 has an important role in immune tolerance, and lower production of IL-10 has been associated with Th2-type immunology, and accordingly, with increased asthma risk. *IL10* rs1800896, *IFNG* rs2430561, and *IL18* rs1872387 polymorphims and their associations with asthma and allergy were studied in 135 preschool-aged children hospitalized for bronchiolitis at age 0–6 months. Parents were interviewed to record asthma and allergy from infancy to present. At age 6.4 years (mean), asthma was present in 17(12.6%), atopic eczema in 47(34.8%) and allergic rhinitis in 36(26.7%) children. *IL10* rs1800896 SNP associated significantly with asthma; only 1/32 (3.1%) of those with G/G genotype had asthma ($P = 0.04$). In logistic regression adjusted for gender, age and atopy, the carriage of allele A (rs1800896) was a significant risk factor for preschool asthma. *IFNG* rs2430561 or *IL18* rs1872387 SNP's had no associations with asthma or allergy. In conclusion, *IL10* rs1800896 SNP was significantly associated with preschool asthma after severe lower respiratory tract infection in early infancy.
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INTRODUCTION

Acute bronchiolitis is a common cause for hospitalization among infants, and in follow-up studies, 30–40% of hospitalized post-bronchiolitis patients have developed chronic asthma continuing until adulthood.[1] On the other hand, the great majority of early-life viral infections do not present with respiratory distress or have no association with increased wheezing or asthma risk in later life. For example, seroconversion to respiratory syncytial virus (RSV) takes place in 69% of the children during the first year and in 83% during the second year of life.[2] Thus, there evidently are marked individual variations in genetic factors and genetically regulated immune responses to viral infections with an impact on both the clinical picture of infection and on the later outcomes. An early viral infection may impair regulatory T cells (Treg) which leads to Th2-oriented immunity and further to increased asthma susceptibility.[3]

Interleukin-10 (IL-10) polymorphism has been linked with the etiology and severity of bronchiolitis.[4–6] Further, polymorphisms in different cytokines, including both pro- and anti-inflammatory cytokines, have been linked to increased asthma prevalence.[7,8] IL-10 is a key factor in the development of immune tolerance with a regulatory role in both inflammatory and anti-inflammatory processes.[7] Polymorphism in *IL10* gene affects the level of IL-10 production so that the carriage of allele G (rs1800896) is

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associated with higher IL-10 production. A low amount of mononuclear cell secreting IL-10 has increased the risk of later wheezing[9,10] and in line, rs1800896 *IL10* promoter polymorphism has been connected to asthma and atopic dermatitis in several studies.[11,12]

Interferon-gamma (IFN-γ) is a pro-inflammatory cytokine associated with Th1-type responses especially when protecting against viral pathogens. The production of IFN-γ is genetically controlled so that the rs2430561 A allele is associated with lower cytokine production.[13] In ex vivo studies, diminished production of IFN-γ in early life has been associated with an increased risk of later atopy and/or asthma.[14,15] Interleukin-18 (IL-18) is a pro-inflammatory cytokine modulating immunologic responses into allergic and autoimmune directions through both Th1-type and Th2-type pathways.[16] Previous studies on *IL18* rs187238 polymorphism and asthma risk have reported inconsistent results,[17] but the most recent study demonstrated a protective association between *IL18* rs187238 polymorphism and adult asthma.[18]

We demonstrated earlier that during the first 1.5 years infants who were homozygous for the allele A *IL10* (rs1800896) were at greater risk for rhinovirus-bronchiolitis at less than 0.5 years of age[5] and the carriage of allele A *IFNG* (rs2430561) was protective from repeated respiratory infections.[19]

The aim of the present study was to evaluate the associations between preschool-age asthma and polymorphisms in *IL10* rs1800896, *IFNG* rs2430561, or *IL18* rs1872387 after hospitalization for bronchiolitis in early infancy. We hypothesized that promoter variants in cytokines that change the Th1/Th2 balance into the Th2 direction, are associated with higher preschool asthma risk. The main focus was in the single nucleotide polymorphism (SNP) *IL10* rs1800896.

MATERIAL AND METHODS

Full-term infants under 6 months of age, who were hospitalized for bronchiolitis in the Department of Pediatrics, Tampere University Hospital, Finland, were included in the study. The enrollment periods were between December 1, 2001 and May 31, 2002 and between October 28, 2002 and May 31, 2004. Bronchiolitis was defined as lower respiratory infection (LRI) with rhinitis, cough, and diffuse wheezes or crackles.[20]

A total of 166 children attended the follow-up visit at 5–7 years of age, and parents were interviewed using a structured questionnaire that reviewed asthma and allergic symptoms from early infancy to present. The diagnosis of asthma and age when the diagnosis had been settled was recorded, as well as the use of bronchodilators and inhaled corticosteroids (ICS). Doctor-diagnosed asthma was considered to be present if the child was on continuous ICS for asthma medication, or if there had been at least one period of doctor-diagnosed wheezing, prolonged cough or night cough without infection during the preceding 12 months and in addition, bronchial hyperreactivity (BHR) was detected in exercise challenge test.[20] Parent-reported allergic rhinitis and atopic eczema were asked, and only doctor-diagnosed cases symptomatic during the preceding 12 months were registered. Atopy was defined by the presence of either allergic rhinitis or atopic eczema. Allergic rhinitis was defined as episodes of watery nasal discharge not accompanied by fever or by other symptoms of respiratory tract infection.[21] This study was approved by the Ethics Committee of the Tampere University Hospital District and informed consent was obtained from parents before enrolling the children.

Genotyping of Cytokine Polymorphisms

A total of 135 (81.3%) frozen whole blood samples were available for cytokine genotyping. DNA was extracted from whole blood using a commercially available kit (Qiagen Inc., CA) according to the manufacturer's instructions at the Department of Clinical Microbiology, Tampere University, Finland. The rest of the genomic DNA was frozen at −70°C. Genotyping of the IL10 rs1800896 and IL18 rs187238 gene polymorphism was performed using the ABI PRISM® 7000 Sequence Detection System for both PCR and allelic discrimination (Applied Biosystems, Carlsbad, CA). For IL18 rs187238 analysis, 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 IL10 rs1800896, a commercial kit was used (Assay on Demand, C_1747360_10 IL10). The universal PCR thermal cycling conditions from ABI were as follows: 50°C for 2 min, then 95°C for 10 min, and then 40 cycles at 95°C for 15 sec and 60°C for 1 min. The PCR reaction was done in a 25 μl volume containing TaqMan® Universal PCR Master Mix with AmpErase® UNG (ABI), 1× Assay Mix (primers and probes: ABI) and 10–100 ng of template DNA. The genotypes were selected manually from the allelic discrimination tab.

IFNG rs2430561 polymorphism was analyzed by the amplification refractory mutational system-PCR method.[19] 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 of reaction and the

conditions were as follows: 95°C for 1 min, then 10 cycles of 95°C for 15 sec, 62°C for 50 sec, and 72°C for 40 sec, followed by 20 cycles of 95°C for 20 sec, 56°C for 50 sec, and 72°C for 50 sec. The amplified products were analyzed by Agilent DNA 1000 Kit according to the manufacturer's instructions.

