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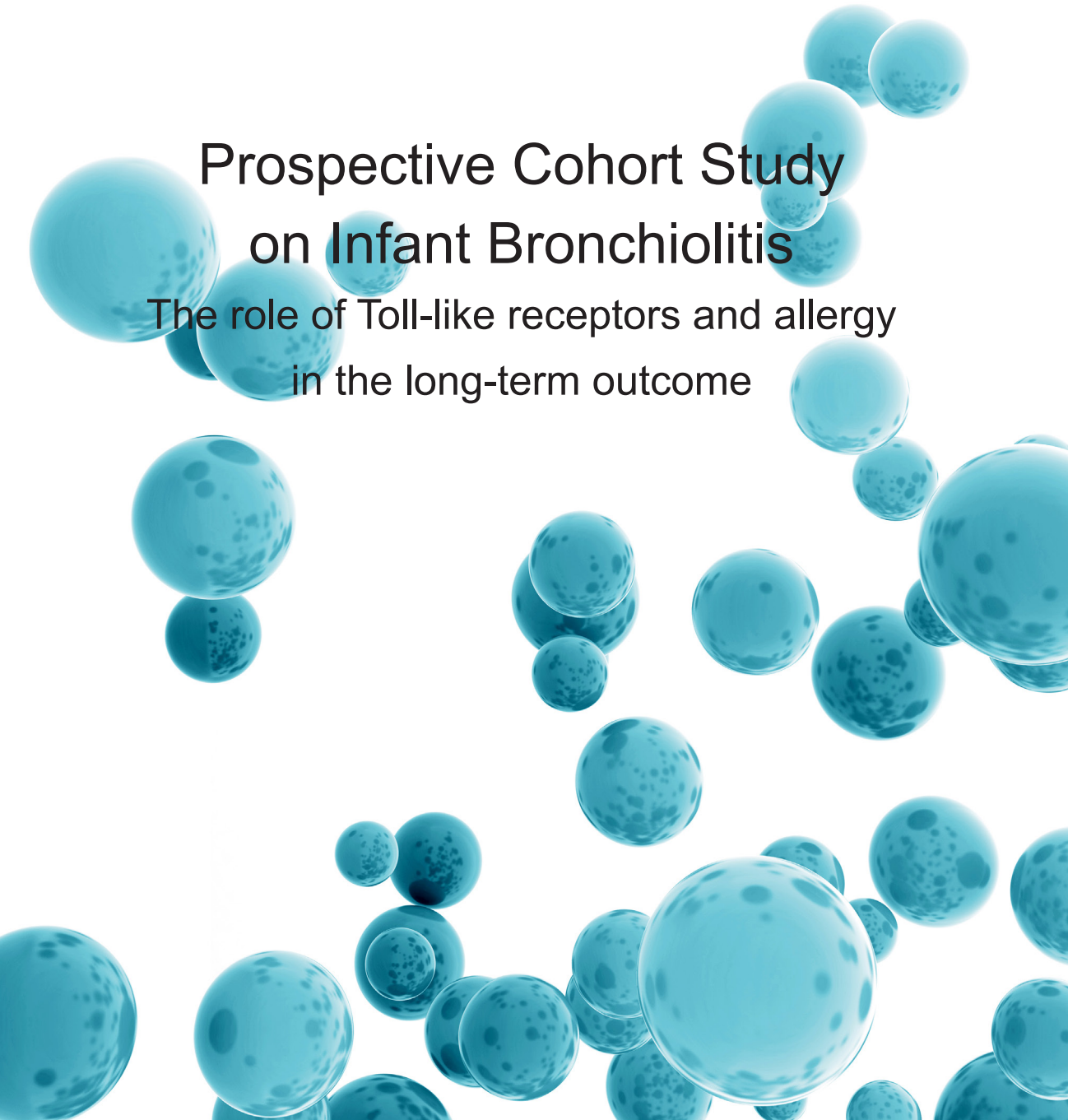
SARI TÖRMÄNEN

Prospective Cohort Study on Infant Bronchiolitis

The role of Toll-like receptors and allergy in the long-term outcome



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ACADEMIC DISSERTATION
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UNIVERSITY OF TAMPERE

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To my Mom

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original articles, which are referred to in the text by numerals I-IV.

I Törmänen S, Lauhkonen E, Riikonen R, Koponen P, Huhtala H, Helminen M, Korppi M, Nuolivirta K. Risk factors for asthma after infant bronchiolitis. Allergy 2018; 73(4): 916-922.

II Törmänen S, Korppi M, Teräsjärvi J, Vuononvirta J, Koponen P, Helminen M, He Q, Nuolivirta K. Polymorphism in the gene encoding toll-like receptor 10 may be associated with asthma after bronchiolitis. Scientific Reports 2017; 7(1): 2956.

III Törmänen S, Teräsjärvi J, Lauhkonen E, Helminen M, Koponen P, Korppi M, Nuolivirta K, He Q. TLR5 rs5744174 gene polymorphism is associated with the virus etiology of infant bronchiolitis but not with post-bronchiolitis asthma. Health Science Reports, in press.

IV Törmänen S, Korppi M, Lauhkonen E, Koponen P, Teräsjärvi J, Vuononvirta J, Helminen M, He Q, Nuolivirta K. Toll-like receptor 1 and 10 gene polymorphisms are linked to postbronchiolitis asthma in adolescence. Acta Paediatrica 2018; 107(1): 134-139.

ABBREVIATIONS

AE	Atopic eczema
API	Asthma predictive index
AR	Allergic rhinitis
BHR	Bronchial hyperreactivity
CpG	2'-deoxyribo-cytidine-phophate-guanosine
DNA	Deoxyribonucleic acid
dsRNA	Double-stranded ribonucleic acid
FEV1	Forced expiratory volume in 1 second
HRMA	High resolution melting analysis
HWE	Hardy-Weinberg equilibrium
ICS	Inhaled corticosteroid
IgE	Immunoglobulin E
IL-1Ra	Interleukin-1 receptor antagonist
IOS	Impulse oscillometry
IRF	Interferon factor
LPS	Lipopolysaccharide
LRR	Leucine-rich repeats
LRTI	Lower respiratory tract infection
MAF	Minor allele frequency
MyD88	Myeloid differentiation primary response 88
NF-κB	Nuclear factor of activated B cells
PAMP	Pathogen associated molecular patterns
RSV	Respiratory syncytial virus
RV	Rhinovirus
SPT	Skin prick test
ssRNA	Single-stranded ribonucleic acid
Th	T-helper
TIR	Toll/ Interleukin-1 receptor domain
TLR	Toll-like receptor
TRAM	Thyreoid receptor activator molecule

TRIF TIR domain inducing interferon

ABSTRACT

BACKGROUND: Bronchiolitis is a lower respiratory tract infection caused by a virus and often leading to hospitalisation, especially in the youngest infants. Recurrent wheezing episodes are common in the years following the contraction of bronchiolitis, and long-term follow-up studies have shown that children infected as infants are at high risk of developing asthma, which may be persistent at least until early adulthood. Established risk factors for post-bronchiolitis asthma include atopic predisposition, exposure to tobacco smoke in infancy, blood eosinophilia during bronchiolitis and rhinovirus as a causative agent of bronchiolitis. However, these risk factors do not fully explain the differences in individual outcomes. Variations in innate immunity, such as Toll-like receptors (TLRs), may alter the susceptibility to infectious and allergic diseases and, thus, contribute to a person's outcome. The function of TLRs is genetically determined, and the encoding genes are highly polymorphic.

AIMS: This study had five aims. The first was to investigate the incidence of asthma and related allergic diseases at 11–13 years of age after bronchiolitis in infancy. The second was to compare the prevalence of asthma, allergic rhinitis and atopic eczema in the original bronchiolitis group and age- and sex-matched control group. The third was to evaluate the clinical factors in early childhood and at preschool age that could have value in predicting the long-term prognosis. The fourth objective was to evaluate the role of single nucleotide polymorphisms (SNPs) of *TLR 3*, *4*, *5*, *7*, *8*, *9* and *10* genes in the post-bronchiolitis outcome at 5–7 years of age. The fifth and final aim was to confirm the associations between the SNPs of *TLR1* and *TLR10* genes and post-bronchiolitis asthma at 11–13 years of age.

MATERIAL AND METHODS: In all, 166 children hospitalised for bronchiolitis under 6 months of age were invited to a follow-up visit at 11–13 years of age, and 138 of them attended. Earlier follow-up visits were arranged at 1.5 and 5–7 years of age. In addition, a control group of 112 children, matched with age and gender and with no history of bronchiolitis in infancy, were recruited for the follow-up study of 11–13-year-olds. Data on current and past doctor-diagnosed asthma and allergies

were collected by structured questionnaires identical for both groups. Also, the questionnaire collected data on asthma medications, asthma presumptive symptoms and early-life exposures to tobacco smoke. Lung function was tested with flow-volume spirometry and a bronchodilation test. Blood samples for genetic studies were available only for the bronchiolitis group as the samples were obtained during earlier phases of this cohort study.

RESULTS: The prevalence of asthma was 13.0% in the bronchiolitis group and 10.7% in the control group at 11–13 years of age. Thus, the prevalence of asthma was rather similar in both groups, as was the prevalence of allergic rhinitis and atopic eczema. Early-life tobacco smoke exposure was significantly more common among the former bronchiolitis patients than in the control group, but it was not associated with the later asthma risk. Respiratory syncytial virus (RSV) was the major causative agent of bronchiolitis, but viral aetiology was not significantly associated with the long-term outcome. Maternal asthma was a significant early-life risk factor for post-bronchiolitis asthma (adjusted odds ratio (OR) 3.45, 95% confidence interval (95% CI) 1.07–11.74). Allergic rhinitis and skin prick test (SPT) positivity, which is an indicator of allergic sensitisation, were risk factors at preschool age (adjusted ORs 4.06, 95% CI 1.35–12.25, and 5.01, 95% CI 1.47–17.10, respectively). The *TLR10* rs4129009 variant genotype was significantly associated with current asthma at 5–7 years of age (adjusted OR 4.30, 95% CI 1.30–14.29) and, supporting this finding, with both current inhaled corticosteroid (ICS) use and persistent asthma at 11–13 years of age (adjusted ORs 7.02, 95% CI 1.56–31.53, and 7.69, 95% CI 1.35–43.95, respectively). In addition, the *TLR1* rs5743168 variant genotype was marginally associated with current ICS use at 11–13 years of age (adjusted OR 4.04, 95% CI 0.99–16.54).

CONCLUSIONS: In those children 11–13 years of age who were hospitalised for bronchiolitis before 6 months of age, the 13.0% prevalence of asthma means a two-fold asthma risk compared to the general school-aged child population. The percentage is lower than in previous post-bronchiolitis studies performed at the same age. Maternal asthma, as well as allergic rhinitis and SPT positivity at preschool age were predictors of post-bronchiolitis asthma at early teenage years. Variations in the *TLR1* and *TLR10* genes were significantly associated with post-bronchiolitis asthma, assessed with an asthma surrogate—specifically, ICS use for asthma at 11–13 years of age. This result confirms the earlier exploratory observations made when the children were 5–7 years of age.

TIIVISTELMÄ (ABSTRACT IN FINNISH)

TAUSTA: Bronkioliitti on viruksen aiheuttama alahengitystieinfektio, joka usein johtaa sairaalahoitoon, erityisesti nuorimmilla imeväisillä. Toistuvat hengenahdistukset ovat yleisiä bronkioliitin jälkeisinä vuosina ja pitkäaikaiset seurantatutkimukset ovat osoittaneet, että näillä lapsilla on korkea riski astman kehittymiseen, joka voi olla pysyvä ainakin varhaiseen aikuisikään saakka. Tunnettuja riskitekijöitä bronkioliitin jälkeisen astman kehittymiselle ovat atooppinen alttius, varhainen tupakansavulle altistuminen, veren eosinofilia bronkioliitin aikana ja rinovirus bronkioliitin aiheuttajana. Nämä riskitekijät eivät kuitenkaan täysin selitä yksilöllisiä eroja bronkioliitin jälkeisessä sairastavuudessa. Variaatio synnynnäisen immuniteetin geeneissä, kuten Tollin kaltaisia reseptoreita (TLR) koodaavissa geeneissä, voi muuttaa alttiutta infektiotaudeille sekä allergisille sairauksille ja siten osaltaan vaikuttaa bronkioliitin ennusteeseen. TLR:ien toiminta on geneettisesti määräytynyttä ja niitä koodavissa geeneissä esiintyy runsaasti polymorfiaa.

TUTKIMUKSEN TAVOITTEET: Tämän väitöskirjatutkimuksen tarkoitus oli tutkia astman ja siihen liittyvien allergisten sairauksien esiintyvyyttä imeväisiän bronkioliitin jälkeen ja verrata astman, allergisen nuhan ja atooppisen ihottuman esiintyvyyttä bronkioliittiryhmän sekä iän ja sukupuolen vakioidun verrokkiryhmän välillä. Tarkoitus oli myös arvioida niitä varhaislapsuuden ja esikouluiän riskitekijöitä, joilla voisi olla merkitystä taudin pitkäaikaisennusteessa. Tutkimuksen tavoitteena oli myös tutkia *TLR3*, *4*, *5*, *7*, *8*, *9* ja *10* geenien yksittäisten nukleotidien polymorfismien merkitystä 5–7-vuoden ennusteen kannalta bronkioliitin jälkeen ja lisäksi vahvistaa havaittu *TLR1* ja *10* geenien polymorfismien yhteys bronkioliitin jälkeiseen astmaan 11–13-vuotiaana.

AINEISTO JA MENETELMÄT: Kaiken kaikkiaan 166 lasta, jotka olivat joutuneet sairaalahoitoon bronkioliitin vuoksi alle 6kk iässä, kutsuttiin seurantakäynnille 11–13 vuoden iässä ja 138 heistä osallistui. Aiemmat seurantakäynnit on järjestetty 1,5 ja 5–7 vuoden iässä. Lisäksi tutkimukseen rekrytointiin 112 iän ja sukupuolen suhteen vakioitua kontrollia, jotka eivät olleet sairastaneet bronkioliittia imeväisiässä. Tiedot nykyisistä ja aiemmista lääkärin toteamista astmasta ja allergioista sekä astmaan

viittaavista oireista, astmalääkityksistä ja varhaisvaiheen tupakan savulle altistumisesta kerättiin strukturoiduilla kyselykaavakkeilla, jotka olivat identtiset molemmille ryhmille. Keuhkojen toimintaa testattiin virtausspirometrialla ja bronkodilataatiokokeella. Verinäytteet geneettisiä tutkimuksia varten oli saatavilla ainoastaan bronkioliittiryhmän osalta, koska ne oli kerätty tämän kohortin aiemmassa vaiheessa.
TULOKSET: Bronkioliitin jälkeisen astman ilmaantuvuus oli 13,0% bronkioliittiryhmässä ja 10,7% kontrolliryhmässä 11–13 vuoden iässä. Astman ilmaantuvuus oli siis varsin samankaltainen molemmissa ryhmissä, kuten oli myös allergisen ja nuhan ja atooppisen ihottuman ilmaantuvuus. Varhaisvaiheen tupakan savulle altistuminen oli merkittävästi yleisempää bronkioliittiryhmässä kuin kontrolliryhmässä, mutta se ei ollut yhteydessä myöhempään astmariskiin. Respiratory syncytial virus (RSV) oli bronkioliitin yleisin aiheuttaja, mutta virusetiologialla ei ollut merkittävää yhteyttä pitkän aikavälin ennusteeseen. Äidin astma oli merkittävä varhaisvaiheen riskitekijä bronkioliitin jälkeiselle astmalle (vakioitu vetosuhde, englanniksi odds ratio, OR 3.45, 95% luottamusväli, englanniksi confidence interval CI 1.07–11.74). Allerginen nuha sekä ihotestipositiivisuus, joka viittaa allergiseen herkistymiseen, olivat merkittäviä esikouluiän riskitekijöitä (vakioidut ORt 4.06, 95% CI 1.35–12.25 ja 5.01, 95% CI 1.47–17.10). *TLR10* rs4129009 variantti genotyyppi oli yhteydessä astmaan 5-7 vuoden iässä (vakioitu OR 4.30, 95% CI 1.30–14.29) ja löydöstä tukien, sekä hengitettävän kortisonilääkityksen käyttöön että pysyvään astmaan 11-13 vuoden iässä (vakioidut ORt 7.02, 95% CI 1.56–31.53 ja 7.69, 95% CI 1.35–43.95). *TLR1* rs5743168 variantti genotyyppi oli marginaalisesti yhteydessä hengitettävän kortisonilääkityksen käyttöön 11–13 vuoden iässä (vakioitu OR 4.04, 95% CI 0.99–16.54).

JOHTOPÄÄTÖKSET: 13,0%:lla bronkioliitin vuoksi sairaalahoitoon alle 6 kuukauden iässä joutuneista lapsista oli astma 11–13 vuoden iässä, mikä tarkoittaa kaksinkertaista riskiä tavalliseen kouluikäiseen lapsiväestöön verrattuna. Luku on pienempi kuin aiemmissa bronkioliittitutkimuksissa, jotka on tehty saman ikäisillä lapsilla. Äidin astma ja allerginen nuha sekä ihotestipositiivisuus esikouluiässä olivat riskitekijöitä bronkioliitin jälkeiselle astmalle varhaisessa teini-iässä. Variaatio *TLR1* ja *TLR10* geeneissä oli yhteydessä bronkioliitin jälkeiseen astmaan, arvioituna astmaan viittaavan hengitettävän kortisonilääkityksen käytöllä 11–13 vuoden iässä. Tämä tulos vahvistaa aiemmat kokeelliset havainnot, jotka tehtiin lasten ollessa 5–7-vuotiaita.

1 INTRODUCTION

Bronchiolitis is a viral infection of the lower respiratory tract, and it affects infants worldwide. In Europe, the upper age limit for bronchiolitis has been 12 months (1), whereas in the United States it has traditionally been 24 months (2). The viral aetiology is strongly linked with age. Respiratory syncytial virus (RSV) is the most common causative agent in children under 12 months of age, and other viruses, rhinoviruses (RVs) most importantly, predominate in older infants (3, 4). The clinical picture resulting from different viruses is the same, typically characterised by nasal congestion, cough, tachypnoea and expiratory wheezing (2). In most children with bronchiolitis these symptoms are mild and the children can be treated as outpatients. The overall prevalence of bronchiolitis has been 18–32% among infants under 12 months of age and 9–17% among children 12–24 months of age (5-7). In a subset averaging 2–3% of the children under one year old, the infection leads to a more severe clinical picture, and the children need monitoring at a hospital (8, 9). The youngest infants, meaning those under 3 months of age, are at the highest risk for severe disease (10). No curative treatment for bronchiolitis exists. Thus, inpatient care is supportive and includes feeding support, repeated mucus suction and supplemental oxygen if necessary.

There are 6 previous long-term studies that have followed the patients prospectively for 10 years or longer after hospitalisation for bronchiolitis (11-16). These studies have shown, that bronchiolitis has a strong impact on later respiratory morbidity, and that as many as one in every two patients hospitalised for bronchiolitis will suffer from recurrent wheezing episodes in the years leading up to preschool (17). Fortunately, a great proportion of these early wheezers will become unsymptomatic by the time they start school, but some will have more persistent symptoms; 15–40% of all children treated as inpatients will have school-age asthma (18-20).

Factors that have been linked with the persistent phenotype of post-bronchiolitis asthma are parental history of asthma or allergies and the atopic characteristics of the child, as well as the RV aetiology or non-RSV aetiology of bronchiolitis (11, 12, 16, 21, 22). It has been well documented that children with RV bronchiolitis are more likely to have an atopic predisposition; moreover, these children tend to be

older than children with RSV bronchiolitis (3, 23-25). As it seems that the predisposing factors and prognosis differ, some investigators have even proposed that RSV and RV bronchiolitis should be regarded as different entities. The aforementioned clinical risk factors, however, do not solely explain why some of the early wheezers will grow out of their symptoms while some will go on to develop more permanent asthma.

Despite the fact that the association between bronchiolitis and asthma is well established and several predictive markers have been identified, the detailed pathophysiology explaining the link is not yet fully understood. It has been suggested that a severe viral infection at a vulnerable developmental stage may drive the immune development towards T-helper (Th) type 2-oriented responses, which are characteristic for asthma. Alternatively, the infection may cause direct injury to the developing lungs, leading to subsequent lung function impairment. A third explanation that has been offered is, that early bronchiolitis only uncovers children who have an underlying susceptibility to asthma (26, 27).

Since only a proportion of children with bronchiolitis have symptoms severe enough to require hospital care, and not all of them develop asthma, it seems evident that underlying genetic factors, more specifically those regulating immune responses, contribute to the clinical course of bronchiolitis as well as the long-term outcome. It has been suggested that post-bronchiolitis asthma might result from a genetically determined dysfunction of innate immunity and its interaction with the causative virus (28). Toll-like receptors (TLRs) are innate immunity molecules that recognise evolutionarily conserved structures of viruses, bacteria and other microorganisms. After ligand binding, TLRs trigger intracellular signalling cascades leading to cytokine and chemokine release (29). TLRs are expressed in various cell types in all interfaces of the body, including epithelial cells lining the airways and immune cells circulating in the body (29-31). Normal activation of TLRs has been described as important for immune development, especially for balancing the Th1/Th2 responses. Polymorphisms in the encoding genes of these receptors may lead to altered recognition of pathogens and/or sustained Th2-oriented immunity and, thus, increase the susceptibility to infectious and inflammatory diseases, including bronchiolitis, asthma and allergies (32). As the viral aetiology of bronchiolitis seems to have an impact on the prognosis, it is likely that different virus-gene -interactions lead to different outcomes via different pathomechanisms (28).

This thesis aimed to study the clinical outcome at 11–13 years of age among children who had contracted severe bronchiolitis before 6 months of age and to identify the potential risk factors in early life, as well as in preschool age, that might

have relevance to the prognosis. Also, this study aimed to understand the effect of *TLR* gene polymorphisms on post-bronchiolitis outcomes at the preschool age and in the early teenage years. Finding tools for identifying infants, who are at high risk for persistent pulmonary morbidity, and thus, in need for longer follow-up, could help in informing the parents better about the prognosis, and in planning a scheduled follow-up for the child after bronchiolitis.

2 REVIEW OF THE LITERATURE

2.1 Definition and characteristics of bronchiolitis

Bronchiolitis is a lower respiratory tract infection (LRTI) in infants that may be caused by multiple different viruses. The upper age limit has varied from 12 months in Europe (1) to 24 months in the United States (2), but it has been debated whether the definition should be globally limited to 12 months, or even to 6 months, and whether to include only the first wheezing period, as the viral aetiology and prognosis of the disease vary greatly in older infants and recurrent wheezers (3, 33). This stricter definition would help to distinguish bronchiolitis from other wheezing-associated diseases, such as wheezy bronchitis and asthma, that have different backgrounds and prognoses.