The data were analyzed using IBM PASW Statistics 18.0. Exploratory data analysis showed that the continuous variables were normally distributed. The statistical significances of the differences between the groups were calculated with *t*-test for continuous variables, and with chi-square test, or Fisher's exact test when appropriate, for discrete variables. Logistic regression was used to analyze the associations between cytokine genotypes and asthma, first by univariate modeling, and then by multivariate modeling adjusted for age at follow-up (<6 years vs. >6 years), gender and current atopy, presented as adjusted odds ratios (aOR) with 95% confidence intervals (95% CI).

The Hardy–Weinberg equilibrium (HWE) for the genotypic distribution was determined by the HWE exact test. *IL10* rs1800896, *IFNG* rs2430561, and *IL18* rs187238 genotypes were in HWE ($P > 0.05$).

RESULTS

The mean age of the 135 study subjects was 6.4 years (SD 0.48), and 69 (51%) were males and 66 females. Altogether, 69 (51%) children had the *IL10* rs1800896 G/A genotype, and 34 (25%) had A/A and 32 (24%) G/G genotypes. Sixty-eight (50.4%) children had the *IFNG* rs2430561 T/A, 49 (36.3%) the T/T and 18 (13.3%) the A/A genotype. Further, 68(50.4%) children had the *IL18* rs187238 G/C, 60(44.4%) had the C/C and 7(5.2%) the G/G genotype (Table 1).

At age of 5–7 years, asthma was present in 17(12.6%), atopic eczema in 47(34.8%) and allergic rhinitis in 36 (26.7%) children. Those who were homozygous for rs1800896 allele G were rarely asthmatics; only 1/32 (3.1%) had doctor-diagnosed asthma at 5–7 years of age ($P = 0.04$ vs. 15.5% of the 103 allele A carriers; Table 1). Carriage of *IL10* rs1800896 allele A was a significant risk factor for preschool asthma even after adjustments with the most important potential confounders: gender, age on admission, and current atopy (Table 2).

Ten % of those with *IL10* rs1800896 allele G versus 21% of non-carriers had doctor-diagnosed current asthma at preschool age ($P = 0.07$). In line, 19/34 (56%) of allele-G non-carriers versus 38/101 (37.6%) of allele-G carriers had current atopy at the control visit ($P = 0.06$; Table 1). Altogether 19 (14.1%) children had doctor-diagnosed asthma between 1 and 2 years of age, being present in 10/101 (9.9%) *IL10* G-allele carries versus 9/34 (26.5%) non-carriers ($P = 0.02$; Table 3).

IFNG rs2430561 or *IL18* rs1872387 polymorphims had no significant associations with asthma at any age, or with allergic rhinitis or atopic eczema (Table 1).

Ninety-eight (72.6%) patients had been hospitalized for RSV and 18(13%) for rhinovirus bronchiolitis. Among those infected with rhinovirus, five had the homozygous

TABLE 1— Clinical Characteristics of 135 Study Children in Relation to *IL10* rs1800896, *IL18* rs187238, or *IFNG* rs2430561 Genotypes, and Asthma or Atopic Findings During Follow-Up Visit at 5–6 Years of Age

Cytokine genotype	Asthma at follow-up (n = 17)	Asthma between 1 and 6 years (N = 39)	Atopic eczema at follow-up (n = 47)	Allergic rhinitis at follow-up (n = 36)	Atopy at follow-up (N = 57)
IL10 rs1800896					
A/A n=34	7 (41.2%)	11 (28.2)	15 (31.9%)	11 (30.6%)	19 (33.3%)[¥]
G/A n=69	9 (52.9%)	22 (56.4)	20 (42.6%)	16 (44.4%)	25 (43.9%)
G/G n=32	1 (5.9%)[*]	6 (15.3%)	12 (25.5%)	9 (25%)	13 (22.8%)
G allele n=101	10 (58.8)	28 (71%)	32 (68.1%)	25 (69.4%)	38 (66.7%)[¥]
A allele n=103	16 (94.1)[*]	33 (84.6)	35 (74.5)	27 (75)	44 (77.2)
IL18 rs187238					
G/G n=7	1 (5.9%)	3 (7.7%)	3 (6.4%)	2 (5.6%)	3 (5.3%)
G/C n=68	11 (64.7)	18 (46.2%)	27 (57.4%)	21 (58.3%)	32 (56.1%)
C/C n=60	5 (29.4%)	18 (46.2%)	17 (36.2%)	13 (36.1%)	22 (38.6%)
G allele n=75	12 (70.6)	21 (53.8%)	30 (63.8%)	23 (63.9%)	35 (61.4%)
C allele n=128	16 (94.1)	35 (89.7)	42 (89.4)	33 (91.7)	52 (91.2)
IFNG rs2430561					
T/T n=49	7 (41.1%)[†]	15 (39%)	15 (32.6%)	14 (38.9%)	22 (38.6%)
T/A n=68	10 (58.8%)	21 (53.8%)	25 (54.3%)	18 (50%)	29 (50.9%)
A/A n=18	0	3 (7.7%)	6 (13%)	4 (11.1%)	6 (10.5%)
A allele n=86	12 (71%)[†]	24 (61.5%)	31 (67.4%)	22 (61.1%)	35 (61.4%)
T allele n=116	17 (100)	36 (92.3)	40 (87)	32 (88.9)	50 (89.3)

The percentages within brackets: children with the genotype in % out of children with the disease.
Pearson's chi-squared test or Fisher's exact test was used for categorised variables.
[*]$P = 0.04$, [¥]$P = 0.06$, [†]$P = 0.07$, other results are statistically non-significant.

TABLE 2— Logistic Regression: *IL10* rs1800896 Allele A Carriage as a Risk Factor for Preschool Asthma

	n	Asthma	aOR (95% Cl)
Gender (male)	69	12	1.98 (0.73–5.82)
Age (<6 years)	77	8	2.12 (0.82–6.52)
IL10 rs1800896 allele A carriage	103	16	1.84 (1.2–3.87)
Atopy at follow-up	57	12	3.25 (1.65–7.84)

IL10 rs1800896 allele A carriage, gender, age at follow-up (<6 years), and atopy at follow-up in the same analysis; aOR, adjusted odd ratio; 95% CI, 95% confidence interval.

A/A genotype of *IL10* rs1800896, and 4/5 (80%) had doctor-diagnosed asthma at some time during childhood ($P = 0.014$ vs. 12.5% of the 13 allele-G carriers; Table 4).

DISCUSSION

The main result of the present post-bronchiolitis long-term follow-up study is that the children who are non-carriers of *IL10* rs1800896 allele G are at increased risk for preschool asthma. This means that the presence of the major allele G, either as homozygous or heterozygous, may be protective from asthma. Though there is increasing evidence on an association of *IL10* rs1800896 with susceptibility to asthma and certain infections,[7] the associations of *IL10* polymorphisms with post-bronchiolitis asthma has not been studied previously.