The diagnosis of bronchiolitis is clinical. The same viruses that cause bronchiolitis are also common in mild upper respiratory infections and, in fact, the infection leading to bronchiolitis usually begins with upper respiratory tract symptoms. In some children, however, the virus spreads into lower airways and causes inflammation characterised by oedema and excessive mucus secretion. This, in turn, leads to obstruction of the lower airways, including the bronchioles, that is, the smallest and most distal airways (2, 34). Clinical manifestations usually include rhinitis, nasal congestion, tachypnoea and cough, but apnoea may be among the first signs of bronchiolitis especially in the youngest infants under 2 months of age (2, 35). Obstruction of the lower airways leads to laboured respiration. Further fine diffuse crackles or expiratory wheezes are often present on auscultation, and nasal flaring and chest retractions, in addition to tachypnoea, are marks of increased respiratory effort. Moreover, feeding problems are common. Chest radiograph, although not recommended as a routine examination in bronchiolitis, may show signs of overinflated lungs, local atelectatic areas and perihilar infiltrations (2, 9).

It is worth noting that most children with bronchiolitis develop only mild disease and can be treated as outpatients and that only a small proportion require hospital care. Most infants hospitalised for bronchiolitis are previously healthy, but pre- or postnatal exposure to tobacco smoke, maternal asthma, short duration or lack of breastfeeding, premature birth, low birth weight, underlying chronic disease –

especially hemodynamically significant heart or lung disease, chromosomal abnormality and (8) immunodeficiency—and, most importantly, age less than 3 months old, are risk factors for severe bronchiolitis requiring hospitalisation (8, 36-38). During the last two decades, factors related to innate immunity have been established to play a role in determining the course of bronchiolitis (39, 40).

Bronchiolitis is typically a self-limiting disease, management of which is, most importantly, supportive and includes feeding support, cleaning of the airways and supplemental oxygen if necessary. Hypertonic saline inhalations are often used to increase mucus clearance and decrease mucosal swelling, although the evidence of their effectiveness is weak (2). Only a small proportion of infants need mechanical ventilation and treatment in the intensive care unit. In the most recent Finnish study, low chronological age, low birthweight, prematurity and congenital heart disease were significant risk factors for intensive care and respiratory support of bronchiolitis in infants (10). Mortality due to bronchiolitis is rare in developed countries and is usually associated with preexisting comorbidities (41).

2.2 Epidemiology of bronchiolitis

Bronchiolitis affects children all over the world. In birth cohort studies, the prevalence of bronchiolitis has been 18–32% among infants under one year of age and 9–17% among children 12–24 months of age (5, 6). A retrospective population-based cohort study reported a marked increase in all health care visits due to bronchiolitis in otherwise healthy, low-income children under 12 months of age, from 19% to 27% between the years 1996–2003 (7). Most affected children can be treated as outpatients, but approximately 2–3% of infants worldwide are hospitalised for bronchiolitis during their first year of life (8, 9, 42). In fact, bronchiolitis is a leading cause of hospitalisation in infants under 12 months of age (2). In a two-year study in Finland, 3.7% of all infants under 6 months of age were admitted to the emergency room each year due to bronchiolitis, and 70% of them were hospitalised (35). Only 4–5% of infants hospitalised require treatment in the intensive care unit, and less than 1% need mechanical ventilation (35, 43). The peak occurrence of bronchiolitis takes place during the winter months (1, 7). RSV is the most common cause of bronchiolitis, but the viral aetiology is associated with the age of bronchiolitis patients (44) and the time of the year, since RSV infections occur as annual distinct epidemics. In Finland, RSV epidemics have typically followed a biannual pattern (45).

2.3 Viral aetiology

Originally, bronchiolitis was regarded solely as an RSV infection. Only two decades ago, researchers revealed that RVs are also able to affect lower airways and to cause a similar clinical picture. Since then, the introduction of more sensitive diagnostic methods has led to the discovery of several viruses that were unknown until that time, including metapneumovirus, human bocavirus and some coronaviruses, in the pathogenesis of bronchiolitis. However, RSV remains the most common causative agent, especially in infants younger than 12 months of age (1, 3, 43, 46). Mixed infections are also common, presenting in 5–38% of all bronchiolitis cases (1, 25, 43). Because RSV is the major pathogen in bronchiolitis, many studies have used the classifications of RSV bronchiolitis and RV/non-RSV bronchiolitis to investigate whether they, apart from causing similar clinical pictures, also share the same predisposing factors and a similar prognosis.

2.3.1 Respiratory syncytial virus

RSV is the most common aetiological agent causing bronchiolitis, accounting for 60-80% of all cases needing hospital care (1, 35, 43, 46). RSV infections have typically followed a seasonal pattern with highest incidence during the winter months (47, 48). In Finland, there has typically been a pattern where a minor outbreak occurs every second spring, followed by a major outbreak the next autumn and winter (45).

RSV is an enveloped single-stranded RNA -virus. The envelope around the virus contains G and F glycoproteins that are important for the infectivity and pathogenicity of RSV (49). There are two subtypes of RSV—type A and type B—that alternate in their predominance during epidemics. The classification is based on the variation in the G glycoprotein, but clinically, these two subgroups seem not to differ significantly from each other by their virulence or pathogenicity (50). The attachment of the virus to a target cell occurs with G glycoprotein binding to the cell membrane receptor. After the ligation, RSV-F fusion protein mediates the fusion of the virus and host cell membrane and enables the virus to enter the cell. RSV begins to replicate in the cell cytoplasm, which promotes intracellular signalling pathways that lead to the release of cytokines, chemokines and adhesion molecules and further, activation of immune responses, including the recruitment of inflammatory cells, eosinophils, T-lymphocytes and, most importantly, neutrophils. RSV infection is typically characterised by neutrophil-induced necrotisation of airway epithelial cells that shed and slough into airways. This together with increased mucus production

and oedema, leads to obstruction of the small airways (51, 52). RSV infection does not produce effective trace of memory T cells and therefore does not protect against reinfections (40, 52). Palivizumab immunoprophylaxis is used to prevent RSV infections in high-risk infants with a history of prematurity or congenital heart disease (34).

Approximately 80–90% of children are infected by RSV before their second birthday, the peak occurrence being in children under 2 months of age (8, 48). Moreover, young age is the most important risk factor for severe disease (43). Most RSV infections are limited to upper airways, but approximately 30% of infected subjects develop more severe disease when the virus spreads into the lower airways and causes bronchiolitis (40). It has been estimated that 10% of children with RSV bronchiolitis have symptoms severe enough to warrant hospital care (42). In a Swedish register-based study, 1.7% of children in the age group of under 12 months were admitted to hospital because of a verified RSV infection, and the median length of hospital stay was three days (48). RSV is associated with a more severe clinical course and length of hospital stay than bronchiolitis caused by other viruses (4, 35, 43, 53). Children with RSV are also younger than other bronchiolitis patients; RSV predominates in infants under 12 months of age, with RV becoming more frequent thereafter (3, 4).

2.3.2 Rhinoviruses

RVs are non-enveloped single-strand RNA viruses that belong to the genus Enterovirus in the Picornaviridae family. RVs are common in upper respiratory infections at all ages but are also able to cause more severe infections like bronchiolitis in infants. In fact, they are the most common cause of bronchiolitis outside RSV epidemics (54). More than 160 serotypes of RVs are classified into RV-A, RV-B and RV-C species; RV-A and RV-C have been most commonly detected in bronchiolitis (55). RV-C is the most recently recognised RV type (56), and RV-C seems to be associated more often than other types with wheezing after bronchiolitis (57). A Finnish study that investigated the distribution of RV species in infants who were 3–23 months old during their first wheezing period reported that RV-C was the most frequently detected type, followed by RV-A, while RV-B was found relatively seldomly (58). In contrast to RSV, RVs occur more steadily throughout the year (47), except for RV-C, which seems to peak in spring and fall (58).

RV bronchiolitis and RSV bronchiolitis have different predisposing factors. RV bronchiolitis or RV-induced wheezing typically occurs in older infants than does

RSV bronchiolitis (3, 25, 59, 60). In addition, several studies have reported that RV bronchiolitis or RV-induced wheezing is more frequent in infants who are atopic by themselves or have a family history of asthma (4, 23-25). Low interferon responses have also been associated with a predisposition to RV infections (61). Moreover, RV involvement has been linked to a more chronic type of respiratory illness, since these are the most often detected viruses in young children with recurrent wheezing episodes (3), and the risk of recurrent wheezing after bronchiolitis (23) and asthma in later childhood (62, 63) has been greater after RV bronchiolitis than after RSV bronchiolitis. Interestingly, RVs are also the most commonly detected viruses in asthma exacerbations. This correlation has been suggested to be due to abnormal antiviral responses and preexisting inflammation of the airways (64). There is also evidence of preexisting abnormalities in lung function in infants who later developed rhinovirus LRTIs (65). Compared to RSV and the majority of other respiratory viruses, RVs are less capable of cell destruction (52). Thus, the host's immune response to RV infection seems to determine the severity and outcome of the infection more than the direct injury caused by the virus, which is characteristic for RSV infections (66).

2.3.3 Other viruses

In addition to the two major pathogens—RSV and RVs—several other viruses can induce bronchiolitis. When a virus causes a symptomatic upper respiratory infection in infants, it may also spread to the lower respiratory tract and ultimately cause infant bronchiolitis. Viruses that have been detected in patients hospitalised for bronchiolitis include human bocavirus, enteroviruses, parainfluenza viruses, adenovirus, metapneumovirus, coronaviruses and influenza A and B virus (25, 36). Similarly to RVs, these viruses become more prevalent with increasing age, but each causes fewer than 10% of bronchiolitis cases in patients less than 24 months of age (58). Human bocavirus has been found in up to 25% of wheezing cases, but usually as mixed infection, and its role as a causative agent is still obscure (3). Similarly to RSV, influenza A and B viruses are causative agents only during the annual epidemics (67). Viruses other than RSV and influenza have been reported to cause mainly minor local outbreaks (68).

2.4 Long-term outcome after bronchiolitis

Both birth cohort studies and hospital-based cohort studies are applicable and reliable in the assessment of the long-term outcome after bronchiolitis in infancy, as the results in both are based on prospective and repetitive data collection. However, neither the definition of bronchiolitis nor the terminology used in these studies is completely established, which complicates the comparisons between the studies. Some studies have used the terms "wheezing" or "virus-induced wheezing" or even simply "lower respiratory tract infection" instead of bronchiolitis. Wheezing has been linked especially to RV-induced symptoms, whereas in RSV infection, the lung sounds on auscultation may be more variable (25). This thesis focuses on studies in which the definition has included only the first episode of wheezing and the age limit has been under 12 or 24 months.

Both types of long-term cohorts—birth cohorts and hospital-based cohorts—have shown that severe bronchiolitis in infancy requiring treatment in hospital is associated with an increased risk of subsequent wheezing and asthma both later in childhood and in adolescence. The overall prevalence of doctor-diagnosed asthma at school age is about 5% (69-71), and the prevalence of asthma-like symptoms without diagnosed asthma is an additional 5–7% (71). In hospital-based cohorts, the prevalence of post-bronchiolitis asthma has been 23–50% before school age (17, 21, 72-74), 15–40% at early school age (18-20) and 14–39% at 10–14 years of age (Table 1) (11-16). In the present cohort, 13% of former bronchiolitis patients had current asthma at the 5–7 years control visit (62). In birth-cohort studies, which have evaluated the incidence of asthma after bronchiolitis severe enough to warrant hospitalisation, the prevalence has varied 22–28% in preschool age (75, 76), but thus far no data is available regarding the later school-age outcome. Moreover, there is evidence that an increased risk of asthma after bronchiolitis persists into adulthood. A Swedish hospital-based study reported a 37% prevalence of asthma at 25–28 years of age after RSV bronchiolitis in infancy (77), and a Finnish hospital-based study reported a 23–28% prevalence at 27–29 years of age (78). Most longitudinal studies have reported that the prevalence of post-bronchiolitis asthma decreased with age until puberty (11, 13, 14), but after puberty, the prevalence started to increase (79-81). There is a secular trend, since asthma prevalence was lower in older studies (12, 18) and higher in newer studies (11, 15, 20).

In addition to asthma, some investigators have reported an increased risk for other allergic diseases after bronchiolitis. The Swedish follow-up study found an increased risk for allergic rhinitis and allergic sensitisation in skin tests at the age of

13 years after RSV bronchiolitis in infancy (15). However, the control group in that study may be regarded as selected, as the rate of asthma was only 3%, atopic eczema 7% and allergic rhinitis 15%, thus lower than in the general population. Several other studies (13, 16, 75, 82) have reported no association between bronchiolitis and later atopy, and it has been suggested that atopic mechanisms do not form the link between bronchiolitis and asthma (83).

It is still unclear whether bronchiolitis itself causes alterations in the immune responses leading to allergic sensitisation, causes direct injuries in the developing lungs that lead to airway remodelling and subsequent alterations in lung function or is just the first sign of already preexisting abnormalities in lung function or immune responses that cause ineffective antiviral response and/or promote allergic sensitisation (84, 85). A substantial proportion of children do not develop pulmonary sequelae after bronchiolitis, some tend to suffer from repeated wheezing but grow out of their symptoms before school age, and some develop permanent asthma, which may be further classified into atopic or non-atopic, depending on the concomitant manifestation of other atopic diseases.

Table 1. Prevalence of post-bronchiolitis asthma at school age and identified risk factors in hospital-based cohort studies with a follow-up time over 10 years.

Study	RSV (%)	Age at admission (months)	Age at re-evaluation (years)	Asthma (%)	Risk factors for asthma
Noble (13) United Kingdom 1979-1981 n=61/101*	66	< 12	10	39	None identified
Hyvärinen (11) Finland 1981-1982 n=65/81*	55	< 24	13-16	14-23	Repeated wheezing, atopic eczema, blood eosinophilia, allergic rhinitis
Wennergren (14) Sweden 1984-1985 n=92/101*	28	< 24	10	30	Wheezing <6 months of age, other atopic disease during 4 previous years, continuous medication for asthma >6 months as a young child, exposure to tobacco smoke in infancy
Sigurs (15) Sweden 1989-1990 n= 46/47*	100	< 12	13	28	Parental asthma and atopy **
Hyvärinen (12) Finland 1992-1993 n=81/100*	30	< 24	11-14	40	Atopic eczema, sensitisation to inhalant allergens
Mikalsen (16, 22) Norway 1997-1998 a)121/131* b)93/105*	a) 74 b) 67	a) <12 b) < 12	a) 11 b) 11	a) 21 b) 23	a)Male gender and non-RSV aetiology b)Wheezing, wheezing combined with parental atopy or asthma or atopic eczema

*number of patients reevaluated/ originally recruited

** evaluated when bronchiolitis and control groups were combined

2.5 Early-life predictive factors of post-bronchiolitis asthma

Wheezing after bronchiolitis is common, but only a subset of children with recurrent wheezing periods have symptoms continuing at school age. To differentiate those children who require more persistent medication and intensive follow-up from those whose symptoms are more likely transient, investigators have tried to find factors that could predict asthma after bronchiolitis. Long-term follow-ups of children with bronchiolitis have helped to determine those possible risk factors (Table 1). It seems that post-bronchiolitis outcome results from a combination and interaction between host, viral and environmental factors.

2.5.1 Atopic predisposition

A number of longitudinal follow-up studies have identified atopic predisposition, such as a familial history of asthma or allergies, or personal atopic characteristics, or both, as a strong risk factor for post-bronchiolitis asthma, while children without this predisposition seem more likely to grow out of their symptoms (11, 12, 14, 15, 22). The prevalence of atopic characteristics increases with age (86). In early infancy, the markers of inherited atopic predisposition are usually atopic eczema or familial asthma or allergies, especially maternal asthma and/or allergies, whereas manifestation of other atopic diseases occurs later in life: allergic sensitisation to food and inhalant allergens typically after 6 and 12 months of age, respectively, and allergic rhinitis even later, at preschool age (86). A Norwegian hospital-based study found the combination of recurrent wheeze and either atopic eczema, parental asthma or parental atopy to predict asthma at school age after bronchiolitis before 12 months of age (22). The association between atopic predisposition and asthma after bronchiolitis was also seen in an earlier publication from the present cohort: maternal asthma and atopic eczema before 12 months of age were independent risk factors for asthma at 5–7 years of age, as was skin prick test (SPT) positivity tested at the same follow-up visit (62). However, two long-term bronchiolitis studies had contradictory results, reporting no association between atopic tendency and asthma after bronchiolitis. However, this may be due to strict definitions for atopy used: SPT positivity in one (16) and SPT positivity or a first-degree relative being atopic in the other (13). A selection bias is likely in the former study, as 38% of the controls were atopic, which is more than in the general population. Interestingly, a prospective multicentre study reported that RSV prophylaxis with palivizumab decreased the relative risk of recurrent wheezing in non-atopic late-preterm children,

but not in children with a family history of atopy, suggesting that respiratory sequelae caused by RSV are not related to atopic characteristics of the host (87).

2.5.2 Eosinophilia

A decrease in circulating eosinophils is considered a normal immunological response to virus infections (88, 89). Eosinophilia and the presence of markers of eosinophilic activity, such as eosinophil cationic protein, during bronchiolitis have been associated with an increased risk for subsequent wheezing (74) and later asthma (90).

2.5.3 Other host-related factors

Several studies have reported male gender to be associated with an increased risk for asthma before the teenage years, and this is also true in post-bronchiolitis asthma (76, 91, 92). The balance shifts after puberty, however, and women are more likely than men to be asthmatics in adulthood (77, 93). In the prospective hospital-based follow-up study from Norway, the risk of asthma at 11 years of age after bronchiolitis was increased only in boys when stratified by gender (16). Overweight and obesity have also been reported to affect the pulmonary outcome after bronchiolitis (94), and an earlier publication from the present cohort found this to apply to post-bronchiolitis asthma at preschool age as well (95). However, the published results are not consistent, since another Finnish hospital-based cohort reported controversial results (96).

Birth cohort studies that have had the opportunity to assess lung function prior to the development of bronchiolitis have reported that inherited or prenatally induced abnormalities in lung function may precede bronchiolitis and explain the different outcomes (97, 98).

Postnatal bronchial hyperresponsiveness has been associated with the occurrence of severe bronchiolitis and development of post-bronchiolitis asthma (99). However, the median age of the patients at the time of bronchiolitis was 11 months. Thus, it is possible that bronchiolitis was not the correct diagnosis in all cases but, instead, the symptoms were due to asthma. It is also possible that the preceding abnormalities in lung function may predispose infants only to certain virus infections. In a recent study, reduced lung function was reported in infants who later developed rhinovirus LRTIs (65).

Age at admission has also been reported to be relevant to bronchiolitis outcome, reflecting that the developmental stage of immune responses plays a role. Interestingly, some investigators have found the youngest infants (aged under 6 months) to be at the highest risk for asthma (14), while another hospital-based cohort suggested that an age of more than 12 months during the first wheezing period would be a risk factor for later asthma (11). In a high-risk birth cohort of children in an outpatient setting, RV-induced wheezing before 12 months of age increased the risk of asthma threefold at 6 years of age, whereas wheezing with RV between 2 and 3 years of age increased the risk 32-fold. Interestingly, RSV-related wheezing before age 2 did not predict asthma at preschool age, whereas wheezing with RSV between 2 and 3 years of age increased the risk of preschool-age asthma 13-fold (91). In the present cohort, the prevalence of asthma at preschool age was 13% after hospitalisation for bronchiolitis under 6 months of age (62). Thus, the prevalence is significantly lower than in other prospective studies that have used higher age limits for bronchiolitis. Age is strongly linked with the virus aetiology of bronchiolitis, which also explains the differences in the results between the cohorts: in the Swedish cohort, RSV accounted for only 28% of cases (14), compared to more than 70% in the present cohort (62). The differences in the results between the studies may be due to different phenotypes of asthma: the youngest bronchiolitis patients might have an immunologic predisposition for developing severe bronchiolitis leading to hospitalisation; alternatively, the timing of bronchiolitis at a crucial stage of immune and/or lung development might lead to permanently biased immune responses or lung injury. In older children bronchiolitis might be the first sign of underlying genetic susceptibility towards asthma, and the virus infection simply enhances the preexisting airway inflammation (61, 84). This is supported by the observation that children with atopic tendency are more prone to certain virus infections, especially RV infections (100).

2.5.4 The role of viruses

In addition to host-related factors, the causative virus has been established to have a strong impact in the outcome after bronchiolitis. There is evidence that the risk of subsequent asthma is significantly higher after bronchiolitis (or wheezing) caused by a virus other than RSV, and particularly high when RV has been the causative virus (11, 16). Some investigators have reported that the impact of RSV LRTI on later asthma risk seems to decrease over time (101), while non-RSV—or RV bronchiolitis, more specifically—seems to be a risk of more permanent asthma (102). One long-

term hospital-based cohort study found that the prevalence of asthma 11 years after hospitalisation for bronchiolitis was significantly increased in patients with non-RSV bronchiolitis, whereas RSV bronchiolitis did not significantly increase the risk when compared to a control group (16). This was also seen in the present cohort at 5–7 years of age: the prevalence of asthma was 8.2% after RSV bronchiolitis and 24% after non-RSV-bronchiolitis (62).

Some investigators have assessed risk factors separately for atopic and non-atopic asthma after early wheezing. Atopic asthma at school age has been associated with RV-related first wheezing period, early sensitisation and atopic eczema, whereas the risk of non-atopic asthma has been related to RSV, early exposure to tobacco smoke and first wheezing episode under 12 months old (75, 92). It is interesting to note that RV bronchiolitis has been more frequent in infants with atopic predisposition (24, 25, 60). As inherited atopic characteristics are also strongly linked with asthma in patients without any history of bronchiolitis in infancy, it is possible that RVs cause bronchiolitis particularly in patients who are genetically predisposed to asthma, with bronchiolitis being the first sign of this predisposition. More specifically, attenuated Th (T helper) 1–type immune responses, and lower production of interferons most importantly, have been associated with increased susceptibility to RV infections (44, 103). In the present cohort, however, sensitisation to inhalant allergens tested by SPTs was equally common in former RSV and non-RSV patients, although the prevalence of asthma was significantly higher in the non-RSV group (62). It has also been suggested that RV amplifies the preexisting inflammation and Th2–type immune responses in the airways and may increase airway reactivity. These preexisting abnormalities in immune responses might provide an explanation for why RVs, which are usually restricted to upper airways, sometimes cause LRTIs (61). The fact that RVs often cause asthma exacerbations supports this idea (104). Moreover, repeated wheezing, often related to respiratory infection caused by RVs, before 2 years of age is a risk factor for subsequent asthma after bronchiolitis (12). The severity of wheezing episodes before age 2 also predicts later asthma (105). It is possible that the interaction of causative viruses with different predisposing factors leads to separate outcomes (28). Further, it has been discussed, whether RSV and RV/non-RSV bronchiolitis are completely different entities with different predisposing factors and different outcomes (106).