In this post-bronchiolitis cohort, the absence of rs1800896 allele G, that is the A/A genotype, was associated with rhinovirus etiology of bronchiolitis,[5] but not with subsequent wheezing at age 6–18 months.[19] In the most recent study, detection of multiple SNPs in the IL10 gene, including the SNP rs1800893 which is in total linkage with rs1800896 we applied in this study, was associated with wheezing and atopic eczema at age 3 years in a birth cohort of 200 infants.[22] In addition, we found two other noteworthy but this far preliminary associations. The carriage of rs2430561 *IFNG* allele-A resulted in slightly lower asthma prevalence but the finding did not reach statistical significance. In our subgroup analyses, the occurrence of pre-school asthma was as high as 80% in children with low producing *IL10* rs1800896 genotype A/A hospitalized for rhinovirus bronchiolitis in early infancy.

TABLE 3— Doctor-Diagnosed Asthma in 135 Study Children During First 5 Years of Age in Relation to AlleleG Carriage of *IL10* rs1800896

IL10 rs1800896	G-allele carrier, n = 101	G-allele non-carrier, n = 34	*P*-value
Asthma at 1–2 years, n = 19	10 (9.9%)	9 (26.5%)	0.02
Asthma at 2–3 years, n = 23	15 (14.9%)	8 (23.5%)	0.244
Asthma at 3–4 years, n = 18	13 (12.9%)	5 (14.7%)	0.775*
Asthma at 4–5 years, n = 11	7 (6.9%)	4 (13.3%)	0.47*

Chi-squared test and *Fisher's exact test.

IL10 rs1800896 polymorphism has been shown to affect IL-10 protein production,[23] and a recent meta-analysis of 18 case-control studies revealed a significant association with asthma susceptibility. The low-producing genotype A/A carried a 1.27-fold (95% CI 1.01–1.60) asthma risk compared with genotypes A/G and G/G.[24] This is in line with our observations on asthma in children after early-life bronchiolitis; doctor-diagnosed asthma at preschool age was present only in 3.1% of children who were homozygous for allele G in *IL10* rs1800896, compared to 15.0% of those with other genotypes. Further, the carriage of allele A was significantly associated with increased asthma risk even after adjustment for potential confounding factors gender, age and atopy at follow-up. *IL10* SNPs were studied in 330 children, including 110 atopic and 110 non-atopic asthmatics with a mean age of 8.5 years and 110 controls with a mean age of 10.5 years.[11] In accordance with our results, children with G/G genotype of rs1800896 were almost 50% less likely to have atopic asthma than children with other genotypes.

IL-10 is an immune regulatory cytokine and down-regulates both Th1 and Th2 responses. Low IL-10 production in infancy may lead to a delayed shift of the Th2-oriented immunity to Th1-oriented immunity, which may be the mechanism beyond the increased asthma risk due to *IL10* polymorphisms.[25] Lower levels of secreted IL-10 with less Th1-type responsiveness allow more invasive virus infections, also in the case of rhinovirus. The continuation of Th2-type responsiveness leads to less immunological tolerance with an increased risk of asthma and allergy.[25] In a recent study, *IL10* rs1800893 polymorphism was associated with down-regulation of the markers of Treg cells, and presence of wheezing and/or symptoms of atopic eczema at age 3 years.[22] According to the international HapMap data, rs1800893 is in total linkage with rs1800896 which was determined in the present study. These results are in agreement with the main observation of the present study, as discussed recently.[26]

In an earlier follow-up of this cohort at 1.5 years of age, the presence of allele A of *IFNG* rs2430561 was associated with less repeated viral respiratory infections and also with less corticosteroid use after hospitalization for bronchiolitis.[19] Thus, earlier results showed preliminary evidence that the presence of allele A, meaning a low producing genotype,[13] could be

TABLE 4— *IFNG* and *IL18* Polymorphisms, and Pre-School Asthma and Atopy, in Five Children With *IL10* rs1800896 Genotype Hospitalized for Rhinovirus Bronchiolitis in Infancy

IL10 rs1800896	*IFNG* rs2430561	*IL18* rs187238	Asthma at follow-up[1]	Atopy at follow-up[2]	Allergic rhinitis at follow-up[3]
A/A	T/T	C/C	−	−	−
A/A	T/A	C/C	+	−	−
A/A	T/T	G/G	+	+	+
A/A	T/A	G/C	+	+	+
A/A	T/A	G/C	+	+	+

[1]Doctor-diagnosed asthma at follow-up.
[2]Doctor-diagnosed atopic eczema during last 12 months.
[3]Doctor-diagnosed allergic rhinitis during last 12 months.

protective from the development of wheezing after severe respiratory infection in infancy. In line, the present results suggest that the carriage of *IL10* rs 2430561 allele A increased asthma risk at preschool age. The associations between *IFNG* polymorphisms and asthma in studies not related to early-life respiratory infections have been inconsistent, including both indifferent results[27,28] and asthma risk favoring[29,30] findings. In our cohort, a strong immune reaction provoked by an early viral infection can have acted as a catalyst that modified inflammatory reactions to promote later wheezing tendency and even chronic asthma. Accordingly, those children with lower IFN-γ production may present with more controlled reactions to early-life infections, which may even protect them from later disorders.

IL-18 has a regulatory role in both Th1- and Th2-mediated diseases.[16] IL-18 serum levels were higher among Japanese adult asthmatics than among controls.[31] However, there were no significant differences between German asthmatic and non-asthmatic children in *IL18* SNPs.[17] In a recent study in 410 Indian adults with asthma and in 414 healthy controls, the homozygous *IL18* rs187238 C/C genotypes were significantly more prevalent in controls,[12] in accordance with the present results where C/C genotype was 1.5 times more frequent among controls than asthmatics.

IL-10 is a Treg-type anti-inflammatory cytokine, and IL-10 secretion is elevated during acute virus infections.[25] Therefore it seems that high amounts of IL-10 are needed to control virus- inflammatory responses. In many studies, rhinovirus has been the most common virus triggering wheezing in children with asthma,[32,33] and subsequent wheezing and asthma have been more common after rhinoviral than after other viral wheezing in infancy.[34,35] In the present study, nearly all infants with low IL-10 producing genotype had asthma before school age if they had been hospitalized for rhinovirus bronchiolitis. A low capacity to control rhinovirus-induced inflammation in the airways in infancy seems to play even a pathogenetic role for asthma. In addition, low IL-10 production may favor early allergic inflammation in the airways which may further increase rhinovirus-induced inflammation. Adult asthma patients had an inverse association between IL-10 secretion and respiratory symptoms or pulmonary function during rhinovirus infection.[36] In a study from Japan, IL-10 levels in cultured peripheral blood mononuclear cells were lower if obtained from asthmatic children during rhinovirus infection and, interestingly, five children who outgrew their wheezing symptoms at age 2–6 years had higher IL-10 levels after rhinovirus exposure than those eight with persistent wheezing.[37] Thus, based on earlier and our present findings, IL-10 plays an important role in development of childhood asthma.

The main strengths of the present study are the relatively long follow-up time that extended up to 7 years, and the homogeneity of the study subjects. All children were of Finnish origin, and hospitalized for bronchiolitis at age under 6 months. The main weakness of our study was the lack of a control group. However, the distributions of *IL10* rs 1800896, *IFGN* rs2430561, and *IL18* rs187238 SNPs in our cohort were similar to the Finnish population data.[5] In addition, the focus of the study was in the within-study comparison between preschool asthmatics and non-asthmatics. Genetic data were not available from 19% of follow-up attendees and due to the low age of bronchiolitis patients, only 13% had rhinovirus bronchiolitis.

In conclusion, IL-10 plays an important role in the development of asthma, at least in former bronchiolitis patients. Based on the present results, IFN-γ and IL-18 seem to have less impact in this context. The interaction between the *IL10* gene and early-life rhinovirus exposure may be a key question when solving the pathogenesis of childhood asthma.

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Original Article

Association of *MBL2* polymorphism with asthma after bronchiolitis in infancy

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Abstract ***Background***: Mannose-binding lectin (*MBL*) is a component of innate immunity and has been linked with the pathogenesis of asthma. The aim of the present study was to evaluate the association of *MBL* genotypes with preschool asthma and allergy in children with bronchiolitis in early infancy.

Methods: In all, 205 infants were hospitalized for bronchiolitis at <6 months of age. Asthma and allergy were studied from a total of 166 children at 6.4 years (mean). A total of 141 (85%) frozen whole blood samples were available for *MBL* genotyping and *MBL2* gene mutations were determined on pyrosequencing for detection of three single-nucleotide polymorphisms.

Results: Ninety-five children (67.4%) had the wild-type *MBL* genotype A/A and 46 had A/O or O/O genotypes. Asthma was present in 16 children (11.3%) at 5–7 years of age. Nine children (19.6%) with non-AA genotype had asthma (vs 7.4% of those with genotype AA, $P = 0.03$). The result remained significant after adjustment for age, gender and atopy. There were no significant associations between *MBL* genotypes and asthma at any age before the study. Atopic dermatitis, allergic rhinitis or paternal and/or maternal asthma had no significant associations with *MBL* genotypes.

Conclusions: The variant non-A/A *MBL* genotype is associated with asthma after bronchiolitis in infancy, but not earlier than at 5–7 years of age.

Key words bronchiolitis, mannose-binding lectin, pediatric asthma, pulmonology, respiratory syncytial virus.

Birth cohort and post-bronchiolitis follow-up studies have shown an elevated risk for later asthma after bronchiolitis in infancy.[1–3] Evidently, both genetic and environmental factors play a role in asthma development. *Mannose-binding lectin* (*MBL*), a recognition molecule of the lectin pathway of the complement cascade, is an important component of innate immunity and seems to be involved in the pathogenesis of many acute and chronic infectious and inflammatory diseases.[1,2] In previous studies, however, the results on the association between asthma and serum *MBL* levels, *MBL* genotypes or *MBL* gene polymorphisms have been conflicting.[4–9] *MBL* gene polymorphisms have also been linked with atopy[3,10] and in mouse studies, with the development of airway responsiveness.[11]

Innate immunity is particularly important during the first year of life when the antibodies of maternal origin disappear, and when the child's own adaptive immunity is not yet fully established.[4,5] Polymorphisms in the *MBL*-regulating structural gene (*MBL2*) may cause reduced *MBL* production, which further can lead to various consequences such as increased infection susceptibility.[6,7] The wild-type allele is referred to as A, with variants collectively named as O.[4] Individuals with homozygous O/O or heterozygous A/O genotypes may be classified as deficient or low *MBL* producers, and those carrying the homozygous wild-type A/A genotype are considered to be higher *MBL* producers.[10]

We prospectively followed until 5–7 years of age a group of children hospitalized for bronchiolitis at <6 months of age.[12] The aim of the present study was to evaluate the association of MBL genotypes with preschool asthma and allergy in this child group with bronchiolitis in early infancy.

Methods

In all, 205 full-term infants aged <6 months who were hospitalized for bronchiolitis in the Department of Pediatrics, Tampere University Hospital (Finland) were enrolled in the study between 1 December 2001 and 31 May 2002, and between 28 October 2002 and 31 May 2004. Bronchiolitis was defined as lower respiratory infection (LRI) with rhinitis, cough and diffuse wheezes or crackles.[8] Altogether 139 infants (68%) were diagnosed with respiratory syncytial virus (RSV) infection.

Asthma and allergy histories, covering the time from hospitalization at <6 months of age until the study visit in 2008–2009 at 5–7 years of age, were charted by interviewing the parents using structured questionnaires.[12] Doctor-diagnosed asthma, the age when asthma had been diagnosed and the use of inhaled corticosteroids (ICS) as maintenance medication for asthma were

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Table 1 Asthma and allergy at 5–7 years of age, vs *MBL* genotype[†]

MBL genotype	A/A (n = 95) n (%)	A/O or O/O (n = 46) n (%)	P[‡]
Asthma diagnosis at 1–7 years of life	24 (25.3)	14 (30.4)	0.516[‡]
Asthma diagnosis at the control visit	7 (7.4)	9 (19.6)	0.032[‡]
Allergic rhinitis	28 (29.5)	12 (26.1)	0.676[‡]
Atopic dermatitis	35 (36.8)	12 (26.1)	0.2[‡]
Atopy at the control visit	45 (47.4)	19 (41.3)	0.188[‡]
Asthma at the control visit, no atopy	0	3 (6.5)	0.03[§]

[†]In children hospitalized for bronchiolitis at <6 months of age. [‡]Pearson's χ^2 test; [§]Fisher's exact test.
For definitions of allergic rhinitis, atopic dermatitis and atopy, see the text. MBL, mannose-binding lectin.

registered by year. At the control visit, asthma was considered to be present if the child was on continuous asthma medication, or if the child had suffered from doctor-diagnosed wheezing or from prolonged (>4 weeks) cough or night cough apart from infection during the preceding 12 months, and bronchial hyperreactivity was documented on exercise challenge test.[12] Atopy was considered if the patient had allergic rhinitis or atopic dermatitis; although parent reported, only doctor-diagnosed cases and those symptomatic during the preceding 12 months were included.

Skin prick tests (SPT) were done in 118 children for eight allergens: birch, timothy grass and mugwort pollens, cat and dog dander, house dust mites (*Dermatophagoides pteronyssimus* and *D. farinae*) and spores of the mold *Alternaria alternata*. As published recently, wheals with a mean diameter of ≥3 mm were regarded as positive, and no reactions were accepted to negative control.[13]

Genotyping of MBL2 *gene polymorphism*

In all, 141 frozen whole blood samples (85%) were available for *MBL* genetics. The genotyping of *MBL2* structural gene mutations was done on pyrosequencing for simultaneous detection of three single-nucleotide polymorphisms (SNP). In short: the genomic DNA was purified from peripheral blood using the commercial kit (QiagenR, Hilden, Germany), 200 μL DNA was collected and 1 μL (20 ng) was used for amplification of the first exon of the *MBL2* gene on polymerase chain reaction (PCR). The PCR product was used for pyrosequencing,[9] and NTP was sequentially added in the following order: CTCTGTGTCATCACAGC. Three functional SNP in exon 1 of the *MBL2* gene can be detected in the same pyrosequencing reaction, resulting in pyrograms with unique patterns for each allele combination.