2.5.5 Other environmental factors

Pre- or postnatal exposure to tobacco smoke has been associated with an increased risk for bronchiolitis (37, 38, 107) and wheezing before 12 months of age (65), and it has been established to be a general risk factor for asthma (108, 109). Interestingly, though, it seems to have lesser effect on long-term pulmonary outcome after bronchiolitis or early wheezing (11, 13, 15, 91). However, one long-term hospital-based study reported a positive association between tobacco smoke exposure in early life and asthma at 10 years of age after bronchiolitis, even though this type of relationship was not seen in the previous follow-up investigation of that same cohort at preschool age (14, 21). Later in the same cohort, maternal smoking was associated with asthma and bronchial hyperresponsiveness, but paternal smoking was associated with one's own predilection to smoke and, via that, to asthma (79).

The Norwegian group assessed clinical risk factors present at 2 years of age that would predict later asthma in children who had been hospitalised for bronchiolitis under 12 months old. The investigators found recurrent wheezing to be the strongest risk factor for asthma at 11 years of age, although the researchers stated that this variable would be more accurate in excluding than in predicting asthma. The predictive accuracy of this tool was found to improve when recurrent wheezing was combined with atopic eczema or with parental atopy or asthma (22). Another—and perhaps the most distinguished—tool to predict persistent wheezing is the Asthma Predictive Index (API) originally built based on the results of the large Tucson birth cohort study (110). The API index stresses the role of atopic characteristics and eosinophilia and is less useful in predicting non-atopic asthma.

The exact pathogenesis of post-bronchiolitis asthma is still unclear, and it seems that asthma results from a complex interaction between environmental factors and inherited genetic predisposition that alters immune responses and/or lung function. In fact, "two-hit-hypothesis" of asthma development has been suggested (111). This means that at least two predisposing factors, namely genetic or environmental, are required for the development of asthma. A Finnish study that evaluated risk factors for later asthma in children under 2 years old with their first wheezing period reported that the risk of asthma increased cumulatively if the child had more than one risk characteristic (92). Thus, bronchiolitis would lead to asthma only in combination with other risk factors, such as genetic predisposition.

2.5.6 Genetic factors

A substantial number of genes have been investigated as possible candidate genes for asthma as well as in relation to bronchiolitis severity or susceptibility, but genetics behind post-bronchiolitis asthma is markedly less studied. Variations in genes that regulate innate immunity may explain the different outcomes between individuals. TLRs and their encoding genes form one suggested link between bronchiolitis and asthma and are reviewed more thoroughly in the following chapters.

2.6 Toll-like receptors

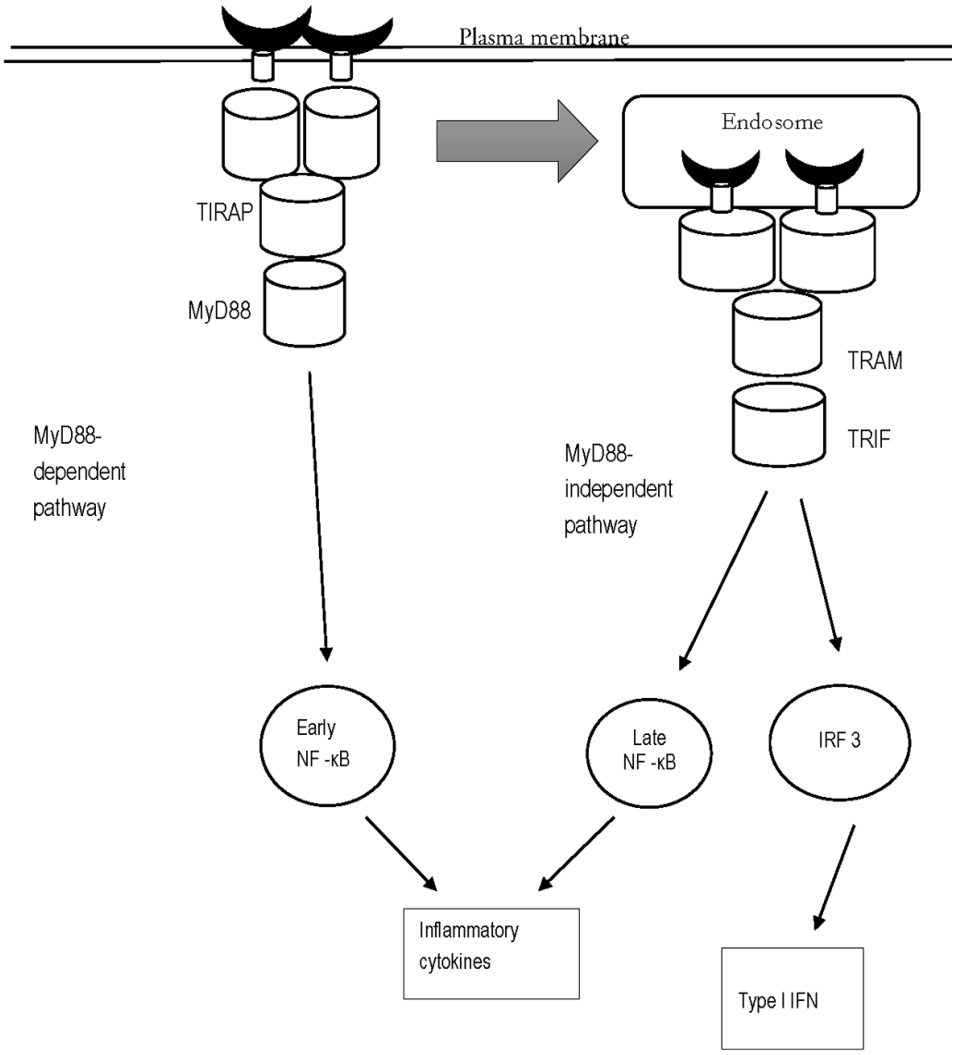
TLRs, a human homologue of *Drosophila* Toll, are evolutionarily conserved transmembrane glycoproteins. They are crucial for innate immunity, as they initiate the primary host defence against pathogens. TLRs recognise pathogen-associated molecular patterns (PAMPs) that are conserved structures of various microbes but are not present on host cells. Once activated, TLRs trigger the production and secretion of inflammatory cytokines and chemokines and, further, the activation of adaptive immunity (112). TLRs are expressed in various cell types, including neutrophils, macrophages, dendritic cells, killer cells and epithelial and endothelial cells, as well as in T- and B-lymphocytes. Eleven TLRs have been identified in humans so far, but only 10 of them (TLR1–10) are functional (113). TLR1, TLR2, TLR4, TLR5, TLR6 and TLR10 are located on the cell surface and are able to detect, e.g., peptidoglycans, lipoproteins and lipopolysaccharides (LPS), whereas TLR3, TLR7, TLR8 and TLR9 are found in intracellular compartments, such as endosomes and lysosomes, and are able to detect nucleic acids from bacteria, viruses and fungi after endocytosis of the pathogen (114).

All TLRs share the same basic structure with ligand binding ectodomain, transmembrane domain and cytoplasmic Toll/ Interleukin-1 receptor (TIR) domain. The ectodomain contains varying numbers of leucine-rich repeats (LRRs), the arrangement of these LRRs being distinct for each TLR, thus enabling the recognition of specific PAMPs. After detecting and binding a ligand, TLRs form homo- or heterodimeric complexes and, subsequently, recruit adaptor molecules that interact with the TIR domain of TLRs and induce intracellular signalling. Roughly, there are two main pathways for downstream signalling. All TLRs except TLR3 use signalling pathway, where myeloid differentiation primary response protein MyD88 serves as an adaptor molecule and quickly activates transcription factors such as the nuclear factor of activated B cells (NF-κB) and mitogen-activated protein kinases

(MAPKs) to induce inflammatory cytokines and chemokines. TLR3 in turn uses MyD88-independent pathway where the TIR domain inducing-interferon (TRIF) is an adaptor protein and where activation of transcription factors—interferon factor 3 (IRF3) and late phase NF-κB— induce the release of type I interferon and inflammatory cytokines (Figure 1). TLR4 is the only TLR able to use both of these signalling routes. In the MyD88 independent pathway, TLR4 is translocated to an endosome where it uses TRIF and thyroid receptor activator molecule (TRAM) to induce downstream signalling (Figure 1) (32, 113, 115).

The 10 functional TLRs in humans are discussed in more detail in the following sections.

Figure 1. A simplified theoretical model of the two TLR4 signalling pathways. Modified from Kawai and Akira (115).



2.6.1 Toll-like receptors 1, 2, 6 and 10 (TLR2 subfamily)

TLR 1, 2, 6 and *10* genes are located in chromosome 4 in close proximity to each other. The corresponding proteins of these *TLR* genes form TLR1/2, TLR6/2 and TLR10/2 receptor dimers after they recognise a ligand (116). Unlike other receptors

within the TLR2 subfamily, TLR10 does not always require TLR2 for activity, but it does form heterodimers with TLR1 (31). In addition, TLR10 is the only receptor of these four that is also capable of homo-dimerisation (31). TLR1/2 and TLR2/6 were originally reported to sense triacylated and diacylated lipopeptides, components of bacterial cell walls, but there is accumulating evidence that these receptor dimers can also be activated by viruses, RSV and RV included (117, 118). The specific ligand for TLR10 is currently unknown, but it seems to compete for the same ligands as TLR1 (119) and TLR2 (116). TLR10 also differs from the other TLR2 subfamily members in several other ways. TLR10 is the only TLR with an anti-inflammatory function established partly by the release of interleukin-1 receptor antagonist (IL-1Ra), which inhibits cytokine production, and partly by the competition for ligands and for the formation of heterodimers with TLR2 (116). TLR10 also has a more restricted expression profile than the other receptors in the TLR2 subfamily, as it is limited to granulocytes, B cells and dendritic cells (31). In addition, TLR10 has a distinct signalling pathway in the sense that it uses MyD88 as an adaptor molecule, but does not induce activation of NF-κB or other classical reporter genes (119).

TLR1 rs5743618 polymorphism leads to a non-synonymous mutation located at the junction of the transmembrane and cytoplasmic domain of the receptor (120). The location is not common for other *TLR* polymorphisms. This polymorphism has been associated with a decreased cytokine response, although the detailed mechanism remains unclear (120). *TLR2* rs5743708 is a polymorphism leading to a non-synonymous mutation located in the intracellular domain of the receptor and may affect its signalling (121, 122). *TLR6* rs5743810 polymorphism leads to a non-synonymous mutation located in the extracellular domain of the receptor and may affect ligand binding or, alternatively, alter TLR2/6 hetero-dimerisation. It has been shown to be functional and to alter NF-κB signalling (123). *TLR10* rs4129009 polymorphism leads to a non-synonymous mutation located in the cytoplasmic TIR-domain and is thus likely to affect intracellular signalling but not ligand binding. The latter cannot be ruled out, however, as the specific ligand for *TLR10* is unknown (124).

2.6.2 Other Toll-like receptors

TLR3 recognises double-stranded RNA (dsRNA) during virus replication, including RSV and RV (125). TLR3 gene is located on chromosome 4 (29). TLR3 has been reported to be important for host defence in RV infection: the expression of *TLR3*

gene increased in response to RV infection and blocking TLR3 led to an increased RV replication in bronchial epithelial cells (126)

TLR4 was the first TLR identified in humans. It recognises the LPS of Gram-negative bacteria in addition to those of other microbial structures, including the F glycoprotein of RSV. *TLR4* gene is located on chromosome 9 (29).

TLR5 recognises bacterial flagellin, but it may have a modulating role in virus infections (127). In addition, flagellin has been used as an adjuvant in influenza vaccines, as it triggers immune responses via TLR5 (128, 129). *TLR5* gene is located on chromosome 1 (29).

TLR7 and TLR8 recognise single-stranded viral RNA (ssRNA), including RV ssRNA (117). *TLR7* and *TLR8* genes are located on chromosome X (29).

TLR9 detects unmethylated 2'-deoxyribo-cytidine-phophate-guanosine (CpG) DNA motifs from bacteria and viruses. *TLR9* gene is located on chromosome 3 (29).

2.6.3 Polymorphisms of *TLR* genes

Genetic variation within the human population is common and is thought to result from adaptation to changes in environment requiring thousands of years (130, 131). This potentially leads to protection from certain diseases but also predisposition to other diseases (132). Single nucleotide polymorphism (SNP) is the term used when one base in the genome is replaced with another. If SNP does not lead to an amino acid change in the encoded protein, it is called a synonymous mutation. If it does, it is called a missense mutation (114, 130). A synonymous mutation may have functional relevance if it affects messenger RNA splicing and subsequent protein expression (133). Non-sense mutations cause a codon change and subsequently lead to larger changes in the structure or production of encoded proteins. (114). The consequence of a mutation in the TLR protein is dependent on its location. If the SNP affects the structure of the extracellular domain, it may lead to altered detection or binding of a pathogen (134). If the mutation affects the cytoplasmic domain, it may result in an altered downstream signalling (119).

Variation in innate immunity genes, such as in *TLR* genes, may lead to differences in host defence that further alter susceptibility to infectious diseases. There also is evidence that TLRs play a pivotal role in the normal development of immune responses, whereas mutations in the encoding genes may lead to biased immunity and, further, a predisposition to inflammatory or atopic diseases (32). However, it is possible, that SNPs are not disease-causative alone, but that distinct gene-

environment or gene-gene interactions are required for the development of certain diseases. Thus, a polymorphism in a certain *TLR* gene would be pathological only in interaction with other genes or with environmental factors, such as viruses, for example (28). Minor allele frequency (MAF) means the frequency of the variant (minor) allele in the population.

2.7 Toll-like receptors, bronchiolitis, allergy and asthma

Asthma is the most common chronic illness in children worldwide. Hygiene hypothesis, presented almost three decades ago, stated that early-life contacts with microbes are essential for the maturation of adaptive immunity and to decrease the risk of asthma and allergies (135). In line is the observation that the prevalence of asthma and allergies has increased in Western countries in the last decades, concurrently with improved hygiene in living conditions. This has been confirmed by studies reporting that children living on farms had lower asthma prevalence than urban children (136, 137). However, severe virus infections in early life, bronchiolitis as one of the most important, have been strongly associated with the development of asthma later in childhood and in adolescence (27). The exact mechanisms beyond asthma development are still obscure, but it seems that the development and maturation of immunity play a key role in this process.

Genetic factors, most importantly variations in the genes that regulate innate immunity (such as TLRs encoding genes), have been suggested to be associated with asthma susceptibility. Activation of TLRs has been reported to modify immune development, and their normal function seems to protect from asthma, whereas mutations in these receptors may predispose towards asthma and other atopic diseases (32, 138). Several polymorphisms in *TLR* genes have been linked with asthma in exploratory studies, but since the results and study settings have been highly heterogenic, confirmative studies are usually lacking. The confirmation of the results means that the findings can be repeated in identical verification populations and in other different populations. In addition, the biological function of the polymorphism should be verified in experimental studies.

The normal activation of innate immunity via TLRs probably modifies development of adaptive immunity as TLRs induce naïve T-cells to differentiate into Th1-cells or, in some circumstances, into Th2-cells, the direction depending on the timing, dose and nature of the ligand, as well as genetic factors of the host (139). Existing Th2 responses and weak Th1 responses are characteristic for immature

adaptive immunity at birth; normally, the maturation of immune system shifts the balance from Th2 towards Th1 prominence. It has been suggested that an environmental insult, such as a virus infection in early infancy when the immune system is still Th2-skewed, may disturb the normal maturation process and lead to the persistence of Th2-oriented immunity (85, 103). The consequence can be chronic inflammation in the airway mucosa that is characteristic of asthma and respiratory allergy (27). As only a minority of children with bronchiolitis will go on to develop asthma, however, it is likely that this happens only in predisposed children. Thus, post-bronchiolitis asthma would result from a distinct gene-environment interaction—a distinct combination of genetic susceptibility and environmental exposures, such as viral LRTIs like bronchiolitis (140). The link between bronchiolitis and asthma may be in innate immunity genes. As described earlier, TLRs are in first line in inducing adequate immune responses; therefore, mutations in *TLR* genes may alter susceptibility to severe bronchiolitis via attenuated anti-viral responses. This may further lead to biased immunity and, finally to asthma. Another possibility is that mutations in *TLR* genes do not affect susceptibility to bronchiolitis, but instead susceptibility to asthma, and thus virus infection is an essential trigger in this process. Asthmatic subjects have been reported to have attenuated antiviral responses (100), and it is therefore possible that bronchiolitis leading to hospitalisation is the first sign of impaired TLR responses, thus unmasking the asthma-predisposed individuals.

2.7.1 Toll-like receptors 1, 2, 6 and 10 (TLR2 subfamily)

There is evidence of the role of genes encoding the TLR2 subfamily and their polymorphisms in asthma development, but the results have not been at all unanimous. Thus far, TLR2 is the most studied member of the subfamily. Several different polymorphisms in the *TLR2* gene (rs3804100, rs4696480, rs1898830, rs3804099, rs7656411) have been associated with childhood asthma (141-144) or with asthma severity (rs5743708)(122), but the associations usually have not been strong and the direction of the association regarding distinct polymorphisms has varied between studied populations. In most cases, there is a lack of studies repeating the positive results in other cohorts or populations. Most of the studies are not directly comparable with each other because the study designs have varied in both ethnicity of the study subjects and in environmental exposures. A few studies have reported gene-gene or gene-environment interactions where *TLR2* gene polymorphism has been associated with childhood asthma only in combination with

other gene polymorphism (142) or with environmental factors, such as living on a farm or exposures to air pollution (145, 146). In German children, *TLR2* rs5743708 polymorphism was found to increase the risk of atopic sensitisation assessed by increased total serum immunoglobulin E (IgE) level, allergen-specific serum IgE levels and SPT positivity, but the SNP was not associated with the risk of asthma or atopic eczema (147). It has also been reported that the activation of TLR2 shifts adaptive immune responses towards Th2-polarisation and possibly results in asthma (148) .

In the case of TLR10, two polymorphisms (rs4274855 and rs4129009) in the *TLR10* gene were associated with an increased risk for asthma in an American case-control study in adults, and these findings were replicated in an independent sample of children from a family-based cohort (124). Interestingly, the latter polymorphism rs4129009 was associated with a decreased risk for atopic asthma in a German study, although the result lost its statistical significance after correction for multiple testing (143). In a large Canadian-Australian study that aimed to replicate previous findings from four genetic association studies regarding asthma and atopy candidate genes, a weak association between two *TLR10* polymorphisms (rs10776483 and rs11096957, respectively) and a decreased risk for atopic asthma were found in one of the included studies, and the rs11096957 polymorphism retained its statistical significance in the analysis where all four studies were combined (149). There were no significant associations between four other polymorphisms in *TLR10* and asthma or with polymorphisms in *TLR2* or TLR6 and asthma, nor were any of the examined polymorphisms associated with atopy (149).

Only a few studies have reported an association between *TLR1* polymorphisms and asthma. In the German study, two polymorphisms in *TLR1* (rs5743595 and rs4833095, respectively) were found to protect from atopic asthma in childhood (143). A recent Taiwanese study found no association between *TLR1* rs4833095 and asthma or other atopic diseases. The variant genotype, however, was associated with lower total serum IgE levels in allergic children (150). Another *TLR1* polymorphism rs4543123 was associated with a decreased risk for atopy in a study analysing the data from four independent cohort studies (140). Interestingly, in the SNP-virus interaction analyses, the same polymorphism seemed to increase the risk for both atopy and atopic asthma after RSV infection before 12 months of age, and this interaction with RSV infection was also seen for several other polymorphisms in *TLR1*, *TLR6* and *TLR10* genes, respectively (140).

TLR6 gene polymorphism rs5743789 was reported to have protective effects from atopic asthma in childhood similar to the two *TLR1* polymorphisms in the

German study (143). In the same study, however, another *TLR6* polymorphism (rs5743810) was associated with an increased risk for atopic asthma in childhood (143). This was in line with both another study in German children (151) and a recent Australian study that reported the wild allele in the rs5743810 to protect children with farm exposure from asthma development before 13 years of age (152). In contrast, a study in African-American adults found the minor allele to have an opposite effect, and thus an association with a decreased risk for asthma (153). There are also studies with negative results regarding the association between *TLR6* rs5743810 polymorphism and childhood asthma (142, 149, 154, 155). Two other *TLR6* polymorphisms (rs6531666 and rs5743798) were associated with atopic asthma and/or atopic eczema in Dutch children, with the minor allele increasing the risk (154).

Because of their proximity to each other, *TLR2* subfamily genes are likely to interact. Therefore, it is appropriate to evaluate the impact of the examined polymorphisms in combined analyses. A study examining two *TLR6* polymorphisms and seven *TLR10* polymorphisms did not find any of the polymorphisms to be associated with childhood asthma, but the analysis combining both genes, however, revealed some significant associations (155).

Some experimental studies have shown preliminary evidence that TLRs might be used in the future as therapeutic tools for asthma. A recent study on mice reported that treatment with *TLR1* and *TLR2* mRNA, or with *TLR2* and *TLR6* mRNA, improved lung function and reduced airway inflammation *in vivo* (156).

2.7.2 TLR2 subfamily in the present cohort until 7 years of age

In this cohort, *TLR1* rs5743618, *TLR2* rs5743708, *TLR6* rs5743810 or *TLR10* rs4129009 polymorphisms were not associated with the occurrence or severity of bronchiolitis or repeated wheezing until 1.5 years of age following bronchiolitis in infancy (157, 158). However, the *TLR10* rs4129009 variant genotype was associated with elevated serum total IgE levels and *TLR6* rs5743810 variant genotypes showed a weak association with allergy (a doctor-diagnosed food allergy or atopic eczema) at 18 months of age (158, 159). In line, at the 5–7-years control visit, the carriage of the minor allele of *TLR6* rs5743810 was associated with an increased risk for atopic eczema and allergic rhinitis (160). *TLR1* rs5743618 variant genotypes were associated with post-bronchiolitis asthma between 1 and 6 years of age; in a further haplotype analysis, having the wild genotype in all three aforementioned genes (*TLR1, TLR2* and *TLR6*, respectively) protected from asthma during the first 6 years

of life, compared to variant haplotypes in at least one of the genes (160). In another study from this cohort with a focus on lung function after infant bronchiolitis, the *TLR6* rs5743810 variant genotype was associated with bronchial hyper-reactivity, and having a wild genotype in all four *TLR2* subfamily genes was associated with less bronchial hyper-reactivity (161).

2.7.3 Other Toll-like receptors

In an animal model, activation of TLR3 with a viral ligand and a simultaneous inhalation of allergen resulted in allergic airway disease (162). Previously in the present cohort, the *TLR3* rs3775291 variant genotypes were found to be protective against recurrent wheezing after bronchiolitis. Interestingly, the minor allele T was found to be overrepresented in this study population, suggesting that polymorphism in the TLR3 gene may predispose towards severe bronchiolitis leading to hospitalisation. The investigated polymorphism had no association with early atopic eczema (163).