Ethics

The Ethics Committee of the Tampere University Hospital District approved the study. An informed consent was obtained from parents before enrolling the children.

Statistics

The data were analyzed using IBM SPSS 18.0 (SPSS, Chicago, IL, USA). The statistical significance of differences between the groups was calculated with *t*-test, chi-squared test and Fisher's exact test. Logistic regression was used to analyze the associations between *MBL* genotypes and asthma, first on univariate analysis and then on multivariate analysis adjusted for age at control visit (<6 years vs >6 years), gender and current atopy (allergic rhinitis or atopic dermatitis). Odds ratios (OR) with 95% confidence intervals (95%CI) are reported from adjusted OR analysis.

Results

The mean age of the 141 attending children was 6.4 ± 0.48 years. Ninety-five patients (67.4%) had the wild-type *MBL* genotype A/A, and 43 (30.5%) had variant A/O and three (2.1%) variant O/O genotypes (Table 1). Among 282 alleles, allele A comprised 82%, B 10%, C 0.7% and D 6% (Table 2). Maternal asthma, atopic dermatitis in infancy and the association of bronchiolitis with viruses other than RSV were the early-life risk factors for asthma at 5–7 years of age.[13] The proportion of the wild-type A/A genotype was 54.5% in the case of maternal asthma, 58.1% in the case of atopic dermatitis in infancy and 65.9% in the case of non-RSV bronchiolitis, respectively. There were no significant associations between any of these risk factors and the *MBL* genotype or the presence of A, B, C or D alleles (data not shown).

Asthma was considered to be present in 16 children (11.3%) at the control visit at 5–7 years of age. Nine children (19.6%) with non-AA genotype had asthma (vs 7.4% of those with genotype AA, P = 0.032; Table 1). There were no significant clinical differences between current asthmatic children according to *MBL* genotype (Table 3). In addition, there were no significant associations between *MBL* genotype and asthma at any age from 1 to 5 years before the study (Table 4). The association between asthma at 5–7 years of age and non-AA genotype was robust with regard to adjustments with potential confounding factors of gender, age at control visit and current atopy; adjusted OR was 3.15 (95%CI: 1.04–9.56; Table 5). Current atopy was present in 47.4% of the children with AA genotype and in 41.3% of those with non-AA genotype (Table 1). Asthma with no atopy was

Table 2 *MBL2* alleles vs presence of asthma at the control visit

MBL2 alleles	Asthma (n = 16) n (%)	No asthma (n = 125)	P
A (n = 137)	16 (11.7)	121	0.468[†]
B (n = 28)	3 (10.7)	25	0.906[†]
C (n = 2)	2 (100)	0	0.12[‡]
D (n = 18)	4 (22.2)	14	0.119[†]

[†]Pearson's χ^2 test; [‡]Fisher's exact test. MBL2, *MBL*-regulating structural gene.

Table 3 Asthma subject characteristics vs *MBL* genotype at control visit

MBL genotype	Asthma with A/A genotype (n = 7/95) n (%)	Asthma with A/O or O/O genotype (n = 9/46) n (%)	P
Age (years), mean ± SD	6.24 ± 0.38	6.45 ± 0.4	0.35†
Gender (male)	4 (57.1)	6 (66.7)	0.52†
Atopic dermatitis	5 (71.4)	6 (66.7)	0.07†
Allergic rhinitis	5 (71.4)	5 (55.6)	0.3‡
≥1 Skin prick test positivity	5 (71.4)	5 (55.6)	0.42†
Maternal asthma	4 (57.1)	5 (55.6)	0.61‡

†Pearson's χ^2 test; ‡Fisher's exact test. MBL, mannose-binding lectin.

present in only three children, and none of them had the A/A genotype (Table 1). Only two carriers of allele C were found and both of them had current atopic asthma.

The 118 children with SPT results available were included in a supplementary analysis. Seventy-eight had A/A genotype and 21 of them (26.9%) were SPT positive (vs 22.5% of those with non-A/A genotype, P = 0.5). Thirteen children had asthma, and six of them were SPT positive; the figures are too low for statistical analysis.

Discussion

The main result is the finding of an association between the non-wild variant *MBL* genotype and asthma at the mean age of 6.4 years after bronchiolitis in early infancy. The finding was reliable because the association was robust with regard to potential confounding factors such as atopy. The previous studies have reported conflicting results on the association between *MBL* gene polymorphisms and asthma in children and in adults.[6,7,14–17] Instead, there were no significant associations between *MBL* genotype and allergic rhinitis, maternal and/or paternal asthma, atopic dermatitis or SPT positivity. In line with this, none of the non-atopic asthmatic children had the wild-type A/A genotype. These observations are in disagreement with earlier findings, which have suggested a link between *MBL* polymorphism and the development of atopy.[3,10] The distribution of *MBL* genotypes among children hospitalized for bronchiolitis in early infancy was identical with that in the Finnish population, in that two-thirds of children had A/A genotype and one-third had A/O or O/O genotype,[6] and the association between the variant non-A/A genotype and asthma was not present before the age of 5 years. Thus, *MBL* production seems not to be involved with bronchiolitis in infancy nor with post-bronchiolitis wheezing.

Table 4 Asthma according to age vs *MBL* genotype

Asthma diagnosis†	A/A (n = 95)	A/O or O/O (n = 46)	P
1–2 years, n = 18	12	6	0.95‡
2–3 years, n = 22	15	7	0.93‡
3–4 years, n = 18	12	6	0.94§
4–5 years, n = 14	10	4	0.43‡

†Doctor-diagnosed asthma. ‡Pearson's χ^2 test; §Fisher's exact test. MBL, mannose-binding lectin.

Table 5 Risk factors for asthma at preschool age (logistic regression; n = 141)

	n	P	OR (95%Cl)
Gender (male)	74	0.219	1.78 (0.55–5.75)
Age (<6 years)	82	0.09	1.77 (0.73–6.72)
Genotype (non-A/A)	46	0.025	3.15 (1.04–9.56)
Current atopy	64	0.009	3.7 (1.22–9.75)

CI, confidence interval; OR, odds ratio.

The *MBL* deficiency has been associated with susceptibility to repeated respiratory tract infections.[6,18] In children, wheezing episodes are usually associated with viral respiratory infections, and LRI with or without wheezing in early childhood often precede the development of asthma.[19–21] Thus, one might expect to find reduced *MBL* production, caused by polymorphisms in the *MBL*-regulating genes, in children with asthma. In accordance, we found that more than half, 56%, of children with asthma at 5–7 years of age had the variant non-A/A genotype, compared with 30% in the present non-asthmatic children and with 33% in the Finnish non-selected population.[22] Only three children had the O/O genotype, which is always associated with deficient *MBL* production.[23] The present results support the hypothesis of reduced *MBL* production in children with asthma.

Nagy *et al*. documented an association between *MBL* gene polymorphisms, past *Chlamydophila pneumoniae* infection and the development of asthma in children.[16] They argued that *C. pneumoniae* infection might lead to chronic respiratory symptoms in genetically predisposed children with variant *MBL* gene alleles. Contradictory results have been reported from Africa and India, where higher concentrations of *MBL* have been detected in patients with asthma versus healthy controls.[17,24] The different findings in different studies can be explained, at least partly, by genetic differences due to ethnicity and by the worldwide variation of *MBL* haplotypes.[25] In addition, infections in asthmatic airways may stimulate *MBL* production regardless of the genotype, which can confound the results.[17]

The *MBL* deficiency can cause an imbalance in the normal immune system and may, at least in theory, lead to the development of atopy.[3] The present results are in disagreement with this hypothesis because the prevalence of allergic rhinitis and atopic dermatitis and the proportion of SPT-positive cases were similar in children with wild-type A/A and variant non-AA genotypes. Moreover, asthma was closely associated with atopy, but asthma without atopy was present only in children with the non-A/A *MBL* genotype. In a Brazilian birth cohort, *MBL* gene polymorphisms

were associated with higher prevalence of atopic dermatitis,[10] whereas in Iceland, low MBL serum levels did not predispose to allergy among 2–4-year-old children.[13] In Finland, *MBL* genotypes had no significant association with the occurrence of atopy in adults,[14] and low *MBL* gene expression increased the risk for asthma in non-atopic men, but not in atopic men and women or in non-atopic women. The present findings in children are in agreement with this observation, given that the three non-atopic asthmatic subjects were male and had non-A/A genotype.

The main strengths of the present study are the prospective, >5 year follow up, careful data collection from infancy to preschool age, and homogeneity of the subject group. All 141 children were of Finnish origin, lived in the area that the hospital serves and were < 6 months of age when enrolled. The main shortcoming is the lack of a control group. The distribution of *MBL* polymorphisms in the present cohort was nearly identical with that in the Finnish population, and therefore, the use of internal controls within the study was considered sufficient. Serum MBL concentration was not studied and no methods were available for *MBL* promoter gene polymorphisms. In a meta-analysis, *MBL2* genotype O/O was always associated with *MBL* deficiency, and *MBL2* A/O genotype with either low or deficient MBL serum concentrations depending on promoter gene genotypes.[23] Although 141 children were recruited in infancy, only 16 had asthma at 5–7 years of age, which is a small number for genetic studies.

The great diversity among previous results concerning MBL levels or *MBL* genes and asthma can be partly explained by the heterogeneity of asthma and partly by worldwide variation of *MBL* haplotype. We conclude that the variant non-A/A *MBL* genotype is associated with asthma after bronchiolitis in infancy, but not earlier than at 5–7 years of age.

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The Association of Genetic Variants in Toll-like Receptor 2 Subfamily With Allergy and Asthma After Hospitalization for Bronchiolitis in Infancy

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Background: Toll-like receptors (TLRs) are a pivotal part of the innate immunity system. Variations in *TLR* genes have been connected to autoimmune conditions, such as allergy and asthma. The TLR2 subfamily comprises TLR1, TLR2, TLR6 and TLR 10. We hypothesized that polymorphism of the TLR2 subfamily may be associated with prevalence of post-bronchiolitic asthma and/or atopy.

Methods: TLR1rs5743618, TLR2rs5743708 and TLR6rs5743810 single nucleotide polymorphisms of 133 children who had been hospitalized for bronchiolitis at <6 months of age were analyzed. Doctor-diagnosed asthma and atopy as well as their occurrence during the first 6 years of life were evaluated during a follow-up visit.

Results: At the mean age of 6.4 years, asthma was present in 17 (13%) patients, there was asthma diagnosis during the first 6 years of life in 39 (29%) and current doctor-diagnosed allergic rhinitis in 57 (43%) patients. Twenty-four (24%) children with G/G genotype in TLR1 rs5743618 were diagnosed to have asthma between 1 and 6 years of age (vs. 13 (38%) of those with G/T or T/T genotypes; $P = 0.04$). In addition, 11/60 (18%) children with TLR6 rs5743810 C/T versus 36/73 (49%) children of other genotypes had atopic eczema at follow up. Only 2 children (8%) with wild genotype in all investigated single nucleotide polymorphisms had asthma during the first 6 years of life (vs. 30% in those with variant genotype of TLR1, TLR2 and/or TLR6).

Conclusion: In this study, we demonstrated that TLR1 rs5743618 was associated with asthma and atopic eczema during the first 6 years of life after early bronchiolitis. In addition, TLR6 rs5743810 was associated with present atopy at preschool age.

Key Words: bronchiolitis, asthma, respiratory syncytial virus, cytokines, polymorphism

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Viral bronchiolitis is a common cause for hospitalization among infants. Despite of an active research, the exact mechanisms why some children develop only mild common cold while others require even intensive care has remained largely unknown. Subsequent wheezing and asthma are common among post-bronchiolitis patients.[1,2] It is under debate if viral respiratory infection or only severe respiratory infection during infancy contributes to development of asthma or if early-life viral infection serves only as a marker of children already prone to asthma.[3,4]

Early microbial interaction is important for postnatal maturation of immune system, which means a shift from original Th2-dominated immunity leading to atopy towards Th1-dominated immunity.[5] Thus, the persistence of Th2-dominated balance is 1 of the key factors for later asthma development.[3] Increasing evidence has been generated in recent years that alterations in innate immunity, not in adaptive immunity, seem to be linked with development of chronic inflammatory conditions, such as asthma and allergy.[6]

Toll-like receptors (TLRs) are able to recognize molecular patterns from microorganisms but not in the host.[7,8] TLRs are of great importance for initiating and regulating immune signaling and following adaptive immune responses.[9] TLR2 subfamily consists of TLR1, TLR2, TLR6 and TLR10.[10] TLR1, TLR2 and TLR6 are considered as co-receptors, but TLR10 seems to a separate signaling pathway.[11]

Polymorphisms of *TLR* genes regulating *TLR2, TLR6, TLR9* and *TLR10* have been shown to associate with childhood asthma[12–15] and to a lesser extent with allergy.[16–18] The role of *TLR2* subfamily has not been studied in relation to early-life viral bronchiolitis and post-bronchiolitis outcome. In earlier studies from this same cohort, we were able to confirm some association between *TLR3* and post-bronchiolitis wheezing,[15] but were not able to confirm any association between *TLR4* and post-bronchiolitis wheezing, at the mean age of 1.5 years.[19]

We hypothesized that polymorphisms in genes regulating *TLR2* subfamily play a significant role in determining which children are in elevated risk for asthma and/or atopy after bronchiolitis in early infancy.