TLR4 rs4986790 polymorphism leads to a missense mutation that has been described as resulting in attenuated immune responses to LPS (164) . This mutation was associated with an increased risk of severe RSV bronchiolitis (165). However, in the present cohort, *TLR4* rs4986790 was not associated with bronchiolitis, atopic diseases or post-bronchiolitis wheezing during the 1.5 years follow-up (159, 166). Accordingly, no association was found between the same polymorphism and asthma in a meta-analysis of nine case-control studies (167).

The role of TLR5 in bronchiolitis has been investigated in only one study thus far. In that study, the *TLR5* rs5744174 polymorphism was not associated with the risk of RSV bronchiolitis (168). This polymorphism results in a missense mutation and has been reported to attenuate TLR5 signalling (169). A few studies have reported a decreased expression or impaired function of TLR5 in asthmatic patients. However, a German study found no association between the TLR5 rs5744174 or two other polymorphisms and asthma (143).

Variation in the *TLR7* gene (rs179008 polymorphism more precisely) has been shown to be protective for recurrent RV infections, whereas the *TLR8* rs2407992 polymorphism had the opposite effect, increasing the risk of recurrent RV infections (170). In addition, variations in *TLR7* and *TLR8* genes have been associated with an increased risk for asthma and allergic rhinitis, and *TLR8* polymorphism has been linked with atopic eczema (133, 171), although contradictory results have been reported (143). In line, normal TLR7 function has been suggested to protect from

the development of Th2-type airway inflammation (172) and to prevent airway hyper-reactivity often caused by respiratory viruses (173). An Australian study reported impaired anti-viral responses following TLR7 stimulation in asthmatic adolescents, whereas TLR3 responses did not vary between asthmatics and controls (174). Although the researchers did not study the genetic background behind these weakened responses, their finding that the expression of *TLR7* in mild to moderate asthma did not differ from controls might implicate that a mutation in the *TLR7* gene might affect the downstream signalling. However, in another study, the expression of TLR7 and TLR5 was normal in patients with moderate asthma but was reduced in severe asthmatics (175). In the present cohort, *TLR7* polymorphism was associated with feeding problems during bronchiolitis, but only in boys. Such differences between males and females are interesting, and worth being studied, as the TLR7 and TLR8 genes are located in the X chromosome (29). The *TLR8* rs2407992 polymorphism was not associated with bronchiolitis, post-bronchiolitis wheezing or atopic diseases in the present cohort (157).

The TLR9 rs187084 polymorphism has been linked to asthma susceptibility in a recent meta-analysis (138). In the present cohort, the same polymorphism was associated with an increased risk of wheezing after bronchiolitis. In the present cohort, no associations with bronchiolitis or atopic eczema were found in infancy (157).

Table 2. Variation in the *TLR* genes and their association to bronchiolitis, post-bronchiolitis wheezing, asthma, allergic rhinitis or atopic eczema before school age in previous publications from the present cohort.

***TLR* gene**	**Bronchiolitis**	**Wheezing between 6 and 18 months of age**	**Wheezing/asthma, AR or AE between 1.5 years and 5-7 years of age**	**Asthma, AR or AE at preschool age**	**Publication**
TLR1 rs574618	NS	NS	Asthma: increased risk AE: increased risk	NS	(158, 160)
TLR2 rs5743708	NS	NS	NS	NS	(158, 160)
TLR3 rs3775291	Increased risk	Decreased risk	--	--	(163)
TLR4 rs4986790	NS	NS	--	--	(159, 166)
TLR6 rs5743810	NS	NS	NS	AR: increased risk AE: increased risk	(158, 160)
TLR7 rs179008	NS	NS	--	--	(157)
TLR8 rs2407992	NS	NS	--	--	(157)
TLR9 rs187084	NS	Increased risk	--	--	(157)
TLR10 rs4129009	NS	NS	--	--	(157)

AR, allergic rhinitis; AE, atopic eczema

NS, non-significant

3 AIMS OF THE STUDY

The aims of this study were to investigate the long-term clinical outcome after bronchiolitis in infancy and to determine clinical and genetic risk factors that would predict asthma after bronchiolitis.

The specific aims were:

1. To assess the incidence of asthma, allergic rhinitis and atopic eczema at 11 – 13 years of age after bronchiolitis under 6 months of age.
2. To compare the prevalence of asthma, allergic rhinitis and atopic eczema at 11–13 years of age in the original bronchiolitis group and the age- and sex-matched control group.
3. To identify clinical early-life and preschool-age risk factors predicting asthma at 11–13 years of age after bronchiolitis in infancy.
4. To explore the role of single nucleotide polymorphisms in TLR 3, 4, 5, 7, 8, 9 and 10 encoding genes in asthma at 5–7 years of age after bronchiolitis in infancy.
5. To confirm the role of single nucleotide polymorphisms in TLR 1 and 10 encoding genes in asthma at 11–13 years of age after bronchiolitis in infancy.

4 MATERIALS AND METHODS

4.1 Enrolment of study subjects and the hospitalisation data

In all, 203 infants were originally recruited in this study when they were hospitalised for bronchiolitis in the Department of Paediatrics, Tampere University Hospital, Finland, between December 1, 2001 and May 31, 2002 and between October 28, 2002 and May 31, 2004. According to the inclusion criteria, the infants had to be less than 6 months of age, previously healthy and born full term; of the 203 infants recruited, 187 children were eligible for the study. Bronchiolitis was defined as first episode of acute LRTI characterised by rhinorrhoea, cough, tachypnoea, feeding problems and diffuse wheezes or inspiratory crackles on auscultation.

During hospitalisation, nasopharyngeal aspirates were taken to determine the viral aetiology of bronchiolitis by using indirect immunofluorescence for antigen detection of RSV, Influenza A and B viruses, adenovirus and parainfluenzaviruses 1, 2 and 3. The analyses were supplemented by reverse transcriptase-PCR (RT-PCR), which detected the same viruses in addition to RV, metapneumovirus and bocavirus. Combined results of these analyses were used to determine the causative agent. The need for supplementary oxygen and feeding support and the length of hospital stay were recorded for the assessment of disease severity. The parents were interviewed using a structured questionnaire and data on parental smoking, including maternal smoking during pregnancy, as well as family histories of asthma and allergy were collected.

4.2 Follow-up visit at the age of 1.5 years

The first follow-up visit was arranged from May 2003 to June 2005, and 129 children attended at an average age of 18 months (range 13–25 months). The parents had been asked to keep a record of all doctor-diagnosed respiratory infections and wheezing episodes after hospitalisation for bronchiolitis. At the control visit, the symptom diaries were checked and the parents were interviewed using a structured questionnaire to evaluate the occurrence of atopic eczema, wheezing episodes and

the use of inhaled corticosteroid (ICS) medication. The children were examined by a paediatrician, and blood samples were taken for genetic studies.

4.3 Follow-up visit at the age of 5–7 years

The second follow-up visit was arranged in October 2008, January and March 2009 and October 2009, when the children were 5–7 years of age. Altogether, 166 children took part in the follow-up: 127 participated in the clinical visit, and parents of 39 children were interviewed by telephone. In the case of an acute respiratory infection during the preceding two weeks, the control visit was rescheduled. Antihistamine medication had to be discontinued for five days and beta-agonists for at least 12 hours before the visit.

A structured questionnaire was sent to homes before the scheduled visit to evaluate the occurrence of atopic eczema, allergic rhinitis and doctor-diagnosed asthma as well as age at the time of asthma diagnosis. The questionnaire also contained current and earlier use of ICSs, and the occurrence of asthma-suggestive symptoms was recorded, including wheezing episodes, prolonged cough or night cough occurring for more than 4 weeks outside infection after the last control visit. Data on family histories of asthma and allergies, as well as parental smoking during pregnancy and/or when the child was under 12 months of age were also collected. A paediatrician reviewed the pre-filled questionnaires with the families at the follow-up visit and performed a clinical examination for the child.

SPTs were performed on 124 children to investigate sensitisation to birch, timothy grass and mugwort pollens, cat and dog dander, house dust mites (*D. pteronyssimus* and *D. farinae*) and spores of the mould *Alternaria alternate*. A wheal diameter of at least 3mm was regarded as positive.

Lung function was tested by impulse oscillometry (IOS, Master Screen IOS; Jaeger, Höchberg, Germany), as published recently (176). In brief, three reliable measurements were required: at baseline, after an exercise challenge test outdoors and after the inhalation of 300ug salbutamol (Ventolin, GSK, London, UK) through a spacer (Babyhaler, GSK, London, UK). At least a 35% increase in 5Hz resistance (Rrs5) after exercise or ≤35% decrease in Rrs5 after salbutamol inhalation was regarded as pathological bronchial reactivity. The IOS data on lung function at preschool age was available for 103 children.

4.4 Follow-up visit at the age of 11–13 years

The 166 children who participated in the previous control visit at 5–7 years of age were invited for the third follow-up visit between June 1, 2014 and January 31, 2015, when the children were 11–13 years of age. The families were asked to complete a structured questionnaire (Appendix 1) before the control visit. Altogether, 138 children took part in the follow-up: 89 attended the clinical follow-up visit, while an additional 49 families just returned the completed questionnaire, supplemented by a telephone interview. The questionnaire data were reviewed at the control visit or during the telephone interview and included questions on doctor-diagnosed atopic eczema, allergic rhinitis and asthma, current medication for asthma and current asthma-suggestive symptoms as well as family history of asthma and allergies and exposure to tobacco smoke during pregnancy or infancy.

Lung function of the 89 children who participated in the clinical visit was tested with a flow-volume spirometry and a bronchodilation test. Measurements were performed before and 15 minutes after the inhalation of 400ug salbutamol (Ventolin, GSK, London, UK); three technically acceptable measurements were required at both times. The best pre- and post-treatment values of forced expiratory volume in one second (FEV1) were analysed and presented as age- and gender-specific and height-adjusted values (predicted FEV1). The bronchodilation test was regarded as positive and showing a reversible bronchial obstruction if the predicted FEV1 increased 12% and 0.2l or more after the administration of salbutamol.

4.5 Control group

For the third follow-up visit, four controls were invited for each of the 166 former bronchiolitis patients. The controls were matched for age and gender and were selected from the population register of the Tampere (Pirkanmaa) Hospital District. Hospitalisation for any medical reason and outpatient treatment for bronchiolitis or any other LRTI during infancy were exclusion criteria. Prior to the visit, the controls received the same questionnaire sent to the bronchiolitis group. In all, 108 controls (out of 664 invited) attended the clinical visit and performed the lung function test. An additional four families only returned the completed questionnaire.

4.6 Outcome variables

At 11–13 years of age, the outcome events of this study were current asthma, continuous ICS use during the last 12 months, persistent asthma, current allergic rhinitis and current atopic eczema. Current asthma was considered to be present if the child had used ICS for asthma regularly during the last 12 months or if the child had suffered from repeated wheezing or from prolonged cough or night cough over four weeks during the last 12 months, and, in addition, had a diagnostic increase of FEV1 in the bronchodilation test. Persistent asthma was defined as the presence of current asthma at both control visits at 5–7 years of age and 11–13 years of age, respectively. Current allergic rhinitis was defined as symptoms of runny or stuffy nose or sneezing outside infection during the last 12 months. Current atopic eczema was defined as doctor-diagnosed allergic eczema that was symptomatic during the preceding 12 months.

At 5–7 years of age, when the associations of *TLR* gene polymorphisms with post-bronchiolitis outcomes were analysed, the outcome variables were current asthma, asthma ever, current atopic eczema or current allergic rhinitis. During the follow-up visit at 5–7 years of age, current asthma was considered to be present if the child had had continuous maintenance medication for asthma during the last 12 months. In addition, children who had suffered from asthma-presumptive symptoms (doctor-diagnosed wheezing or prolonged cough or night cough over four weeks) during the last 12 months and whose IOS was pathological were regarded as current asthmatics. Asthma ever was defined as current or previous asthma.

4.7 Genetic methods

The polymorphisms of the *TLR1* rs5743618 (1805 G/T), *TLR3* rs3775291 (1234 C/T), *TLR4* rs4986790 (299 A/G), *TLR5* rs5744174 (1174 C/T), *TLR7* rs179008 (171 A/T), *TLR8* rs2407992 (2040 C/G), *TLR9* rs187084 (1486 T/C) and *TLR10* rs4129009 (2322 A/G) were determined by the National Institute of Health and Welfare, Turku, Finland, and the Department of Medical Microbiology and Immunology, University of Turku, Finland, as described earlier (158). These SNPs were selected for their previously reported functional properties. Except for *TLR5* rs5744174, all other polymorphisms have been investigated previously in this cohort in relation to bronchiolitis and repeated wheezing after bronchiolitis. In addition,

TLR1 rs5743618, *TLR2* rs5743708 and *TLR6* rs5743810 have been investigated in relation to asthma at 5–7 years of age after bronchiolitis (160). *TLR2* rs5743708 and *TLR6* rs5743810 were not associated with current or previous asthma at 5–7 years of age and thus were not included in the confirmatory studies at 11–13 years of age.

The genotyping of *TLR1* rs5743618, *TLR3* rs3775291 and *TLR8* rs2407992 was performed by pyrosequencing (PSQ™96MA Pyrosequencer, Biotage, Uppsala, Sweden) using a PSQ™96 Pyro Gold Q96 reagent kit according to the manufacturer's protocol, as described earlier (160, 163, 177). The genotyping of *TLR4* rs4986790 was performed by the ABIPRISM 7000 Sequence Detection System (Applied Biosystems, CA)(166) and later supplemented with pyrosequencing (PSQ™96MA Pyrosequencer, Biotage, Uppsala, Sweden) using a PSQ™96 Pyro Gold Q96 reagent kit (178). For *TLR7* rs179008, the PCR products were first purified using the QIA quick PCR Purification Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. After purification, the PCR products were pipetted to a 96-well plate (5µl) together with *TLR7* rs179008 forward primer (1.6µl), and the 96-well plate was sent to the Institute for Molecular Medicine laboratory in Helsinki, Finland, for sequencing (161). *TLR9* rs187084 genotyping was performed using BspTI restriction enzyme (ThermoFischer Scientific, Waltham, USA) for digestion of the PCR product (161).

High resolution melting analysis (HRMA) (Roche Diagnostics Light Cycler 480, Basel, Switzerland) was used for genotyping of *TLR5* rs5744174 and *TLR10* rs4129009 (161, 179). HRMA is a post-PCR melt analysis method based on the analysis of released fluorescence from binding dye of the dsDNA. Primers (forward 5´-ACCTTCCGTGGAAAGAGAGAA-3´ and reverse 5´-TGCAGACATATATTGTGTGTACCCT-3) were designed with the Primer-Blast design tool. Amplicon size was only 70bp which is small enough to maximise the difference between melting peaks in variant genotypes and to avoid the other SNPs. Three samples with known genotypes were used to determine the proper concentration of MgCl2 and the appropriate annealing temperature for assay. In each run reaction, the volume was 20µl, consisting of 3µl of genomic DNA (~8,0ng/µl) and 17µl of master mix, including 10µl of melting master dye and 0.2µM of forward and reverse primers. The master mix provides a final concentration of 3 mM of MgCl2. HRMA reactions were run at 95°C for 10 min followed by 45 cycles of amplification at 95°C for 10 s, at 60°C for 10 s and at 72°C for 15 s. After the PCR process, final melting cycle conditions were as outlined by the manufacturer: first heating to 95°C and hold for 1 min, then cooling to pre-hold temperature (40°C). Melting interval for collecting fluorescence from 60°C -95° at ramp rate 0.02°C per

second. In each run, known *TLR5* rs5744174 and *TLR10* rs4129009 standards (wild, and heterozygous and homozygous variants) were used.

There were 135 samples available for genotyping of the *TLR3*, *TLR4*, *TLR7*, and *TLR8*. For the genotyping of the *TLR9* and *TLR10*, 134 samples were available.

4.8 Statistical analyses

Statistical analyses were performed using SPSS for Windows versions 21.0 and 23.0 (IBM Corp, NY, USA). The Chi-square test was used for categorised variables, but in the case of a small sample size, when the expected frequency for any cell was less than 5, Fisher's exact test was used, as appropriate. Logistic regression was used to analyse the association between early-life risk factors (maternal asthma, maternal allergy, atopic eczema at less than 12 months of age and non-RSV aetiology of bronchiolitis) and preschool-age risk factors (allergic rhinitis and SPT positivity) and current asthma at 11–13 years of age. Those factors, which were significant in the non-adjusted analyses, were included in the adjusted analyses. In addition, logistic regression with adjustments for age and gender was used to analyse the *TLR3, TLR4, TLR5, TLR7, TLR8, TLR9* and *TLR10* genotypes between those with and without current asthma, asthma ever, current allergic rhinitis or current atopic eczema at the preschool-age follow-up visit. Logistic regressions were initially carried out as univariate analyses and then as multivariate analyses, adjusted for gender, age, early-life risk factors (namely, maternal asthma and non-RSV aetiology of bronchiolitis); and current confounder (namely, current atopic eczema). At the 11–13 years follow-up visit, logistic regression with adjustments for gender and age was used to analyse the *TLR1* and *TLR10* genotypes and genotype combinations between those with and without current asthma, persistent asthma, current ICS medication for asthma, current allergic rhinitis or current atopic eczema. In multivariate analyses, adjustments were made with maternal asthma, atopic eczema at less than 12 months of age and non-RSV aetiology of bronchiolitis. The results were expressed as odds ratios (OR) and 95% confidence intervals (95% CI).

The FINETTI programme was used to evaluate the Hardy-Weinberg equilibrium (HWE) of the studied *TLR1, TLR3, TLR4, TLR5, TLR9* and *TLR10* alleles, and all were in the HWE. Since *TLR7* and *TLR8* genes are located in the X chromosome, boys and girls were analysed separately and HWE was not studied.

4.9 Ethics

The study was approved by the Ethics Committee of the Tampere (Pirkanmaa) University Hospital District, Finland. Before enrolment and at follow-up visits at 1.5 and 5–7 years of age, parents gave written consent of their child's participation in the study. At the latest follow-up visit, at 11–13 years of age, written consent was obtained from the study subjects and controls themselves, as they were regarded as able and old enough to understand the given information. The use of genetic data was limited to bronchiolitis and asthma research only, and personal details of the study participants were not given to the genetic laboratories. Before each control visit, an invitation letter with information about the study and a structured questionnaire was sent to homes. The study visit had been scheduled beforehand for each study subject, but it was highlighted in the invitation letter that participation was voluntary and that they could choose not to attend. Those who did so were not obliged to reschedule or to cancel the visit by contacting the study nurse. The families had also the opportunity to participate in the study by answering the questions in a telephone interview. In contrast, the controls had to schedule the visit themselves if they chose to take part in the study, and the contact information of the study nurse was provided in the invitation letter. In case the controls did not react, reminders were not allowed.

5 RESULTS

5.1 Post-bronchiolitis outcome at the age of 11–13 years (Article I)

In all, 138 children from the bronchiolitis group participated in the follow-up study at the mean age of 11.7 years (range 10.2–13.2); 72 (52.2%) of them were boys. Current asthma was present in 18 (13.0%) children, boys being slightly overrepresented (61.1%, p=0.42). Of the 18 current asthmatics, 14 had used ICSs as maintenance medication for asthma during the last 12 months and 4 were diagnosed to have asthma at the study visit (Table 3).

Current atopic manifestations were relatively common in former bronchiolitis patients; 60 (43.5%) children had current allergic rhinitis and 35 (25.4%) had current atopic eczema.

There were no significant differences in the preschool risk factors, such as allergic rhinitis, atopic eczema or the use of bronchodilators or ICSs, or in the prevalence of current asthma reported at the 5–7 years follow-up visit between the 138 participants and the 28 drop-outs. Likewise, the 89 children who attended the clinical follow-up differ did not differ from those 49 who only answered the questionnaire in terms of the risk factors or the occurrence of current asthma or asthma from 5–7 years until 11–13 years of age.

Current asthma was present in 12 (10.7%) children in the control group. Of them, 9 had used ICSs during the last 12 months and 3 were diagnosed to have asthma at the study visit. Thus, the prevalence of asthma was almost the same in the cases and the controls. The cases and controls did not differ for the current atopic manifestations (allergic rhinitis or atopic eczema) or for their parental history of asthma or allergy (Table 3). Asthma in fathers was marginally more common in controls than in cases. Early-life tobacco smoke exposure was significantly more common in the bronchiolitis group than in the control group: 13.8% (vs. 2.7%, p=0.002) of the mothers in the bronchiolitis group had smoked during pregnancy, and 21.7% (vs. 5.4%, p<0.001) of the mothers and 36.2% (vs. 23.2%, p=0.026) of the fathers in the bronchiolitis group had smoked when the children were under 12 months old (Table 3).

Table 3. Baseline characteristics in 138 children hospitalised for bronchiolitis before 6 months of age and in 112 age- and sex–matched population-based controls, based on questionnaires completed at the follow-up visits at the age of 11–13 years.

	Bronchiolitis group n=138		Control group n=112		
	n	%	n	%	p value*
Males	72	52.2	62	55.4	0.616
Current allergy					
Atopic dermatitis	35	25.4	26	23.2	0.694
Allergic rhinitis	60	43.5	50	44.6	0.854
Family asthma and allergies					
Asthma in mothers	18	13.0	15	13.4	0.953
Asthma in fathers	9	6.5	16	14.3	0.044
Allergy in mothers	49	35.5	46	41.1	0.391
Allergy in fathers	38	27.5	38	33.9	0.291
Early smoking exposure					
Maternal smoking (pregnancy)	19	13.8	3	2.7	0.002
Maternal smoking (<12 months of age)	30	21.7	6	5.4	<0.001
Paternal smoking (<12 months of age)	50	36.2	26	23.2	0.026
Current asthma and ICS use					
Current asthma	18	13.0	12	10.7	0.573
Current ICS use	14	10.1	9	8.0	0.566

*Calculated by Chi-square test or Fisher´s exact test.

5.2 Risk factors for post-bronchiolitis asthma at the age of 11–13 years (Article I)

5.2.1 Atopy

Atopic manifestations in infancy and at preschool age were associated with an increased risk for post-bronchiolitis asthma at 11–13 years of age. Of the 18 current asthmatics, 15 had current and/or earlier atopic manifestations, such as atopic eczema at <12 months of age, or allergic rhinitis or SPT positivity at 5–7 years of age (Table 3). These participants were regarded as having atopic asthma.