MATERIAL AND METHODS

Full-term infants <6 months of age and hospitalized due to bronchiolitis in the Department of Pediatrics, Tampere University Hospital, Finland were included in the study. Study participants were recruited during December 1, 2001, and May 31, 2002, and between October 28, 2002, and May 31, 2004. Bronchiolitis was defined as lower respiratory infection with rhinitis, cough and diffuse wheezes or crackles.[20]

In all, 166 children attended the follow-up visit at 5–7 years of age. Asthmatic, other respiratory and allergic symptoms with the used medications and asthma or allergy diagnoses by doctors included, during the 6 first years of life, were reviewed with an extensive questionnaire. Doctor-diagnosed asthma was considered to be present if the child was on continuous inhaled corticosteroids medication for asthma or if there had been at least 1 period of doctor-diagnosed wheezing, prolonged cough or night cough without infection during the preceding 12 months and in addition, if bronchial hyper-reactivity was detected in the exercise challenge test.[20] Doctor-diagnosed cases of allergic rhinitis and atopic eczema were registered and, further, participants were categorized as being atopic if they had been doctor diagnosed with allergic rhinitis or atopic eczema. Diagnose was considered as present if the diagnoses had been made during last 12 months.

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Ethics

The study was approved by the Ethics Committee of the Tampere University Hospital District. An informed consent was obtained from parents before enrollment.

Genotyping

A total of 133 whole blood samples were available for further analyses. The genotyping of single nucleotide polymorphisms (SNPs) *TLR1* rs5743618, *TLR2* rs5743708 and *TLR6* rs5743810 was performed by pyrosequencing. The genomic DNA was extracted from peripheral blood with Qiagen QiAmp DNA blood Mini Kit 250 (Qiagen, Hilden, Germany) according to the instructions of the manufacturer.

Polymerase chain reactions (PCRs) with selected primer pairs for the TLRs studied and the PCR amplifications were carried out as described earlier.[21]. The pyrosequencing assay was performed using an automated pyrosequencer (PSQ 96MA Pyrosequencer, Biotage, Uppsala, Sweden). PCR products with potential SNP were recognized as the template in the pyrosequencing reactions, using PSQ 96 Pyro Gold Q96 reagent kit according to the manufacturer's protocol. To guarantee the specificity of pyrosequencing, 3 negative controls (water only, annealing buffer including sequencing primers together with water; PCR products together with water without annealing buffer including sequencing primer) were included in each run. The personal data of the study subjects were not given to the laboratory which performed the genetic studies (National Institute of Health and Welfare, Turku, Finland).

Statistical Analyses

All statistical analyses were done using SPSS package version 19 (IBM Corp., NY). χ^2 and Fisher exact tests were used for categorized variables, and student *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. $P < 0.05$ was considered to be statistically significant.

Deviations from Hardy-Weinberg equilibrium were evaluated using the FINETTI program. $P > 0.05$ was considered to be statistically significant. Logistic regression with adjustments for age and sex was used to analyze the *TLR* genotype versus allergy. The results are expressed as adjusted odds ratios (aOR) and their 95% confidence intervals.

RESULTS

The mean age of the 133 study children was 6.4 (standard deviations: 0.48) years. Current doctor-diagnosed asthma was present in 17 (13%) patients, asthma diagnosis during the 6 years of life in 39 (29%) and current doctor-diagnosed allergic rhinitis in 57 (43%) patients.

The genotypes of *TLR1* rs5743618 were GG in 99 (74%), GT in 29 (22%) and TT in 5 (4%) cases; the frequency of allele G (major) was 0.85 and allele T (minor) 0.15. The genotypes of *TLR2* rs5743708 were GG in 123 (93%) and GA in 10 (5%) cases; the frequency of allele G (major) was 0.96 and allele A (minor) 0.04. The genotypes of *TLR6* rs5743810 were CC 42(31%), CT 60(45%) and TT 31(24%); the frequency of allele C (major) was 0.54 and allele T (minor) 0.46. The observed genotype frequencies of *TLR1, TLR2* and *TLR6* were in Hardy-Weinberg equilibrium.

Twenty-four (24%) children homozygous for major allele G at *TLR1* rs5743618 were diagnosed to have asthma between 1 and 6 years of age [vs. 13(38%) of those with G/T or T/T genotypes; $P = 0.04$]. This association was robust to further statistical adjustments with age and sex (aOR: 0.48, 95% confidence intervals: 0.2–0.9; Table 1). There were no significant differences between the *TLR2* rs5743708 genotypes and clinical characteristics of the children (Table 2).

Of 60 children, 11 (18%) with *TLR6* rs5743810 C/T genotype versus 36/73(49%) of other genotypes had atopic eczema at follow up. After adjustment with gender and age, the finding remained statistically significant (aOR: 0.25; 95% confidence intervals: 0.11–0.56; Table 3). Of the 102 *TLR6* rs5743810 allele

TABLE 1. Clinical Characteristics of 133 Study Children in Relation to TLR1 rs5743618 Genotype and Asthma or Atopic Findings During Follow-up Visit

TLR1 rs5743618	G/G (n = 99)	G/T (n = 29)	T/T (n = 5)	G Allele (n = 126)	T Allele (n = 34)
Asthma between 1 and 6 years (n = 39)	24*†	11	2	35	13 ≠
Present asthma (n = 17)	12	3	2	15	5
Present nonatopic asthma (n = 3)	1	2	0	3	2
Present atopic eczema (n = 47)	38	6‡	3*§	44*	9
Present allergic rhinitis (n = 57)	43	11	3	54	14

Other associations P = NS.
All P values versus other genotypes.
*$P = 0.04$.
†aOR (sex, age): 0.49 (0.2–0.9).
‡$P = 0.08$.
§aOR: 0.19 (0.14–1.2).

TABLE 2. Clinical Characteristics of 133 Study Children in Relation to TLR2 rs5743708 Genotype and Asthma or Atopic Findings During Follow-up Visit

TLR2 rs5743708	G/G (N = 123)	G/A (10)	G Allele	A Allele
Asthma between 1 and 6 years (n = 39)	37	2	39	2
Present asthma (n = 17)	17	0	17	0
Present nonatopic asthma (n = 3)	3	0	3	0
Present atopic eczema (n = 47)	43	4	47	4
Present allergic rhinitis (n = 57)	53	4	57	4

P = NS in all cases
All P values versus other genotypes.

TABLE 3. Clinical Characteristics of 133 Study Children in Relation to TLR6 rs5743810 Genotype and Asthma or Atopic Findings During Follow-up Visit

TLR6 *Rs5743810*	C/C (n = 42)	C/T (n = 60)	T/T (n = 31)	C Allele (n = 102)	T Allele (n = 91)
Asthma between 1 and 6 years (n = 39)	10	21	8	31	29
Present asthma (n = 17)	5	8	4	13	12
Present nonatopic asthma (n = 3)	1	2	0	3	2
Present atopic eczema (n = 47)	16	11*†	20*‖	27*	31
Present allergic rhinitis (n = 57)	16	22	19‡§	38§¶	41

Other associations P = NS.
All P values versus other genotypes.
*$P < 0.001$.
†aOR (sex,age): 0.25 (0.11–0.56).
‡aOR (sex,age): 4.5(1.87–10.3).
§$P = 0.01$.
¶aOR: 0.36 (0.16–0.83).
‖aOR: 2.78 (1.2–6.4).