Half of the current asthmatics had a history of atopic eczema before 12 months of age, and atopic eczema in infancy was a significant early-life risk factor for post-bronchiolitis asthma (Table 4). Allergic rhinitis and SPT positivity when present at preschool age predicted current asthma at 11–13 years of age (Table 5). However, in a multivariate analysis, atopic eczema in infancy lost its significance as an early-life risk factor (Table 6, Model 1). When allergic rhinitis was included in the analysis as a preschool-age risk factor, its association with post-bronchiolitis asthma remained significant (Table 6, Model 2). Data on SPT positivity were available only for 109 of 138 children. In a supplementary analysis, where allergic rhinitis was replaced with SPT positivity as a preschool-age risk factor, SPT positivity retained its significance (Table 6, Model 3). Asthma before school age predicted current asthma at 11–13 years of age (Figure 2). Eleven of the 18 (61.1%) with current asthma, also had asthma at the earlier follow-up visit at 5–7 years of age ($p<0.001$). All children with asthma at both the 5–7 and 11–13 years follow-up visits presented with current allergic rhinitis; and were thus considered to have atopic asthma.

Table 4. Early-life risk factors and current asthma at 11–13 years of age after severe bronchiolitis before 6 months of age.

	Current asthma				
	Yes		No		
	n	%	n	%	p value*
Male gender	11	61.1	61	50.8	0.420
Age at admission					
< 3 months (n=82)	7	38.9	75	62.5	
>3 months (n=56)	11	61.1	45	37.5	0.057
RSV (n=91)	10	55.6	81	67.5	
Non-RSV (n=47)**	8	44.4	39	32.5	0.320
Rhinovirus (n=18)	2	11.1	16	13.3	1.0
Atopic dermatitis (<12 months of age) (n=40)	9	50.0	31	25.8	0.035
Maternal smoking (pregnancy) (n=24)	2	11.1	22	18.3	0.740
Maternal smoking (<12 months of age) (n=37)	5	27.8	32	26.7	1.00
Paternal smoking (<12 months of age) (n=56)	7	38.9	49	40.8	0.880
Asthma in mothers (n=22)	8	44.4	14	11.7	0.002
Asthma in fathers (n=8)	0	0.0	8	6.7	0.600
Allergy in mothers (n=58)	13	72.2	45	37.5	0.005
Allergy in fathers (n=34)	4	22.2	30	25	1.000
Pets at home (n=45)	4	22.2	41	34.2	0.310

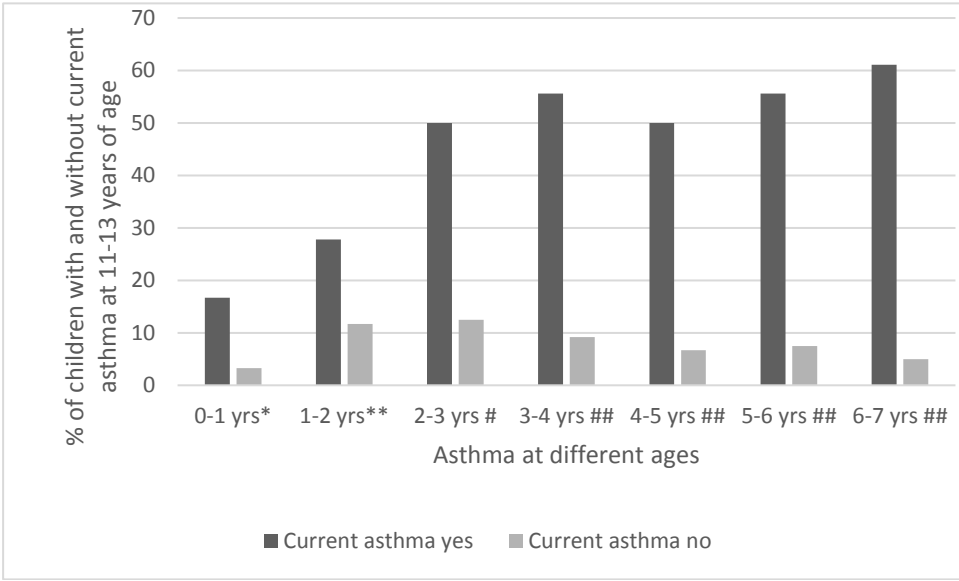
*Calculated by Chi-square test or Fisher´s exact test. **Non-RSV group consisted of 18 rhinovirus-positive and 10 virus-negative cases and of 19 cases caused by other viruses.

Table 5. Preschool-age risk factors and current asthma at 11–13 years of age after severe bronchiolitis before 6 months of age.

	Current asthma				
	Yes		No		
	n	%	n	%	p value*
Allergic rhinitis n=39	11	61.1	28	23.3	0.001
SPT positivity n=32**	9/14	64.3	23/95	24.2	0.002
Asthma n=20	11	61.1	9	7.5	<0.001

*Calculated by Chi-square test or Fisher´s exact test. **Skin prick test (SPT) results were available from 109 children (14 of those with present asthma), other data available from 138 children. SPT positivity was defined as a wheal diameter of 3mm or more as a reaction to at least one of the tested allergens.

Figure 2. Current asthma in relation to doctor-diagnosed asthma before school age after hospitalisation for bronchiolitis before 6 months of age.



*p=0.047, **p=0.076, #p=0.001, ##p<0.001

5.2.2 Parental atopy and asthma

Parental history of asthma was common in the study subjects. Eighteen (13.0%) of the mothers and 9 (6.5%) of the fathers reported having asthma, and 49 (35.5%) of the mothers and 38 (27.5%) of the fathers reported having allergy (Table 3). In univariate analysis, maternal asthma and allergy were significantly associated with post-bronchiolitis asthma at 11–13 years of age, whereas paternal asthma and allergy were not (Table 4). In multivariate analyses, maternal asthma was the only significant early-life risk factor for post-bronchiolitis asthma. It also retained its significance when allergic rhinitis as a preschool-age risk factor was included in the analyses (Table 6, Model 2). In the supplementary analysis including only those 109 children with SPT data available, maternal asthma marginally lost its significance (Table 6, Model 3).

Table 6. Logistic regression: Early-life and preschool-age risk factors for asthma at age 11–13 years after hospitalisation for bronchiolitis in early infancy.

	Univariate		Multivariate					
			Model 1		Model 2		Model 3	
	OR	95% CI	OR	95% CI	OR	95% CI	OR	95% CI
AE at < 12 months of age	2.87	1.05-7.89	1.92	0.65-5.69	2.00	0.65-6.13	1.34	0.38-4.61
Allergy in mother	4.33	1.45-12.96	2.45	0.72-8.32	1.95	0.56-6.80	1.99	0.56-7.04
Asthma in mother	6.06	2.05-17.91	3.45	1.07-11.74	3.50	1.04-11.78	1.99	0.47-8.38
AR at age 5-7 years	5.16	1.83-14.56			4.06	1.35-12.25		
SPT positivity at age 5-7 years*	5.64	1.72-18.52					5.01	1.47-17.01

Model 1 includes early-life risk factors (AE at <12 months of age, maternal allergy, maternal asthma) and Models 2 and 3 both early-life and preschool-age (AR at age 5–7 years or SPT positivity at age 5–7 years) risk factors.
* Data available from 109 children.
AE=atopic eczema AR=allergic rhinitis

5.2.3 Age and environmental factors

Children who were older than 3 months on admission for bronchiolitis were more likely to have current asthma than were younger infants, but this difference did not reach statistical significance (19.6% vs. 8.5%, p=0.057) (Table 4.).

Altogether, 42.9% of children in the bronchiolitis group were exposed to parental smoking either during pregnancy or in infancy. However, pre- or postnatal tobacco smoke exposure was not associated with post-bronchiolitis asthma at 11–13 years of age (Table 4).

Bronchiolitis was caused in 65.9% of the cases by RSV and in 13.0% by rhinovirus (Table 4). In 13.8% of the cases, another virus was responsible (influenza A virus in 7, metapneumovirus in 3, human bocavirus in 1, adenovirus in 3 and parainfluenza type 1, 2 or 3 in 5). In 7.2% of the cases the aetiological agent was not identified. Of the children with current asthma, 11% had a history of RSV bronchiolitis, 11% had a history with rhinovirus and 17% of had non-RSV bronchiolitis in infancy (Table 4). The impact of RSV vs. non-RSV bronchiolitis on the asthma outcome did not reach statistical significance (p=0.22) (Table 4). All eight children with a history of non-RSV bronchiolitis presented with atopic asthma, whereas all three non-atopic asthmatics belonged to the RSV group.

5.3 Polymorphisms in the *TLR3, TLR4*, *TLR5*, *TLR7*, *TLR8*, *TLR9* and *TLR10* genes and post-bronchiolitis asthma at 5–7 years of age (Articles II, III)

Blood samples for exploratory studies regarding *TLR3* rs3775291, *TLR4* rs4986790, *TLR7* rs179008 and *TLR8* rs2407992 polymorphisms were available from 135, regarding *TLR9* rs187084 and *TLR10* rs4129009 from 134 and regarding *TLR5* rs5744174 from 139 of the 166 children who participated in the follow-up study at 5–7 years of age. The genotype distributions and minor allele frequencies are presented in Table 7. Wild genotype is the synonym for major-major genotype, whereas major-minor and minor-minor genotypes are called variant genotypes. In *TLR3*, 45.9% of the children had the wild (CC) genotype, and variant (TC or TT) genotype was found in 54.1% of the cases. Wild (AA) *TLR4* genotype was found in 83.7% and variant (AG) in 16.3% of the cases. In *TLR5*, 19.6% of the children had the wild (CC) and 80.4% had the variant (CT or TT) genotype. The *TLR9* genotype was wild (TT) in 32.1% and variant (TC or CC) in 67.9% of the participants. The minor allele frequencies did not differ significantly when compared to the Finnish

population data from the 1000 Genomes Project Consortium (Table 7). There were no significant associations between the *TLR3*, *TLR4*, *TLR5* or *TLR9* genotypes and asthma ever, current asthma, current atopic eczema or current allergic rhinitis (Article II, Article III).

TLR7 and *TLR8* genes are located in the X chromosome, and therefore the genotype distributions and the associations of genotypes with post-bronchiolitis outcomes were analysed separately for boys and girls. As boys possess only one X chromosome, they have only one allele of the *TLR7* and *TLR8* genes (major, wild-type, or minor, variant-type).

In *TLR7*, 60.6% of the girls had the wild genotype (AA) and 39.4% had the variant genotype (AT or TT), while 79.4% of the boys had the major (wild-type) allele A and 20.6% had the minor (variant-type) allele T of the *TLR7* gene. Asthma ever was significantly more common in girls with the variant genotype AT or TT of the *TLR7* gene (34.6% vs. 12.5%, p=0.03). When adjusted for age, gender, maternal asthma, non-RSV aetiology of bronchiolitis and current atopic eczema, the association remained significant (aOR 3.93, 95% CI 1.06–14.58). This kind of association was not seen in boys (Article II). Neither girls nor boys showed any significant associations between the *TLR7* genotypes and current asthma, current atopic eczema or allergic rhinitis (Article II).

The *TLR8* genotype was wild (GG) in 34.8% and variant (GC or CC) in 65.2% of the girls. In boys, the major (wild-type) allele G was present in 50.7% and the minor (variant-type) allele C in 49.3%. No significant associations were seen between the *TLR8* genotypes and asthma ever, current asthma, current atopic eczema or current allergic rhinitis in either girls or boys (Article II).

The wild (AA) *TLR10* genotype was present in 84.3% and the variant (AG or GG) genotype in 16.7% of the cases. Current asthma was significantly more common in 21 children with the variant genotype (AG or GG) (28.6% vs. 10.6% of those 113 with the wild genotype AA, p=0.03). The OR adjusted for age and gender was 3.74 (95% CI 1.19–11.78). The association remained significant in logistic regression adjusted for age, gender, early-life risk factors of maternal asthma and non-RSV aetiology of bronchiolitis, and current atopic eczema (current confounder) in the same model (adjusted OR 4.30, 95% CI 1.30–14.29). No statistically significant associations were found between *TLR10* gene polymorphisms and asthma ever, current atopic eczema or current allergic rhinitis (Article II).

Table 7. *TLR 3*, *4*, *5*, *7*, *8*, *9* and *10* genotypes and minor allele frequencies (MAFs) at 5–7 years of age.

Gene	SNP	Genotype distribution		MAF	FIN*
		wild	variant		
TLR3	rs3775291 C >T	CC 0.46	CT 0.41 TT 0.13	0.33	0.33
TLR4	rs4986790 A>G	AA 0.84	AG 0.16 GG 0.0	0.08	0.12
TLR5	rs5744174 C>T	CC 0.26	CT TT		0.50
TLR7	rs179008 A>T	AA 0.61 (girls) A 0.79 (boys)	AT 0.35 (girls) TT 0.04 (girls) T 0.21(boys)	0.22 (all)	0.31 (all)
TLR8	rs2407992 G>C	GG 0.35 (girls) G 0.51 (boys)	GC 0.48(girls) CC0.17(girls) C 0.49 (boys)	0.45 (all)	0.36 (all)
TLR9	rs187084 T>C	TT 0.32	TC 0.43 CC 0.25	0.46	0.45
TLR10	rs4129009 A>G	AA 0.84	AG 0.15 GG 0.01	0.08	0.08

*Data from the 1000 Genomes Project (180)

5.4 Polymorphisms in the *TLR1* and *TLR10* genes and post-bronchiolitis asthma at 11–13 years of age (Article IV)

In the present cohort, *TLR1* rs5743618 gene polymorphism (160) and *TLR10* rs4129009 gene polymorphism (Article II) were associated with post-bronchiolitis asthma at 5–7 years of age, when all 10 *TLR* gene polymorphisms were included in the exploratory analyses.

Blood samples for confirmatory analyses of the association between *TLR1* rs5743618 gene polymorphism or *TLR10* rs4129009 gene polymorphism and post-bronchiolitis asthma at 11–13 years of age were available for 125 of those 138 children who took part in the follow-up visit at that age. There were no significant differences in the minor allele frequencies between the subjects of the present cohort and Finnish controls from the 1000 Genomes Project Consortium (Article IV).

The wild genotype GG in *TLR1* rs5743618 was present in 73% and the variant genotype GT or TT in 27% of the children attending the 11–13 years follow-up. The need of continuous ICS medication in the last 12 months was significantly more common in children with the variant genotype GT or TT (OR 3.70, 95% CI 1.04–13.01) than in those with the wild genotype GG (Table 8). The associations between *TLR1* genotypes and current asthma at 11–13 years of age or persistent asthma from 5–7 to 11–13 years of age failed to reach statistical significance (Table 8).

The wild genotype AA in the *TLR10* rs4129009 was present in 83% and variant genotype AG or GG in 17% of the children attending the 11–13 years follow-up. The need for continuous ICS medication in the last 12 months was significantly more common in children with the variant genotype AG or GG (OR 5.05, 95% CI 1.38–18.53) than in those with the wild genotype AA (Table 8). In addition, the variant genotype increased the risk of persistent asthma from 5–7 to 11–13 years of age (OR 4.61, 95% CI 1.12–18.93). There was a parallel but statistically non-significant trend between the variant genotype and the risk of current asthma at 11–13 years of age (Table 8).

There were no significant associations between *TLR1* or *TLR10* genotypes and doctor-diagnosed allergic rhinitis or doctor-diagnosed atopic eczema (Article IV).

Multivariate logistic regression with adjustments for age, gender, non-RSV aetiology of bronchiolitis and atopic eczema at less than 12 months of age was used to evaluate the impact of *TLR1* and *TLR10* variant genotypes, first separately and then in combination, to the presence of current or persistent asthma or current ICS use at 11–13 years of age. In these adjusted analyses, the *TLR1* variant genotype marginally lost statistical significance as a risk factor for current ICS use (adjusted OR 4.04, 95% CI 0.99–16.54), but the *TLR10* variant genotype retained statistical significance as a risk factor for persistent asthma (adjusted OR 7.69, 95% CI 1.35–43.95) and current ICS use (adjusted OR 7.02, 95% CI 1.58–31.89).

Children with the variant genotype both in *TLR1* and *TLR10* genes, presented with persistent asthma and reported current ICS use significantly more often than children with the combination of wild genotypes in these genes; the adjusted OR was 7.1 (95% CI 1.58–31.9) for persistent asthma and 7.7 (95% CI 1.36–44.3) for ICS use, respectively.

Table 8. Genotypes of *TLR1 rs*5743618 and *TLR10 rs4129009* genes, classified as wild and variant as risk factors for ICS use, current asthma and persistent asthma at 11–13 years of age in children hospitalised for bronchiolitis under 6 months of age.

	ICS use in previous 12 months n=11			Current asthma n=15			Persistent asthma n=9		
	n	%	OR (95%CI)	n	%	OR (95%CI)	n	%	OR (95%CI)
TLR1 rs5743168									
GG (wild) n=91	5	5.5		9	9.9		4	4.4	
GT/TT (variant) n=34	6	17.6	3.69 (1.04-13.01)	6	17.6	1.95 (0.64-6.00)	5	14.7	3.75 (0.94-14.91)
TLR10 rs4129009									
AA (wild) n=103	6	5.8		10	9.7		5	4.9	
AG/GG (variant) n=21	5	23.8	5.05 (1.38-18.53)	5	23.8	2.91 (0.88-9.63)	4	19.0	4.61 (1.12-18.93)

6 DISCUSSION

6.1 Post-bronchiolitis outcome at the age of 11–13 years

In this hospital-based long-term follow-up study, the prevalence of doctor-diagnosed asthma after hospitalisation for bronchiolitis under 6 months of age was 13.0% for young teenagers. The prevalence of asthma in the general Finnish population in school-aged children is 7% (69). This means that bronchiolitis in infancy increased the risk of asthma nearly twofold. The prevalence of asthma in the age- and sex-matched control group of children with no history of bronchiolitis in infancy was 10.7%; thus, it did not differ from the bronchiolitis group. The prevalence of allergic rhinitis in former bronchiolitis patients was 43%, and the prevalence of atopic eczema was 25.4%. This occurrence of allergic rhinitis was more common than the reported 6–14% in the general population of school-aged children in Finland (69). The figure was 44.6% in the controls of the present study. The occurrence of atopic eczema was rather similar, about 25%, in both study groups and in the Finnish child population (69). It is worth noting, that the participation rate of the controls was only 16.1%, and a selection bias was evident. It is well known that symptomatic subjects are more motivated to take part in clinical studies than are those who are symptom free (181, 182).

The 13% prevalence of asthma in this cohort is markedly lower compared to other hospital-based post-bronchiolitis follow-up studies, in which the prevalence has varied between 30% and 40% at school age (11-16). Age at the time the infants were hospitalised for bronchiolitis is an evident reason to explain the differences in these figures. In the present cohort, all infants were under 6 months of age, whereas the upper age limit in all other post-bronchiolitis follow-up studies has been 12 or 24 months. Younger age on admission has predicted a more beneficial outcome of bronchiolitis. In a Finnish prospective bronchiolitis study, the occurrence of asthma was 31% at 12 years of age in those who were admitted to the hospital before 12 months of age and 55% in those who were admitted at 12–24 months of age (11). Accordingly, the beneficial effect associated with younger age was also seen in the current study; only 8.5% of those who were 3 months or younger at admission had asthma at the age of 11–13 years, compared to 19.6% of those aged 3–6 months,

although this difference did not quite reach statistical significance. It is intriguing that although the youngest infants are at the greatest risk for severe disease, they seem more likely to experience a more beneficial long-term outcome after bronchiolitis. Thus, children who are over 6 or 12 months of age at the time of hospitalisation for bronchiolitis probably have inherited predisposition to asthma, and virus-induced wheezing may be the first mark of this predisposition. In contrast, the youngest infants develop bronchiolitis due to their immature immune responses. If they have no other risk factors for asthma, their long-term respiratory outcome does not substantially differ from that of the general population.

As is well known, age is linked with the virus aetiology of bronchiolitis, RSV being the major virus before and RV after 12 months of age. In this cohort, with bronchiolitis at less than 6 months of age, 66% of infants had RSV and only 13% had RV as a causative agent of bronchiolitis. Only a few studies to date have assessed the impact of viral aetiology on long-term asthma outcome after bronchiolitis. In those long-term studies, as well as in studies with shorter follow-up times, RSV has been associated with more beneficial outcome than RV bronchiolitis or non-RSV bronchiolitis, which fits well with the low asthma prevalence in early teenage years in this study. In the previous Finnish hospital-based cohort study, the prevalence of asthma at 11–14 years of age was 58% after RV bronchiolitis and 20% after RSV bronchiolitis at less than 24 months of age (11). In a Norwegian study, only non-RSV bronchiolitis at less than 12 months of age was related to increased asthma risk at 11 years of age, whereas no significant difference in asthma prevalence was seen between the RSV bronchiolitis group and the unselected control group (16). Similar results were reported from a high-risk birth cohort study that followed children after outpatient wheezing before 3 years of age; only wheezing during RV infection was associated with asthma at 13 years of age, whereas RSV lost its significance by that age as a risk factor for asthma (102). In the present cohort, the risk of asthma at preschool age was significantly higher after non-RSV (7.7%) than after RSV bronchiolitis (24.4%) (62). In the present study, a similar trend was seen at 11–13 years of age, although it no longer reached statistical significance anymore. The lack of a significant association at this age may reflect the effect of other risk factors that may become more important as children grow older. There was no difference in the asthma outcome between RV and RSV bronchiolitis groups, perhaps because of lack of power due to a low number of RV bronchiolitis patients. The non-RSV group also included those who tested virus negative. Since bronchiolitis is always of viral origin and the PCR-based RSV test has an almost 100% specificity (183), it may be that the subset of the virus-negative cases were actually RV-C cases. The RV-C strain

is known to cause 30–60% of all RV infections (55, 58, 184), and there were no tests for RV-C available at the time the viral samples were taken.

Allergy was relatively common in former bronchiolitis patients. In the present cohort, 83% of current asthmatics were regarded as having atopic asthma. In most long-term studies, atopy has been established as a strong risk factor for post-bronchiolitis asthma (11, 12, 14, 22). Only one hospital-based study suggested a causal relationship between bronchiolitis and allergy, based on the observation that allergy was significantly more common in bronchiolitis group than in the control group (15). However, the control group in that study can be regarded as somewhat selected because the prevalence of asthma (3%) and allergic rhinitis (15%) were lower than in the general population. Some studies have suggested that atopy is linked with an increased post-bronchiolitis asthma risk only after RV bronchiolitis (92). In the present cohort, atopic manifestations were slightly more common in those asthmatics who had RV or non-RSV bronchiolitis in infancy (100%), compared to asthmatics who had RSV bronchiolitis (70%), but the difference was not statistically significant.

Based on the observations that atopic manifestations are more common in patients with RV bronchiolitis (25), and supported by the fact that RV is a common agent involved in asthma exacerbations because of impaired antiviral response characteristic for asthma (84, 104), RV bronchiolitis may actually be the first sign of asthma or predisposition for asthma. In contrast, RSV can induce neurogenic inflammation, leading to bronchial hyperreactivity that may persist for several years or, alternatively, can cause direct injury to the lungs, leading to airway remodelling and decreased lung function in later life (49). Without other predisposing factors, children with RSV bronchiolitis have an opportunity to grow out of their asthma symptoms by school age. Since RV is overrepresented among children who wheeze for the first time at over 12 months of age (3), it is possible, that a subset of patients with underlying asthma exists in studies with an upper age limit of 24 months for bronchiolitis, and that this may have led to the overestimation of the post-bronchiolitis asthma risk. Therefore, it has been suggested that the upper age limit for bronchiolitis should be standardised globally to 12 months or even to 6 months (33).

6.2 Early-life predictive factors

Wheezing symptoms are common in preschool years but often relieve with age. The clinical features of early wheezing periods are the same in infants at risk for later asthma as in those who will outgrow their symptoms. The diagnosis of asthma is difficult in young children because of insufficient cooperation to perform objective measurements of lung function. If infants who require more closer follow-up after bronchiolitis could be identified based on clinical factors already present in infancy, it would help to organise scheduled follow-up visits and perhaps help to identify symptoms of asthma earlier in these children.

In this cohort, as in three other hospital-based follow-up studies (11, 12, 22), atopic predisposition was a significant early-life predictor for post-bronchiolitis outcome. Maternal asthma and allergy and atopic eczema before 12 months of age were associated with post-bronchiolitis asthma at 11–13 years of age in the present univariate analyses. Atopic eczema and maternal allergy lost their significance in multivariate analyses, however, whereas maternal asthma was the only independent early-life risk factor for asthma at 11–13 years of age. In accordance, a large German birth cohort study reported that parental asthma, early atopic eczema and early sensitisation to indoor allergens predicted asthma at 13 years of age among children who wheezed before 3 years of age (6). It is interesting to note that maternal allergy and asthma had relevance for the outcome of bronchiolitis, whereas paternal allergy or asthma did not. Although parental atopy has been established as a strong predictor of later asthma after bronchiolitis or early wheezing, only a few studies have studied the impact of maternal and paternal contribution separately. A cross-sectional analysis of a high-risk birth cohort from the U.S. found that the impact of maternal asthma had a stronger association than paternal asthma with childhood asthma before 5 years of age but that the risks were the same after that (185). Interestingly, more than half (61%) of the current asthmatics in the present cohort also had asthma at the preschool-age visit, 44% of them had maternal asthma and 50% of them had early atopic eczema, whereas none of the current asthmatics had paternal asthma. This supports the suggestions represented in the cited birth cohort that inheritance of atopic diseases may preferentially occur through mothers and that maternally inherited atopy typically manifests in early life (185).

A longitudinal study that evaluated the association between maternal asthma and the occurrence of RSV and RV infections reported that RV infections were more common, as well as more severe, than RSV infections in children whose mothers had atopic asthma (24). The majority of patients in the present cohort had a history

of RSV bronchiolitis, and early atopic characteristics were common when compared to the general population. Since atopy also increases the risk of asthma in subjects with no history of bronchiolitis (186), it is possible that asthma after RSV infection actually develops due to other predisposing factors, such as atopy, whereas atopy is not a predisposing factor for RSV bronchiolitis. RV infection, on the contrary, may be a marker of impaired antiviral responses due to underlying asthmatic predisposition.

Clinical characteristics, family history of atopic diseases and early-life exposures were similar in the bronchiolitis group and the controls, which clearly were selected, but an outstanding difference between the cases and the controls was seen in the history of pre- and postnatal tobacco smoke exposure, which was much more common in the bronchiolitis group. Almost half of the children in the bronchiolitis group had tobacco smoke exposure pre- or postnatally. In particular, maternal smoking during pregnancy (13.8%) and before the children had reached 12 months of age (21.7%) was more frequent when compared to the controls (2.7% and 5.4%, respectively). This is in line with previous studies reporting that parental smoking increases the risk of hospitalisation for bronchiolitis (37, 38, 107). It has been reported that maternal smoking in pregnancy leads to impaired neonatal immune responses via attenuated TLR function (187), affects lung growth and reduces lung function (188). In the postnatal period, parental smoking — and especially maternal smoking—was reported to alter the epithelial function and local immunity in the lungs (188). Although early-life exposure to tobacco smoke was common in this cohort of former bronchiolitis patients, it was not associated with asthma risk at the present follow-up at 11–13 years of age or earlier at 5–7 years of age (62). This is in accordance with the results of many other long-term follow-up studies (11, 13, 15, 91). In contrast, a long-term Swedish hospital-based study reported that postnatal exposure to tobacco smoke was significantly associated with asthma 10 years after a first wheezing episode before 24 months of age (14) and later in early adulthood (189). In the same cohort, however, a similar association was yet not seen at the control visit at 6 years of age (21). In a Finnish long-term study, early tobacco smoke exposure was associated with lung function impairment at 5–8 years of age (190), although it was not associated with clinical asthma. Several studies have reported parental smoking in early life as a risk for asthma in general (108, 109). The lack of an association with post-bronchiolitis asthma in this study and many other hospital-based bronchiolitis studies may reflect the fact that smoking exposure is so common in this patient group.

6.3 Preschool-age predictive factors

Atopic manifestations become more prevalent with age. Therefore, reassessment of risk factors at preschool age helps to define more precisely the post-bronchiolitis outcome at later school age.

In this cohort, allergic rhinitis and SPT positivity at preschool age showed a strong association with asthma at 11–13 years of age. As many as 61% of the asthmatics presented with allergic rhinitis at the 5–7 years follow-up visit, and 65% of them were sensitised to one or more allergens in SPTs. The figures for non-asthmatic former bronchiolitis patients were 23% and 24%, respectively, the figures being rather similar in the general child population at school age. In an adjusted analysis with early-life risk factors, allergic rhinitis at preschool age increased the risk of post-bronchiolitis asthma fourfold and SPT positivity fivefold. Accordingly, allergic rhinitis and sensitisation to inhalant or food allergens have also been associated with later asthma in other hospital-based bronchiolitis studies (11, 12, 14, 22). Sixty-one percent of the current asthmatics had asthma at the preschool-age visit; in addition, all of them had current allergic rhinitis. This is in line with the retrospective Finnish study that reported a 61% prevalence of allergic rhinitis in school-aged children with asthma (191). Simultaneous allergic rhinitis may thus predict a more persistent type of asthma, and atopic asthma in childhood has been reported to continue into adulthood more often than non-atopic asthma (192, 193).

The prevalence of asthma in this cohort was the same at both the 5–7 years and 11–13 years follow-up visits. In accordance, several long-term bronchiolitis studies have described the incidence of asthma as declining until school age (14, 18, 20) and thereafter, staying relatively steady during the school years (11, 12). Interestingly, two follow-up studies that followed the patients until adulthood, reported the prevalence of asthma to increase again after the teenage years (79, 81). This means that some of the patients who became non-symptomatic during school years have relapsed.

At both visits, boys were slightly more often asthmatic than girls, although the difference was not statistically significant. In line with this finding, many studies have reported male gender to be associated with an increased risk for asthma after bronchiolitis (16, 76), as in the case of asthma in general (194). This difference was seen only until puberty, after which girls seemed to be at increased risk (77, 93).

6.4 Polymorphisms in the *TLR* genes

At present, 11 TLRs have been identified, and 10 of them seem to be involved in the innate immunity in humans (113). In this post-bronchiolitis cohort, data on the association of *TLR1* rs5743618, *TLR2* rs5743708 and *TLR6* rs5743810 polymorphisms with asthma or allergy at 5–7 years of age has been published previously (160), and the *TLR1* rs5743618 was associated with asthma between 1 and 6 years of age. The *TLR3* rs3775291, *TLR4* rs4986790, *TLR5* rs5744174, *TLR7* rs179008, *TLR8* rs2407992, *TLR9* rs187084 and *TLR10* rs4129009 polymorphisms were included in the exploratory analyses of the present thesis, and *TLR10* rs4129009 was associated with current asthma at 5–7 years of age. The *TLR1* rs5743618 and *TLR10* rs4129009 gene polymorphisms were included in the confirmatory analyses of the present thesis for their associations with asthma at 11–13 years of age. The theory of the exploratory clinical study designs versus the confirmatory clinical study designs was presented recently (195).

6.4.1 *Toll-like receptor 1* and *10* genes

In the present cohort, two *TLR2* subfamily polymorphisms showed significant associations with post-bronchiolitis outcome.

The *TLR10* rs4129009 variant genotype was significantly more common in children with current asthma at 5–7 years of age. Just under one-third (29%) of the children with the variant genotype had current asthma, compared to 11% of those with the wild genotype. The association was robust to adjustments with early-life risk factors and current atopic eczema. To confirm this finding, the association between the *TLR10* rs4129009 and post-bronchiolitis outcome was investigated again at 11–13 years of age. At the early teenage years, the variant genotype was significantly associated with the use of continuous ICS medication for asthma; 24% of children with the variant genotype had been on ICS medication during the last 12 months, compared to 6% of children with the wild genotype. The use of ICSs is an indicator of active asthma requiring continuous controller therapy. In addition, persistent asthma continuing from preschool age until the early teenage years was significantly more common in children with the variant genotype of the *TLR10* rs4129009 gene. These associations remained significant after adjustments with early-life risk factors.

The *TLR1* rs5743618 variant genotype resulted in a 3.7-fold risk for the need of ICS medication at 11–13 years of age; 17.6% of the children with variant genotype had ICS medication for asthma, compared to 5.5% of children with the wild

genotype. However, in multivariate analyses adjusted for early-life risk factors, TLR1 marginally lost its statistical significance. Previously in this cohort, the same polymorphism in *TLR1* was associated with asthma between 1 and 6 years of age (160). The marginal loss of significance may be due to small sample size, as 16.8% of the cases dropped out of the follow-ups after the preschool-age visit. The combination including both the *TLR1* and *TLR10* variant genotypes increased the risk of current ICS use at 11–13 years of age as well as persistent asthma more than sevenfold.

TLRs play a key role in innate immunity, and their normal activation may promote lymphocyte differentiation towards Th1-type cytokines expressing cells (196). In asthma and atopy, the balance between Th1 and Th2 responses is skewed to the Th2-orientation (26). *TLR1* rs5743618 and *TLR10* rs4129009 are both non-synonymous SNPs resulting in an amino acid change (120, 143). If the changed amino acid has relevance on TLR function, early infection may lead to the persistence of Th2-oriented immunity with an elevated risk of asthma and/or atopy. In line with this view is the observation from the present cohort that children with the variant genotype in the *TLR10* rs4129009 gene had an increased risk of both current asthma at preschool age and persistent asthma in the early teenage years. In addition, children with the variant genotype in the *TLR1* rs5743618 gene and/or in the *TLR10* rs4129009 gene were significantly more often on continuous maintenance medication for asthma as early teenagers than were children with the wild genotype.

The prevalence of asthma in the general population of Finnish school-aged children is 5–7% (69), which is the same as in children with *TLR1* and *TLR10* wild genotypes in this cohort. The MAFs of either of these SNPs did not differ from the general Finnish population, according to the FIN data of the 1000 Genomes Project Consortium. Thus, these results suggest that *TLR1* and *TLR10* polymorphisms do not alter susceptibility to bronchiolitis but may instead predispose to post-bronchiolitis asthma. Interestingly, the *ad hoc* analyses confirmed that none of the children with a history of RSV bronchiolitis and bearing the wild genotype in both *TLR1* and *TLR10* genes had persistent asthma, nor had they used ICSs in the last 12 months. In contrast, children with a history of non-RSV bronchiolitis and variant genotypes in both genes, were significantly more likely to have current as well as persistent asthma and continuous ICS use, than children who did not belong to this group.

There are no prior studies regarding the role of *TLR1* rs5743618 in asthma, but two other polymorphisms (rs5743595 and rs4833095) in *TLR1* have been reported to decrease the risk of atopic asthma (143).

In accordance with the findings from the present cohort, the variant *TLR10* genotype rs4129009 was associated with asthma, both in a case-control study and in an independent cohort study (124). In previous studies, TLR10 has been described mainly as an inhibitory receptor; it induces the production of IL-1Ra, which attenuates the release of proinflammatory cytokines (116). In addition, TLR10 has modulatory effects on immune responses, since it competes for ligands with pro-inflammatory TLR1 and for the formation of heterodimers with TLR1 and TLR6 (116, 119). *TLR10* rs4129009 is a polymorphism that has been reported to result in an amino-acid change in the cytoplasmic domain of TLR10 protein (124). This may potentially alter the downstream signalling of the receptor and hamper the IL-1Ra production, further leading to increased inflammatory responses. This theoretical basis remains yet unconfirmed but may partly explain the observed association between that polymorphism and asthma. However, opposite findings on the impact of this polymorphism have also been reported; a German study found the same polymorphism in *TLR10* to be protective for atopic asthma (143). These conflicting findings may result from different gene-environment interactions; the same polymorphism may lead to different consequences in different environments and also in interaction with other host-related risk factors (28, 140).

6.4.2 *Toll-like receptor 3, 4, 5, 7, 8* and *9* genes

TLR7 and *TLR8* genes are located on the X chromosome (29), and therefore boys have only one allele—either wild-type (major) or variant-type (minor)—in both of these genes. It was therefore mandatory to analyse the effects of these polymorphisms separately for boys and girls. We found that *TLR7* rs179008 variant genotype was significantly more common in girls, who had asthma ever (i.e., current or previous asthma), at the follow-up visit at 5–7 years of age. Similar association was not seen in boys. It has been reported that normal activation of TLR7 in airway nerves leads to bronchodilation via nitric oxide production (173). In line with this, an abnormal response of TLR7 to viral ssRNA has been described in adolescents with asthma (174). These findings suggest that normal function of TLR7 may be protective from asthma, whereas polymorphism in the *TLR7* gene may induce airway hyperreactivity and asthma via altered TLR7 function. In a Danish study with two family samples, the *TLR7* rs179008 was associated with asthma, allergic rhinitis,

atopic eczema and IgE sensitisation, but interestingly, and in contrast to our findings, the associations were more significant in boys (133). In the same Danish study, even stronger associations were found regarding the *TLR8* rs2407992 (133), which is in disagreement with our findings; the investigated polymorphism in *TLR8* was not associated with any of the asthma variables, nor with atopy at preschool age. Likewise, the TLR7 rs179008 was not associated with either allergic rhinitis or atopic eczema in the present cohort.

TLR3 rs3775291, *TLR4* rs4986790 and *TLR9* rs187084 polymorphisms were not associated with post-bronchiolitis asthma or other atopic diseases at 5–7 years of age in the present cohort. Accordingly, a meta-analysis found no association between the *TLR4* rs4986790 and asthma (167), nor did another meta-analysis find a link between the *TLR9* rs187084 and asthma (138). In previous publications from this cohort, *TLR3* wild genotype and *TLR9* variant genotype were associated with an increased risk of post-bronchiolitis wheezing (157, 163). The lack of an association with asthma at 5-7 years of age may reflect the beneficial prognosis of recurrent wheezing after RSV bronchiolitis since the majority of these children will outgrow from their symptoms before school age.

TLR5 rs5744174 polymorphism was not associated with post-bronchiolitis asthma at any age. This is in accordance with the findings of a German study, that did not find associations between this or two other polymorphism in the *TLR5* gene and childhood asthma (143).

6.5 Methodological aspects

The strengths of this long-term post-bronchiolitis follow-up study are the prospective design and the carefully collected data during hospitalisation and at subsequent follow-ups. The comprehensive viral testing at admission enabled the assessment of the association between virus aetiology and the outcome of bronchiolitis. The relatively long follow-up time with three clinical reevaluations after hospitalisation enabled a reliable assessment of early-life and preschool-age risk factors for post-bronchiolitis outcome. The study population's Finnish origin was ethnically homogenic, which is beneficial for the genetic studies. Further, the study design is novel, since this is the first study that reports the long-term outcome after hospitalisation for bronchiolitis before 6 months of age. The upper age limit of 6 months further increases the homogeneity of the study population, which in other

prospective post-bronchiolitis studies with higher age limits may have led to inclusion of other wheezing entities that were distinct from bronchiolitis.

This study also has some limitations. The drop-out rate among the bronchiolitis group was 16.8%, and one-third of the participants did not perform the lung function test. Thus, it is possible that some cases of current asthma were missed. Another shortcoming of this study is the evident selection bias in the control group, as they had asthma and allergy rates comparable to those of the former bronchiolitis patients. This complicated the evaluation of post-bronchiolitis outcome in a controlled setting. Another limitation is that the sample size was relatively small for the evaluation of the impact of genetic polymorphisms; in addition, blood samples for genetic studies were not available from all study participants. This may have led to type-2 statistical error. Analysing the association between multiple *TLR* polymorphisms and the post-bronchiolitis outcome without multiplicity adjustments using, for example, the Bonferroni correction may have led to type-1 statistical error. However, the analyses performed at 5–7 years of age were regarded as exploratory, and the significant associations were aimed to be confirmed at the later follow-up at 11–13 years of age. In fact, the associations between *TLR1* and *TLR10* polymorphisms and post-bronchiolitis asthma were seen at both the 5–7 years and 11–13 years controls. As in many other studies on the association of gene polymorphisms and diseases, a clear limitation is that the functionality of the *TLR* genes was not studied, and the data on the functionality from other studies is not always convincing.

6.6 Conclusions

Hospitalisation for bronchiolitis in infants under 6 months of age has an impact on later respiratory morbidity continuing at least until the early teenage years. However, the prognosis was more beneficial than in previous long-term follow-up studies that have used upper age limits of 12 months or 24 months for bronchiolitis. The prevalence of asthma in young teenagers was twofold compared to the asthma prevalence in the general population of Finnish school-children. In addition, allergic rhinitis was overrepresented in the bronchiolitis group, but the prevalence of atopic eczema was about the same as in the general population. The former bronchiolitis and control groups did not differ in terms of asthma, allergic rhinitis or atopic eczema, which can be explained by an evident selection bias in the controls.

Early exposure to tobacco smoke was a significant risk factor for severe bronchiolitis requiring treatment in a hospital but did not associate with later asthma risk within the bronchiolitis group. Maternal asthma was a strong predictor of post-bronchiolitis asthma in the early teenage years. At preschool age, the presence of allergic rhinitis and sensitisation to allergens assessed by SPTs were risk factors for later asthma. RSV was the major causative agent in bronchiolitis, but viral aetiology was not significantly associated with the outcome after bronchiolitis.

Polymorphisms in the *TLR3*, *TLR4*, *TLR5*, *TLR7, TLR8* and *TLR9* genes were not associated with post-bronchiolitis asthma or allergy in exploratory analyses at preschool age, and thus, they were not included in the confirmatory analyses at 11–13 years of age. *TLR10* rs4129009 variant genotype increased the risk of current asthma at preschool age. This association was confirmed by the observation that the same *TLR10* polymorphism was associated with an increased risk of needing continuous ICS medication at 11–13 years of age and with an increased risk of persistent asthma continuing from preschool age until the early teenage years. The *TLR1* rs574618 variant genotype was associated with an increased risk of young teenagers needing ICS medication, thus confirming the earlier exploratory findings in this cohort. The combination of the variant genotypes in *TLR1* and *TLR10* genes predicted current ICS use as well as persistent asthma in the early teenage years.

This study confirms that children with bronchiolitis who have atopic predisposition, particularly maternal asthma, require closer monitoring in later life for an increased risk of subsequent asthma. Reevaluation of risk factors at preschool age helps to define the later prognosis more accurately and children with allergic rhinitis or allergic sensitisation are at risk for a permanent asthma phenotype. Variations in the *TLR* genes, especially in the *TLR1* and *TLR10*, seem to contribute to the prognosis, but larger studies are needed to confirm these preliminary results.

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REFERENCES

1. Cangiano G, Nenna R, Frassanito A, Evangelisti M, Nicolai A, Scagnolari C, et al. Bronchiolitis: Analysis of 10 consecutive epidemic seasons. Pediatr Pulmonol. 2016; 51(12):1330-5.
2. Ralston SL, Lieberthal AS, Meissner HC, Alverson BK, Baley JE, Gadomski AM, et al. Clinical practice guideline: the diagnosis, management, and prevention of bronchiolitis. Pediatrics. 2014;134(5):1474.
3. Jartti T, Lehtinen P, Vuorinen T, Ruuskanen O. Bronchiolitis: age and previous wheezing episodes are linked to viral etiology and atopic characteristics. Pediatr Infect Dis J. 2009;28(4):311-7.
4. Dumas O, Mansbach JM, Jartti T, Hasegawa K, Sullivan AF, Piedra PA, et al. A clustering approach to identify severe bronchiolitis profiles in children. Thorax. 2016;71(8):712-8.
5. Taussig LM, Wright AL, Holberg CJ, Halonen M, Morgan WJ, Martinez FD. Tucson children's respiratory study: 1980 to present. J Allergy Clin Immunol. 2003;111(4):661-75.
6. Matricardi PM, Illi S, Gruber C, Keil T, Nickel R, Wahn U, et al. Wheezing in childhood: incidence, longitudinal patterns and factors predicting persistence. Eur Respir J. 2008;32(3):585-92.
7. Carroll KN, Gebretsadik T, Griffin MR, Wu P, Dupont WD, Mitchel EF, et al. Increasing burden and risk factors for bronchiolitis-related medical visits in infants enrolled in a state health care insurance plan. Pediatrics. 2008;122(1):58-64.
8. Murray J, Bottle A, Sharland M, Modi N, Aylin P, Majeed A, et al. Risk factors for hospital admission with RSV bronchiolitis in England: a population-based birth cohort study. PLoS One. 2014;9(2):e89186.
9. Smyth RL, Openshaw PJ. Bronchiolitis. The Lancet. 2006;368(9532):312-22.
10. Mecklin M, Heikkila P, Korppi M. Low age, low birthweight and congenital heart disease are risk factors for intensive care in infants with bronchiolitis. Acta Paediatr. 2017;106(12):2004-10.
11. Hyvarinen MK, Kotaniemi-Syrjanen A, Reijonen TM, Korhonen K, Korppi MO. Teenage asthma after severe early childhood wheezing: an 11-year prospective follow-up. Pediatr Pulmonol. 2005;40(4):316-23.
12. Hyvarinen M, Piippo-Savolainen E, Korhonen K, Korppi M. Teenage asthma after severe infantile bronchiolitis or pneumonia. Acta Paediatr. 2005;94(10):1378-83.
13. Noble V, Murray M, Webb MS, Alexander J, Swarbrick AS, Milner AD. Respiratory status and allergy nine to 10 years after acute bronchiolitis. Arch Dis Child. 1997;76(4):315-9.
14. Wennergren G, Amark M, Amark K, Oskarsdottir S, Sten G, Redfors S. Wheezing bronchitis reinvestigated at the age of 10 years. Acta Paediatr. 1997;86(4):351-5.