C carriers, 38 (37%) had current allergic rhinitis versus 19(61%) noncarriers. After adjustment with gender and age, the finding remained statistically significant, aOR 0.36 (0.16–0.83; Table 3).

In further haplotype analyses, the children homozygous for the major alleles (wild) were compared with carriers of minor alleles (variant; see Table, Supplemental Digital Content 1, http://links.lww.com/INF/B805). Only 2 (7.7%) children with *TLR1* G/G rs5743618, *TLR2* G/G rs5743708 and *TLR6* CC rs5743810, which were wild genotypes for all 3 SNPs, had asthma during the first 6 years of life (vs. 30% in those with variant *TLR1, TLR2* and/or *TLR6* haplotypes).

Current atopic eczema was common in both groups but significantly more common in non-respiratory syncytial virus (RSV)-infected children (55% vs. 80%, respectively, $P = 0.04$). When atopic eczema was included as an additional covariate in the age- and sex-adjusted analyses, the associations between *TLR1* G/G rs5743618 and asthma between 1 and 6 years lost their statistical significance.

Ninety-one children were infected by RSV during the hospitalization at <6 months of age. When RSV was included as an additional covariate in the age- and sex-adjusted analyses, the associations between *TLR1* GG rs5743618 and asthma between 1 and 6 years remained significant.

DISCUSSION

There are 4 main results in our present study. First, *TLR1* rs5743618 gene polymorphism was associated with asthma prevalence between 1 and 6 years of age after hospitalization for bronchiolitis in infancy. No previous studies have been published on this association. However, Kormann et al[14] found an association between 2 *TLR1* SNPs and asthma prevalence in children 9–11 years of age. Second, atopic eczema was common in *TLR6* rs5743610 minor allele carriers 5–7 years of age. In line, Daley et al[15] (2012) recently confirmed a correlation between atopy and *TLR1* polymorphism in Canadian and Australian children. Third, *TLR6* rs5743810 minor allele T homozygosity correlated with increased allergic rhinitis prevalence at 5–7 years of age. In a recent Dutch study,[22] *TLR6* SNP was linked with atopic gene-environment immune responses. Fourth, *TLR2* rs5743708 did not show any correlation with asthma or atopy at preschool age after early-life bronchiolitis.

Previously, a few reports about *TLR2* subfamily polymorphism and childhood asthma have been published.[14,23,24] Only 1 previous study has confirmed the association between *TLR1* SNP and childhood asthma.[14] They found that SNPs rs5743595 and rs4833095, but not SNP rs5743594, showed significant association with atopic childhood asthma. They also showed that at rs5743595 and rs4833095, the minor alleles correlated with increased mRNA expression of the respective TLR's in children. As rs4833095 is in close linkage disequilibrium with rs5743618 evaluated in our study, the results are comparable with our study. However, in contrast to their findings, we found that *TLR1* rs5743618 major allele G/G homozygosity was associated with fewer cases of doctor-diagnosed asthma during the first 6 years of life. The conflicting results might be explained by differences in study participants. The early severe respiratory infection has most likely had major role in determining future immune responses in our cohort. However, in earlier follow up at 1.5 years of age, no correlation between *TLR* SNPs and post-bronchiolitis wheezing could be demonstrated. The different results are most likely explained by different wheezing genotypes. Those children having respiratory symptoms at very early age are most likely transient wheezers who usually will outgrow from their symptoms before preschool age. Instead, those who start to wheeze later in childhood are more likely to have persistent asthma continuing until adulthood.[25,26]

The development of atopy is caused by a complex interaction between genes and environment. Exposure to microbes in early infancy can be protective from atopy but early severe infection can lead to sustained Th2 type immune responses.[5,27] Lowered immune responses associated with specific *TLR 1*, *2, 4* and *6* polymorphisms have been linked to increased susceptibility to atopy.[14,23,24] In our cohort, *TLR1* rs5743618 polymorphism correlated with atopic eczema at the follow-up visit. In agreement with our findings, *TLR1* rs4543123 was significantly associated with prick test positive atopy at 7 years of age in combined cohort of Canadian and Australian children.[15] Further, we found that *TLR6* rs5743810 was correlated with atopy and allergic rhinitis at preschool age. In a Dutch study evaluating gene-environment associations, *TLR6* rs5743810 was positively associated with immunoglobulin E secretion to indoor allergens at 6–8 years of age.[22] Further, Daley et al[15] examined *TLR6* rs5743794, which is in close linkage equilibrium with rs5743810 we used, but did not find significant associations between TLR6 and atopy. There are no other convincing earlier reports about correlation between TLR6 and atopy.

TLR6 forms heterodimers with another major TLR's involved in allergic responses, such as TLR1 and TLR2, and is also expressed on mast cells, which plays an important role in allergy.[28] The interaction between different genotypes and TLR's probably mean that 1 single SNP can influence the function of the whole TLR2 heterodimer and thus, more studies are needed to evaluate the association between the haplotype of TLR2 heterodimer and atopy.

Despite many studies, asthma pathogenesis in childhood is not yet completely understood. Early viral respiratory infections, especially rhinovirus, are known to be associated with increased

asthma risk.[2,4,29] Infantile immune system can be directed towards Th2-oriented atopic responses by early viral infection or some individuals can already be predisposed to asthma due to impaired lung function and the post-viral immune responses may be only reactive to infection.[3] TLR polymorphism causes impaired immune responses due to lowered recognition of pathogens that have been linked to asthma and atopy.[30] In our present study, children with wild homozygous genotypes in all 3 investigated *TLR* SNPs were significantly less likely to develop asthma after viral bronchiolitis in infancy. This finding provides preliminary evidence that alterations in *TLR* genotypes, at least in the TLR2 subfamily, may increase the later asthma susceptibility due to lowered capability of innate immunity to defend against severe early-life respiratory infection.

Daley et al[15] studied the combined effect of early respiratory virus infection and *TLR* SNPs on asthma and atopy risks among 7-year-old children. Interestingly, atopic asthma risk was associated with *TLR1* rs4543123 SNP and RSV infection during the first year of life. In our subgroup analyses with different SNPs, no association with later asthma prevalence could be found between RSV etiology of bronchiolitis and SNPs of *TLR 1, 2* or *6*. Instead, we found some preliminary evidence that *TLR6* rs5743810 and atopy at preschool age was linked to virus infection in infancy.

We acknowledge that investigating single SNPs may cause biased results especially with rather small sample sizes for genetic studies. However, testing a single SNP is more likely to give false-negative than false-positive results. Our present results, as well as conclusions of gene-gene or gene-environment interactions in general, need to be verified in larger patient samples. Our main strength is our unique patient cohort, nearly 100% virus detection rate and relatively long prospective follow-up period after bronchiolitis in <6 months old infants.

In conclusion, preliminary evidence was found that *TLR1* rs5743618 was associated with asthma prevalence during first 6 years of life after bronchiolitis at age <6 months. In addition, *TLR1* rs5743618 and *TLR6* rs5743810 were associated with allergic rhinitis at preschool age. *TLR2* rs5743708 did not show any correlation with asthma or allergic rhinitis.

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