15. Sigurs N, Gustafsson PM, Bjarnason R, Lundberg F, Schmidt S, Sigurbergsson F, et al. Severe respiratory syncytial virus bronchiolitis in infancy and asthma and allergy at age 13. Am J Respir Crit Care Med. 2005;171(2):137-41.
16. Mikalsen IB, Halvorsen T, Oymar K. The outcome after severe bronchiolitis is related to gender and virus. Pediatr Allergy Immunol. 2012;23:391-8.
17. Kuikka L, Reijonen T, Remes K, Korppi M. Bronchial asthma after early childhood wheezing: a follow-up until 4.5-6 years of age. Acta Paediatr. 1994;83(7):744-8.
18. Korppi M, Kuikka L, Reijonen T, Remes K, Juntunen-Backman K, Launiala K. Bronchial asthma and hyperreactivity after early childhood bronchiolitis or pneumonia. An 8-year follow-up study. Arch Pediatr Adolesc Med. 1994;148(10):1079-84.
19. Sigurs N, Bjarnason R, Sigurbergsson F, Kjellman B. Respiratory syncytial virus bronchiolitis in infancy is an important risk factor for asthma and allergy at age 7. Am J Respir Crit Care Med. 2000;161(5):1501-7.
20. Kotaniemi-Syrjanen A, Reijonen TM, Korhonen K, Korppi M. Wheezing requiring hospitalization in early childhood: predictive factors for asthma in a six-year follow-up. Pediatr Allergy Immunol. 2002;13(6):418-25.
21. Wennergren G, Hansson S, Engstrom I, Jodal U, Amark M, Brolin I, et al. Characteristics and prognosis of hospital-treated obstructive bronchitis in children aged less than two years. Acta Paediatr. 1992;81(1):40-5.
22. Mikalsen IB, Halvorsen T, Eide GE, Øymar K. Severe bronchiolitis in infancy: Can asthma in adolescence be predicted? Pediatr Pulmonol. 2013;48:538-44.
23. Midulla F, Pierangeli A, Cangiano G, Bonci E, Salvadei S, Scagnolari C, et al. Rhinovirus bronchiolitis and recurrent wheezing: 1-year follow-up. Eur Respir J. 2012;39(2):396-402.
24. Carroll KN, Gebretsadik T, Minton P, Woodward K, Liu Z, Miller EK, et al. Influence of maternal asthma on the cause and severity of infant acute respiratory tract infections. J Allergy Clin Immunol. 2012;129(5):1236-42.
25. Turunen R, Koistinen A, Vuorinen T, Arku B, Soderlund-Venermo M, Ruuskanen O, et al. The first wheezing episode: respiratory virus etiology, atopic characteristics, and illness severity. Pediatr Allergy Immunol. 2014;25(8):796-803.
26. Message SD, Johnston SL. The immunology of virus infection in asthma. Eur Respir J. 2001;18(6):1013.
27. Gern JE, Busse WW. The role of viral infections in the natural history of asthma. J Allergy Clin Immunol. 2000;106(2):201-12.
28. Singh AM, Moore PE, Gern JE, Lemanske RFJ, Hartert TV. Bronchiolitis to asthma: a review and call for studies of gene-virus interactions in asthma causation. Am J Respir Crit Care Med. 2007;175:108-19.
29. Akira S, Takeda K, Kaisho T. Toll-like receptors: critical proteins linking innate and acquired immunity. Nat Immunol. 2001;2:675-80.
30. Muir A, Soong G, Sokol S, Reddy B, Gomez MI, Van Heeckeren A, et al. Toll-like receptors in normal and cystic fibrosis airway epithelial cells. Am J Respir Cell Mol Biol. 2004;30(6):777-83.
31. Hasan U, Chaffois C, Gaillard C, Saulnier V, Merck E, Tancredi S, et al. Human TLR10 is a functional receptor, expressed by B cells and plasmacytoid dendritic cells, which activates gene transcription through MyD88. J Immunol. 2005;174:2942-50.
32. Medvedev AE. Toll-like receptor polymorphisms, inflammatory and infectious diseases, allergies, and cancer. J Interferon Cytokine Res. 2013;33:467-84.

33. Korppi M, Koponen P, Nuolivirta K. Upper age limit for bronchiolitis: 12 months or 6 months? Eur Respir J. 2012;39(3):9.
34. Meissner HC. Viral Bronchiolitis in Children. N Engl J Med. 2016;374(1):62-72.
35. Pruikkonen H, Uhari M, Dunder T, Pokka T, Renko M. Infants under 6 months with bronchiolitis are most likely to need major medical interventions in the 5 days after onset. Acta Paediatr. 2014;103(10):1089-93.
36. Ricart S, Marcos MA, Sarda M, Anton A, Munoz-Almagro C, Pumarola T, et al. Clinical risk factors are more relevant than respiratory viruses in predicting bronchiolitis severity. Pediatr Pulmonol. 2013;48(5):456-63.
37. Bradley JP, Bacharier LB, Bonfiglio J, Schechtman KB, Strunk R, Storch G, et al. Severity of respiratory syncytial virus bronchiolitis is affected by cigarette smoke exposure and atopy. Pediatrics. 2005;115(1):7.
38. Lanari M, Vandini S, Adorni F, Prinelli F, Di Santo S, Silvestri M, et al. Prenatal tobacco smoke exposure increases hospitalizations for bronchiolitis in infants. Respir Res. 2015;16:5.
39. Janssen R, Bont L, Siezen CLE, Hodemaekers HM, Ermers MJ, Doornbos G, et al. Genetic Susceptibility to Respiratory Syncytial Virus Bronchiolitis Is Predominantly Associated with Innate Immune Genes. J Infect Dis. 2007;196(6):826-34.
40. Lambert L, Sagfors AM, Openshaw PJ, Culley FJ. Immunity to RSV in Early-Life. Front Immunol. 2014;5:466.
41. Scheltema NM, Gentile A, Lucion F, Nokes DJ, Munywoki PK, Madhi SA, et al. Global respiratory syncytial virus-associated mortality in young children (RSV GOLD): a retrospective case series. Lancet Glob Health. 20175(10):e991.
42. Nair H, Nokes DJ, Gessner BD, Dherani M, Madhi SA, Singleton RJ, et al. Global burden of acute lower respiratory infections due to respiratory syncytial virus in young children: a systematic review and meta-analysis. Lancet. 2010;375(9725):1545-55.
43. Papoff P, Moretti C, Cangiano G, Bonci E, Roggini M, Pierangeli A, et al. Incidence and predisposing factors for severe disease in previously healthy term infants experiencing their first episode of bronchiolitis. Acta Paediatr. 2011;100(7):17.
44. Jartti T, Korppi M. Rhinovirus-induced bronchiolitis and asthma development. Pediatr Allergy Immunol. 2011;22:350-5.
45. Valkonen H, Waris M, Ruohola A, Ruuskanen O, Heikkinen T. Recurrent wheezing after respiratory syncytial virus or non-respiratory syncytial virus bronchiolitis in infancy: a 3-year follow-up. Allergy. 2009;64(9):1359-65.
46. Midulla F, Scagnolari C, Bonci E, Pierangeli A, Antonelli G, De Angelis D, et al. Respiratory syncytial virus, human bocavirus and rhinovirus bronchiolitis in infants. Arch Dis Child. 2010;95(1):35-41.
47. Miller EK, Gebretsadik T, Carroll KN, Dupont WD, Mohamed YA, Morin LL, et al. Viral etiologies of infant bronchiolitis, croup and upper respiratory illness during 4 consecutive years. Pediatr Infect Dis J. 2013;32(9):950-5.
48. Svensson C, Berg K, Sigurs N, Trollfors B. Incidence, risk factors and hospital burden in children under five years of age hospitalised with respiratory syncytial virus infections. Acta Paediatr. 2015;104(9):922-6.
49. Rossi GA, Colin AA. Respiratory syncytial virus-Host interaction in the pathogenesis of bronchiolitis and its impact on respiratory morbidity in later life. Pediatr Allergy Immunol. 2017;28(4):320-31.

50. Laham FR, Mansbach JM, Piedra PA, Hasegawa K, Sullivan AF, Espinola JA, et al. Clinical Profiles of Respiratory Syncytial Virus Subtypes A AND B Among Children Hospitalized with Bronchiolitis. Pediatr Infect Dis J. 2017;36(8):808-10.
51. Pickles RJ, DeVincenzo JP. Respiratory syncytial virus (RSV) and its propensity for causing bronchiolitis. J Pathol. 2015;235(2):266-76.
52. Vandini S, Calamelli E, Faldella G, Lanari M. Immune and inflammatory response in bronchiolitis due to respiratory Syncytial Virus and Rhinovirus infections in infants. Paediatr Respir Rev. 2017;24:60-4.
53. Mansbach JM, Piedra PA, Teach SJ, Sullivan AF, Forgey T, Clark S, et al. Prospective multicenter study of viral etiology and hospital length of stay in children with severe bronchiolitis. Arch Pediatr Adolesc Med. 2012;166(8):700-6.
54. Paul SP, Mukherjee A, McAllister T, Harvey MJ, Clayton BA, Turner PC. Respiratory-syncytial-virus- and rhinovirus-related bronchiolitis in children aged <2 years in an English district general hospital. J Hosp Infect. 2017;96(4):360-5.
55. Miller EK, Williams JV, Gebretsadik T, Carroll KN, Dupont WD, Mohamed YA, et al. Host and viral factors associated with severity of human rhinovirus-associated infant respiratory tract illness. J Allergy Clin Immunol. 2011;127(4):883-91.
56. Lee WM, Kiesner C, Pappas T, Lee I, Grindle K, Jartti T, et al. A diverse group of previously unrecognized human rhinoviruses are common causes of respiratory illnesses in infants. PLoS One. 2007;2(10):e966.
57. Turunen R, Vuorinen T, Bochkov Y, Gern J, Jartti T. Clinical and Virus Surveillance After the First Wheezing Episode: Special Reference to Rhinovirus A and C Species. Pediatr Infect Dis J. 2017;36(6):539-44.
58. Turunen R, Jartti T, Bochkov YA, Gern JE, Vuorinen T. Rhinovirus species and clinical characteristics in the first wheezing episode in children. J Med Virol. 2016;88(12):2059-68.
59. Mansbach JM, Clark S, Teach SJ, Gern JE, Piedra PA, Sullivan AF, et al. Children Hospitalized with Rhinovirus Bronchiolitis Have Asthma-Like Characteristics. J Pediatr. 2016;172:204.e1.
60. Korppi M, Kotaniemi-Syrjanen A, Waris M, Vainionpaa R, Reijonen TM. Rhinovirus-associated wheezing in infancy: comparison with respiratory syncytial virus bronchiolitis. Pediatr Infect Dis J. 2004;23(11):995-9.
61. Jackson DJ, Gern JE, Lemanske RF. The contributions of allergic sensitization and respiratory pathogens to asthma inception. J Allergy Clin Immunol. 2016;137(3):659-65; quiz 666.
62. Koponen P, Helminen M, Paassilta M, Luukkaala T, Korppi M. Preschool Asthma after Bronchiolitis in Infancy. Eur Respir J. 2012;39:76-80.
63. Kotaniemi-Syrjanen A, Vainionpaa R, Reijonen TM, Waris M, Korhonen K, Korppi M. Rhinovirus-induced wheezing in infancy--the first sign of childhood asthma? J Allergy Clin Immunol. 2003;111(1):66-71.
64. Ferreira A, Williams Z, Donninger H, van Schalkwyk EM, Bardin PG. Rhinovirus is associated with severe asthma exacerbations and raised nasal interleukin-12. Respiration. 2002;69(2):136-42.
65. van der Zalm, M M, Uiterwaal CS, Wilbrink B, Koopman M, Verheij TJ, van der Ent, C K. The influence of neonatal lung function on rhinovirus-associated wheeze. Am J Respir Crit Care Med. 2011;183(2):262-7.

66. Gern JE, Vrtis R, Grindle KA, Swenson C, Busse WW. Relationship of upper and lower airway cytokines to outcome of experimental rhinovirus infection. Am J Respir Crit Care Med. 2000;162(6):2226-31.
67. Heymann PW, Carper HT, Murphy DD, Platts-Mills TA, Patrie J, McLaughlin AP, et al. Viral infections in relation to age, atopy, and season of admission among children hospitalized for wheezing. J Allergy Clin Immunol. 2004;114(2):239-47.
68. Jartti T, Lehtinen P, Vuorinen T, Osterback R, van den Hoogen B, Osterhaus AD, et al. Respiratory picornaviruses and respiratory syncytial virus as causative agents of acute expiratory wheezing in children. Emerg Infect Dis. 2004;10(6):1095-101.
69. Ruokolainen L, Paalanen L, Karkman A, Laatikainen T, von Hertzen L, Vlasoff T, et al. Significant disparities in allergy prevalence and microbiota between the young people in Finnish and Russian Karelia. Clin Exp Allergy. 2017;47(5):665-74.
70. Hugg T, Ruotsalainen R, Jaakkola M, Pushkarev V, Jaakkola JK. Comparison of allergic diseases, symptoms and respiratory infections between Finnish and Russian school children. Eur J Epidemiol. 2008;23:123-33.
71. Remes ST, Korppi M, Remes K, Pekkanen J. Prevalence of asthma at school age: a clinical population-based study in eastern Finland. Acta Paediatr. 1996;85(1):59-63.
72. Sigurs N, Bjarnason R, Sigurbergsson F, Kjellman B, Bjorksten B. Asthma and immunoglobulin E antibodies after respiratory syncytial virus bronchiolitis: a prospective cohort study with matched controls. Pediatrics. 1995;95(4):500-5.
73. Murray M, Webb MS, O'Callaghan C, Swarbrick AS, Milner AD. Respiratory status and allergy after bronchiolitis. Arch Dis Child. 1992;67(4):482-7.
74. Reijonen TM, Kotaniemi-Syrjanen A, Korhonen K, Korppi M. Predictors of asthma three years after hospital admission for wheezing in infancy. Pediatrics. 2000;106(6):1406-12.
75. Henderson J, Hilliard TN, Sherriff A, Stalker D, Al Shammari N, Thomas HM. Hospitalization for RSV bronchiolitis before 12 months of age and subsequent asthma, atopy and wheeze: a longitudinal birth cohort study. Pediatr Allergy Immunol. 2005;16(5):386-92.
76. Balekian DS, Linnemann RW, Hasegawa K, Thadhani R, Camargo CA. Cohort Study of Severe Bronchiolitis during Infancy and Risk of Asthma by Age 5 Years. J Allergy Clin Immunol Pract. 2017;5(1):92-6.
77. Goksor E, Amark M, Alm B, Ekerljung L, Lundback B, Wennergren G. High risk of adult asthma following severe wheezing in early life. Pediatr Pulmonol. 2015;50(8):789-97.
78. Backman K, Piippo-Savolainen E, Ollikainen H, Koskela H, Korppi M. Adults face increased asthma risk after infant RSV bronchiolitis and reduced respiratory health-related quality of life after RSV pneumonia. Acta Paediatr. 2014;103(8):850-5.
79. Goksor E, Amark M, Alm B, Gustafsson PM, Wennergren G. Asthma symptoms in early childhood--what happens then? Acta Paediatr. 2006;95(4):471-8.
80. Sigurs N, Aljassim F, Kjellman B, Robinson PD, Sigurbergsson F, Bjarnason R, et al. Asthma and allergy patterns over 18 years after severe RSV bronchiolitis in the first year of life. Thorax. 2010;65(12):1045-52.
81. Piippo-Savolainen E, Remes S, Kannisto S, Korhonen K, Korppi M. Asthma and lung function 20 years after wheezing in infancy: results from a prospective follow-up study. Arch Pediatr Adolesc Med. 2004;158(11):1070-6.

82. Juntti H, Kokkonen J, Dunder T, Renko M, Niinimaki A, Uhari M. Association of an early respiratory syncytial virus infection and atopic allergy. Allergy. 2003;58(9):878-84.
83. Thomsen SF, van der Sluis S, Stensballe LG, Posthuma D, Skytthe A, Kyvik KO, et al. Exploring the association between severe respiratory syncytial virus infection and asthma: a registry-based twin study. Am J Respir Crit Care Med. 2009;179:1091-7.
84. Jartti T, Gern JE. Role of viral infections in the development and exacerbation of asthma in children. J Allergy Clin Immunol. 2017;140(4):895-906.
85. Wennergren G, Kristjansson S. Relationship between respiratory syncytial virus bronchiolitis and future obstructive airway diseases. Eur Respir J. 2001;18:1044-58.
86. Jartti T, Lehtinen P, Vuorinen T, Ruuskanen O. Bronchiolitis: age and previous wheezing episodes are linked to viral etiology and atopic characteristics. Pediatr Infect Dis J. 2009;28:311-7.
87. Simoes EA, Carbonell-Estrany X, Rieger CH, Mitchell I, Fredrick L, Groothuis JR, et al. The effect of respiratory syncytial virus on subsequent recurrent wheezing in atopic and nonatopic children. J Allergy Clin Immunol. 2010;126(2):256-62.
88. Garofalo R, Dorris A, Ahlstedt S, Welliver RC. Peripheral blood eosinophil counts and eosinophil cationic protein content of respiratory secretions in bronchiolitis: relationship to severity of disease. Pediatr Allergy Immunol. 1994;5(2):111-7.
89. Martinez FD, Stern DA, Wright AL, Taussig LM, Halonen M. Differential immune responses to acute lower respiratory illness in early life and subsequent development of persistent wheezing and asthma. J Allergy Clin Immunol. 1998;102(6 Pt 1):915-20.
90. Hyvarinen MK, Kotaniemi-Syrjanen A, Reijonen TM, Piippo-Savolainen E, Korppi M. Eosinophil activity in infants hospitalized for wheezing and risk of persistent childhood asthma. Pediatr Allergy Immunol. 2010;21(1 Pt 1):96-103.
91. Jackson DJ, Gangnon RE, Evans MD, Roberg KA, Anderson EL, Pappas TE, et al. Wheezing rhinovirus illnesses in early life predict asthma development in high-risk children. Am J Respir Crit Care Med. 2008;178(7):667-72.
92. Lukkarinen M, Koistinen A, Turunen R, Lehtinen P, Vuorinen T, Jartti T. Rhinovirus-induced first wheezing episode predicts atopic but not nonatopic asthma at school age. J Allergy Clin Immunol. 2017;140(4):988-95.
93. Sears MR, Greene JM, Willan AR, Wiecek EM, Taylor DR, Flannery EM, et al. A longitudinal, population-based, cohort study of childhood asthma followed to adulthood. N Engl J Med. 2003;349(15):1414-22.
94. Sidoroff V, Hyvärinen M, Piippo-Savolainen E, Korppi M. Lung Function and Overweight in School Aged Children After Early Childhood Wheezing. Pediatric Pulmonology. 2011;46:435-41.
95. Törmänen S, Lauhkonen E, Saari A, Koponen P, Korppi M, Nuolivirta K. Excess weight in preschool children with a history of severe bronchiolitis is associated with asthma. Pediatr Pulmonol. 2015;50(5):424-30.
96. Sidoroff V, Hyvärinen MK, Piippo-Savolainen E, Korppi M. Overweight does not increase asthma risk but may decrease allergy risk at school age after infantile bronchiolitis. Acta Paediatrica. 2011;101:43-7.
97. Turner SW, Young S, Landau LI, Le Souef PN. Reduced lung function both before bronchiolitis and at 11 years. Arch Dis Child. 2002;87(5):417-20.
98. Martinez FD, Wright AL, Taussig LM, Holberg CJ, Halonen M, Morgan WJ. Asthma and wheezing in the first six years of life. The Group Health Medical Associates. N Engl J Med. 1995;332(3):133-8.

99. Chawes BL, Poorisrisak P, Johnston SL, Bisgaard H. Neonatal bronchial hyperresponsiveness precedes acute severe viral bronchiolitis in infants. J Allergy Clin Immunol. 2012;130(2):61.e3.
100. Baraldo S, Contoli M, Bazzan E, Turato G, Padovani A, Marku B, et al. Deficient antiviral immune responses in childhood: Distinct roles of atopy and asthma. J Allergy Clin Immunol. 2012;130(6):1307-14.
101. Stein RT, Sherrill D, Morgan WJ, Holberg CJ, Halonen M, Taussig LM, et al. Respiratory syncytial virus in early life and risk of wheeze and allergy by age 13 years. Lancet. 1999;354(9178):541-5.
102. Rubner FJ, Jackson DJ, Evans MD, Gangnon RE, Tisler CJ, Pappas TE, et al. Early life rhinovirus wheezing, allergic sensitization, and asthma risk at adolescence. J Allergy Clin Immunol. 2017;139(2):501-7.
103. Rossi GA, Colin AA. Infantile respiratory syncytial virus and human rhinovirus infections: respective role in inception and persistence of wheezing. Eur Respir J. 2015;45(3):774-89.
104. Kennedy JL, Turner RB, Braciale T, Heymann PW, Borish L. Pathogenesis of rhinovirus infection. Curr Opin Virol. 2012;2(3):287-93.
105. Devulapalli CS, Carlsen KC, Haland G, Munthe-Kaas MC, Pettersen M, Mowinckel P, et al. Severity of obstructive airways disease by age 2 years predicts asthma at 10 years of age. Thorax. 2008;63(1):8-13.
106. Jartti T, Paul-Anttila M, Lehtinen P, Parikka V, Vuorinen T, Simell O, et al. Systemic T-helper and T-regulatory cell type cytokine responses in rhinovirus vs. respiratory syncytial virus induced early wheezing: an observational study. Respir Res. 2009;10:85.
107. Simoes EA, Carbonell-Estrany X. Impact of severe disease caused by respiratory syncytial virus in children living in developed countries. Pediatr Infect Dis J. 2003;22:20.
108. Grabenhenrich LB, Gough H, Reich A, Eckers N, Zepp F, Nitsche O, et al. Early-life determinants of asthma from birth to age 20 years: A German birth cohort study. J Allergy Clin Immunol. 2014;133:979-88.
109. Burke H, Leonardi-Bee J, Hashim A, Pine-Abata H, Chen Y, Cook DG, et al. Prenatal and passive smoke exposure and incidence of asthma and wheeze: systematic review and meta-analysis. Pediatrics. 2012;129(4):735-44.
110. Castro-Rodriguez JA, Holberg CJ, Wright AL, Martinez FD. A clinical index to define risk of asthma in young children with recurrent wheezing. Am J Respir Crit Care Med. 2000;162(4 Pt 1):1403-6.
111. Lemanske RF. The childhood origins of asthma (COAST) study. Pediatr Allergy Immunol. 2002;13 Suppl 15:38-43.
112. Medzhitov R, Preston-Hurlburt P, Janeway CAJ. A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. Nature. 1997;388(6640):394-7.
113. Skevaki C, Pararas M, Kostelidou K, Tsakris A:,J.G. Single nucleotide polymorphisms of Toll-like receptors and susceptibility to infectious diseases. Clin Exp Immunol. 2015;180:165-77.
114. Lin YT, Verma A, Hodgkinson CP. Toll-like receptors and human disease: lessons from single nucleotide polymorphisms. Curr Genomics. 2012;13:633-45.
115. Kawai T, Akira S. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. Nat Immunol. 2010;11(5):373-84.

116. Oosting M, Cheng S, Bolscher JM, Vestering-Stenger R, Plantinga TS, Verschueren IC, et al. Human TLR10 is an anti-inflammatory pattern-recognition receptor. Proc Natl Acad Sci U S A. 2014;111(42):E4484.
117. Triantafilou K, Vakakis E, Richer EA, Evans GL, Villiers JP, Triantafilou M. Human rhinovirus recognition in non-immune cells is mediated by Toll-like receptors and MDA-5, which trigger a synergetic pro-inflammatory immune response. Virulence. 2011;2(1):22-9.
118. Murawski MR, Bowen GN, Cerny AM, Anderson LJ, Haynes LM, Tripp RA, et al. Respiratory syncytial virus activates innate immunity through Toll-like receptor 2. J Virol. 2009;83(3):1492-500.
119. Guan Y, Ranoa DRE, Jiang S, Mutha SK, Li X, Baudry J, et al. Human TLRs 10 and 1 Share Common Mechanisms of Innate Immune Sensing but Not Signaling. J Immunol. 2010;184(9):5094-103.
120. Hawn TR, Misch EA, Dunstan SJ, Thwaites GE, Lan NTN, Quy HT, et al. A common human TLR1 polymorphism regulates the innate immune response to lipopeptides. Eur J Immunol. 2007;37(8):2280-9.
121. Fischer M, Spies-Weisshart B, Schrenk K, Gruhn B, Wittig S, Glaser A, et al. Polymorphisms of Dectin-1 and TLR2 Predispose to Invasive Fungal Disease in Patients with Acute Myeloid Leukemia. PLoS ONE. 2016;11(3):e0150632.
122. Hussein YM, Awad HA, Shalaby SM, Ali AA, Alzahrani SS. Toll-like receptor 2 and Toll-like receptor 4 polymorphisms and susceptibility to asthma and allergic rhinitis: A case–control analysis. Cell Immunol. 2012;274(1–2):34-8.
123. Shey MS, Randhawa AK, Bowmaker M, Smith E, Scriba TJ, de Kock M, et al. Single nucleotide polymorphisms in toll-like receptor 6 are associated with altered lipopeptide- and mycobacteria-induced interleukin-6 secretion. Genes Immun. 2010;11(7):561-72.
124. Lazarus R, Raby BA, Lange C, Silverman EK, Kwiatkowski DJ, Vercelli D, et al. TOLL-like receptor 10 genetic variation is associated with asthma in two independent samples. Am J Respir Crit Care Med. 2004;170(6):594-600.
125. Slater L, Bartlett NW, Haas JJ, Zhu J, Message SD, Walton RP, et al. Co-ordinated role of TLR3, RIG-I and MDA5 in the innate response to rhinovirus in bronchial epithelium. PLoS Pathog. 2010;6(11):e1001178.
126. Hewson CA, Jardine A, Edwards MR, Laza-Stanca V, Johnston SL. Toll-Like Receptor 3 Is Induced by and Mediates Antiviral Activity against Rhinovirus Infection of Human Bronchial Epithelial Cells. J Virol. 2005;79(19):12273-9.
127. Zhang B, Chassaing B, Shi Z, Uchiyama R, Zhang Z, Denning TL, et al. Prevention and cure of rotavirus infection via TLR5/NLRC4-mediated production of IL-22 and IL-18. Science. 2014;346:861-5.
128. Huleatt JW, Nakaar V, Desai P, Huang Y, Hewitt D, Jacobs A, et al. Potent immunogenicity and efficacy of a universal influenza vaccine candidate comprising a recombinant fusion protein linking influenza M2e to the TLR5 ligand flagellin. Vaccine. 2008;26(2):201-14.
129. Song L, Liu G, Umlauf S, Liu X, Li H, Tian H, et al. A rationally designed form of the TLR5 agonist, flagellin, supports superior immunogenicity of Influenza B globular head vaccines. Vaccine. 2014;32:4317-23.
130. Shastry BS. SNPs in disease gene mapping, medicinal drug development and evolution. J Hum Genet. 2007;52(11):871-80.

131. Wlasiuk G, Khan S, Switzer W, Nachman MW. A history of recurrent positive selection at the toll-like receptor 5 in primates. Mol Biol Evol. 2009;26:937-49.
132. Tesse R, Pandey RC, Kabesch M. Genetic variations in toll-like receptor pathway genes influence asthma and atopy. Allergy. 2010;66:307-16.
133. Møller-Larsen S, Nyegaard M, Haagerup A, Vestbo J, Kruse TA, Børglum AD. Association analysis identifies TLR7 and TLR8 as novel risk genes in asthma and related disorders. Thorax. 2008;63(12):1064-9.
134. Lee W, Yao T, Yeh K, Chen L, Ou L, Huang J. Stronger Toll-like receptor 1/2, 4, and 7/8 but less 9 responses in peripheral blood mononuclear cells in non-infectious exacerbated asthmatic children. Immunobiology. 2013;218(2):192-200.
135. Strachan DP. Hay fever, hygiene, and household size. BMJ. 1989;299(6710):1259-60.
136. Ege MJ, Mayer M, Normand A, Genuneit J, Cookson, William O C M, Braun-Fahrländer C, et al. Exposure to Environmental Microorganisms and Childhood Asthma. N Engl J Med. 2011;364(8):701-9.
137. Von Ehrenstein OS, Von Mutius E, Illi S, Baumann L, Bohm O, von Kries R. Reduced risk of hay fever and asthma among children of farmers. Clin Exp Allergy. 2000;30(2):187-93.
138. Tizaoui K, Kaabachi W, Hamzaoui K, Hamzaoui A. Association of Single Nucleotide Polymorphisms in Toll-like Receptor Genes With Asthma Risk: A Systematic Review and Meta-analysis. Allergy Asthma Immunol Res. 2015;7:130-40.
139. Patel M, Xu D, Kewin P, Choo-Kang B, McSharry C, Thomson NC, et al. TLR2 agonist ameliorates established allergic airway inflammation by promoting Th1 response and not via regulatory T cells. J Immunol. 2005;174(12):7558-63.
140. Daley D, Park JE, He J, Yan J, Akhabir L, Stefanowicz D, et al. Associations and interactions of genetic polymorphisms in innate immunity genes with early viral infections and susceptibility to asthma and asthma-related phenotypes. J Allergy Clin Immunol. 2012;130:1284-93.
141. Bjornvold M, Munthe-Kaas MC, Egeland T, Joner G, Dahl-Jorgensen K, Njolstad PR, et al. A TLR2 polymorphism is associated with type 1 diabetes and allergic asthma. Genes Immun. 2009;10(2):181-7.
142. Bottema RW, Kerkhof M, Reijmerink NE, Thijs C, Smit HA, van Schayck CP, et al. Gene-gene interaction in regulatory T-cell function in atopy and asthma development in childhood. J Allergy Clin Immunol. 2010;126(2):10.
143. Kormann MSD, Depner M, Hartl D, Klopp N, Illig T, Adamski J, et al. Toll-like receptor heterodimer variants protect from childhood asthma. J Allergy Clin Immunol. 2008;122(1):92.e8.
144. Qian F, Zhang Q, Zhou L, Jin G, Bai J, Yin K. Polymorphisms in the Toll-like Receptor 2 Subfamily and Risk of Asthma: A Case-control Analysis in a Chinese Population. J Invest Allergol Clin Immunol. 2010;20(4):340-6.
145. Eder W, Klimecki W, Yu L, von Mutius E, Riedler J, Braun-Fahrländer C, et al. Toll-like receptor 2 as a major gene for asthma in children of European farmers. J Allergy Clin Immunol. 2004;113(3):482-8.
146. Kerkhof M, Postma DS, Brunekreef B, Reijmerink NE, Wijga AH, de Jongste JC, et al. Toll-like receptor 2 and 4 genes influence susceptibility to adverse effects of traffic-related air pollution on childhood asthma. Thorax. 2010;65(8):690-7.
147. Kormann MS, Ferstl R, Depner M, Klopp N, Spiller S, Illig T, et al. Rare TLR2 mutations reduce TLR2 receptor function and can increase atopy risk. Allergy. 2009;64(4):636-42.

148. Redecke V, Hacker H, Datta SK, Fermin A, Pitha PM, Broide DH, et al. Cutting edge: activation of Toll-like receptor 2 induces a Th2 immune response and promotes experimental asthma. J Immunol. 2004;172(5):2739-43.
149. Daley D, Lemire M, Akhabir L, Chan-Yeung M, He JQ, McDonald T, et al. Analyses of associations with asthma in four asthma population samples from Canada and Australia. Hum Genet. 2009;125(4):445-59.
150. Yang CA, Chiang BL. Toll-like receptor 1 N248S polymorphism affects T helper 1 cytokine production and is associated with serum immunoglobulin E levels in Taiwanese allergic patients. J Microbiol Immunol Infect. 2017;50(1):112-7.
151. Hoffjan S, Stemmler S, Parwez Q, Petrasch-Parwez E, Schultze-Werninghaus G, Bufe A, et al. Evaluation of the toll-like receptor 6 Ser249Pro polymorphism in patients with asthma, atopic dermatitis and chronic obstructive pulmonary disease. BMC Med Genet. 2005;6:34.
152. Lau MY, Dharmage SC, Burgess JA, Win AK, Lowe AJ, Lodge C, et al. The interaction between farming/rural environment and TLR2, TLR4, TLR6 and CD14 genetic polymorphisms in relation to early- and late-onset asthma. Sci Rep. 2017;7:43681.
153. Tantisira K, Klimecki WT, Lazarus R, Palmer LJ, Raby BA, Kwiatkowski DJ, et al. Toll-like receptor 6 gene (TLR6): single-nucleotide polymorphism frequencies and preliminary association with the diagnosis of asthma. Genes Immun. 2004;5(5):343-6.
154. Miedema KG, Tissing WJ, Te Poele EM, Kamps WA, Alizadeh BZ, Kerkhof M, et al. Polymorphisms in the TLR6 gene associated with the inverse association between childhood acute lymphoblastic leukemia and atopic disease. Leukemia. 2012;26(6):1203-10.
155. Puthothu B, Heinzmann A. Is toll-like receptor 6 or toll-like receptor 10 involved in asthma genetics--or both? Allergy. 2006;61(5):649-50.
156. Zeyer F, Mothes B, Will C, Carevic M, Rottenberger J, Nurnberg B, et al. mRNA-Mediated Gene Supplementation of Toll-Like Receptors as Treatment Strategy for Asthma In Vivo. PLoS ONE. 2016;11(4):e0154001.
157. Nuolivirta K, Tormanen S, Terasjarvi J, Vuononvirta J, Koponen P, Korppi M, et al. Post-bronchiolitis wheezing is associated with toll-like receptor 9 rs187084 gene polymorphism. Sci Rep. 2016;6:31165.
158. Nuolivirta K, Vuononvirta J, Peltola V, Koponen P, Helminen M, He Q, et al. Toll-like receptor 2 subfamily genotypes are not associated with severity of bronchiolitis or postbronchiolitis wheezing in infants. Acta Paediatr. 2013;102(12):1160-4.
159. Nuolivirta K, Hurme M, Halkosalo A, Koponen P, Korppi M, Vesikari T, et al. Gene polymorphism of IFNG +874 T/A and TLR4 +896 A/G and recurrent infections and wheezing in toddlers with history of bronchiolitis. Pediatr Infect Dis J. 2009;28:1121-3.
160. Koponen P, Vuononvirta J, Nuolivirta K, Helminen M, He Q, Korppi M. The Association of Genetic Variants in Toll-like Receptor 2 Subfamily With Allergy and Asthma After Hospitalization for Bronchiolitis in Infancy. Pediatr Infect Dis J. 2014;33(5):463-6.
161. Lauhkonen E, Koponen P, Vuononvirta J, Terasjarvi J, Nuolivirta K, Toikka JO, et al. Gene Polymorphism of Toll-Like Receptors and Lung Function at Five to Seven Years of Age after Infant Bronchiolitis. PLoS ONE. 2016;11:e0146526.

162. Reuter S, Dehzad N, Martin H, Böhm L, Becker M, Buhl R, et al. TLR3 but Not TLR7/8 Ligand Induces Allergic Sensitization to Inhaled Allergen. The Journal of Immunology. 2012;188(10):5123-31.
163. Nuolivirta K, He Q, Vuononvirta J, Koponen P, Helminen M, Korppi M. Toll-like Receptor 3 L412F Polymorphisms in Infants With Bronchiolitis and Postbronchiolitis Wheezing. Pediatr Infect Dis J. 2012;31(9):920-3.
164. van der Graaf C, Kullberg BJ, Joosten L, Verver-Jansen T, Jacobs L, Van der Meer, J W, et al. Functional consequences of the Asp299Gly Toll-like receptor-4 polymorphism. Cytokine. 2005;30(5):264-8.
165. Tal G, Mandelberg A, Dalal I, Cesar K, Somekh E, Tal A, et al. Association between common Toll-like receptor 4 mutations and severe respiratory syncytial virus disease. J Infect Dis. 2004;189(11):2057-63.
166. Helminen M, Nuolivirta K, Virta M, Halkosalo A, Korppi M, Vesikari T, et al. IL-10 gene polymorphism at -1082 A/G is associated with severe rhinovirus bronchiolitis in infants. Pediatr Pulmonol. 2008;43(4):391-5.
167. Chen S. Association between the TLR4 +896A>G (Asp299Gly) Polymorphism and Asthma: A Systematic Review and Meta-Analysis. J Asthma. 2012;49(10):999-1003.
168. Siezen CLE, Bont L, Hodemaekers HM, Ermers MJ, Doornbos G, van't Slot R, et al. Genetic susceptibility to respiratory syncytial virus bronchiolitis in preterm children is associated with airway remodeling genes and innate immune genes. Pediatr Infect Dis J. 2009;28:333-4.
169. Klimosch SN, Forsti A, Eckert J, Knezevic J, Bevier M, von Schonfels W, et al. Functional TLR5 genetic variants affect human colorectal cancer survival. Cancer Res. 2013;73:7232-42.
170. Toivonen L, Vuononvirta J, Mertsola J, Waris M, He Q, Peltola V. Polymorphisms of Mannose-Binding Lectin and Toll-Like Receptors 2, 3, 4, 7, and 8 and the Risk of Respiratory Infections and Acute Otitis Media in Children. Pediatr Infect Dis J. 2017;36(5):e114-e122.
171. Nilsson D, Andiappan A, Hallden C, De Yun W, Sall T, Tim C, et al. Toll-like receptor gene polymorphisms are associated with allergic rhinitis: a case control study. BMC Med Genet. 2012;13(1):66.
172. Fili L, Ferri S, Guarna F, Sampognaro S, Manuelli C, Liotta F, et al. Redirection of allergen-specific TH2 responses by a modified adenine through Toll-like receptor 7 interaction and IL-12/IFN release. J Allergy Clin Immunol. 2006;118(2):511-7.
173. Drake MG, Scott GD, Proskocil BJ, Fryer AD, Jacoby DB, Kaufman EH. Toll-like Receptor 7 Rapidly Relaxes Human Airways. Am J Respir Crit Care Med. 2013;188(6):664-72.
174. Roponen M, Yerkovich ST, Hollams E, Sly PD, Holt PG, Upham JW. Toll-like receptor 7 function is reduced in adolescents with asthma. Eur Respir J. 2010;35(1):64-71.
175. Shikhagaie MM, Andersson CK, Mori M, Kortekaas Krohn I, Bergqvist A, Dahl R, et al. Mapping of TLR5 and TLR7 in central and distal human airways and identification of reduced TLR expression in severe asthma. Clin Exp Allergy. 2013;44:184-96.
176. Lauhkonen E, Koponen P, Nuolivirta K, Paassilta M, Toikka J, Korppi M. Lung function by impulse oscillometry at age 5-7 years after bronchiolitis at age 0-6 months. Pediatr Pulmonol. 2015;50(4):389-95.
177. Ahmad-Nejad P. Pyrosequencing of toll-like receptor polymorphisms of functional relevance. Methods Mol Biol. 2009;496:73-87.

178. Gröndahl-Yli-Hannuksela K, Vuononvirta J, Barkoff A, Viander M, Van Der Meeren O, Mertsola J, et al. Gene Polymorphism in Toll-like Receptor 4: Effect on Antibody Production and Persistence After Acellular Pertussis Vaccination During Adolescence. J Infect Dis. 2012;205(8):1214-9.
179. Terasjarvi J, Hakanen A, Korppi M, Nuolivirta K, Grondahl-Yli-Hannuksela K, Mertsola J, et al. Rapid detection of functional gene polymorphisms of TLRs and IL-17 using high resolution melting analysis. Sci Rep. 2017;7:41522.
180. The 1000 Genomes Project Consortium. Abecasis, G R, Altshuler D, Auton A, Brooks LD, Durbin RM, Gibbs RA, et al. A map of human genome variation from population scale sequencing. Nature. 2010;467(7319):1061-73.
181. Bornehag CG, Moniruzzaman S, Larsson M, Lindström CB, Hasselgren M, Bodin A, et al. The SELMA Study: A Birth Cohort Study in Sweden Following More Than 2000 Mother-Child Pairs. Paediatr Perinat Epidemiol. 2012;26:456-67.
182. Joseph CLM, Saltzgaber J, Havstad SL, Johnson CC, Johnson D, Peterson EL, et al. Comparison of early-, late-, and non-participants in a school-based asthma management program for urban high school students. Trials. 2011;12:141.
183. Bruning AHL, de Kruijf WB, van Weert, H C P M, Willems WLM, de Jong MD, Pajkrt D, et al. Diagnostic performance and clinical feasibility of a point-of-care test for respiratory viral infections in primary health care. Fam Pract. 2017;34(5):558-63.
184. Peltola V, Jartti T, Putto-Laurila A, Mertsola J, Vainionpaa R, Waris M, et al. Rhinovirus infections in children: a retrospective and prospective hospital-based study. J Med Virol. 2009;81(10):1831-8.
185. Litonjua AA, Carey VJ, Burge HA, Weiss ST, Gold DR. Parental history and the risk for childhood asthma. Does mother confer more risk than father? Am J Respir Crit Care Med. 1998;158(1):176-81.
186. Nissen SP, Kjaer HF, Host A, Nielsen J, Halken S. The natural course of sensitization and allergic diseases from childhood to adulthood. Pediatr Allergy Immunol. 2013;24(6):549-55.
187. Noakes PS, Hale J, Thomas R, Lane C, Devadason SG, Prescott SL. Maternal smoking is associated with impaired neonatal toll-like-receptor-mediated immune responses. Eur Respir J. 2006;28(4):721-9.
188. Prescott SL. Effects of early cigarette smoke exposure on early immune development and respiratory disease. Paediatr Respir Rev. 2008;9(1):3-10.
189. Goksör E, Åmark M, Alm B, Gustafsson PM, Wennergren G. The impact of pre- and post-natal smoke exposure on future asthma and bronchial hyper-responsiveness. Acta Paediatr. 2007;96:1030-5.
190. Kotaniemi-Syrjanen A, Reijonen TM, Korhonen K, Waris M, Vainionpaa R, Korppi M. Wheezing due to rhinovirus infection in infancy: Bronchial hyperresponsiveness at school age. Pediatr Int. 2008;50(4):506-10.
191. Ruokonen M, Kaila M, Haataja R, Korppi M, Paassilta M. Allergic rhinitis in school-aged children with asthma - still under-diagnosed and under-treated? A retrospective study in a children's hospital. Pediatr Allergy Immunol. 2010;21:e154.
192. Stein RT, Martinez FD. Asthma phenotypes in childhood: lessons from an epidemiological approach. Paediatr Respir Rev. 2004;5(2):155-61.
193. Kusel MM, de Klerk NH, Kebadze T, Vohma V, Holt PG, Johnston SL, et al. Early-life respiratory viral infections, atopic sensitization, and risk of subsequent development of persistent asthma. J Allergy Clin Immunol. 2007;119(5):1105-10.

194. Pescatore AM, Dogaru CM, Duembgen L, Silverman M, Gaillard EA, Spycher BD, et al. A simple asthma prediction tool for preschool children with wheeze or cough. J Allergy Clin Immunol. 2014;133(1):13.
195. Korppi M, Nuolivirta K. Exploratory and confirmatory studies have different targets and both are needed in clinical research. Acta Paediatr. 2018;107(5):734-5.
196. Reijmerink NE, Kerkhof M, Bottema RWB, Gerritsen J, Stelma FF, Thijs C, et al. Toll-like receptors and microbial exposure: gene–gene and gene–environment interaction in the development of atopy. Eur Respir J. 2011;38(4):833-40.

APPENDIX 1.

POST-BRONCHIOLITIS OUTCOME AT SCHOOL AGE

Tampere University and University Hospital, Paediatric Department 2014

QUESTIONNAIRE

Name: _____Research number:_____

Personal ID number: _____-_____

1. OBSTRUCTIVE RESPIRATORY SYMPTOMS

Please circle the appropriate answer.	**NO**	**YES**
Has your child experienced any respiratory wheezing symptoms during the last 12 months?	0	1
Has your child experienced any respiratory wheezing symptoms before (but after 5–7-years of age, after the last follow-up visit)?	0	1

*If you answered all questions **"No"**, please proceed to item number 2. If you answered **"Yes"** to either or both questions, please answer the following questions.*

Please answer "No" or "Yes". If you don't know, please draw a line in the box.	**During the last 12 months**	**Before (but after 5–7 years of age)**
The wheezing symptoms have occurred coincidentally.		
The wheezing symptoms have occurred repeatedly.		
The wheezing symptoms have occurred during flu-like illnesses.		
The wheezing symptoms have occurred during pollen season and/or during contact with furry animals.		
The wheezing symptoms have occurred during physical activities.		
The wheezing symptoms have occurred on some other occasion. **If "yes", please, describe the situation:**		

2. COUGH SYMPTOMS

Please circle the appropriate answer.	**No**	**Yes**
Has your child suffered from prolonged (> 4 weeks) coughing symptoms, apart from respiratory infection, during the last 12 months?	0	1
Has your child suffered from repeated night cough apart from respiratory infection, during the last 12 months?	0	1
Has your child suffered from prolonged (> 4 weeks) coughing symptoms apart from respiratory infection, before (but after 5–7 years of age)?	0	1

*If you answered all questions **"No"**, please proceed to item number 3. If you answered **"Yes"** to any question, please answer the following questions.*

Please answer "No" or "Yes". If you don't know, please draw a line in the box.	**During the last 12 months**	**Before (but after 5–7 years of age)**
The cough has continued at least 4 weeks.		
The coughing symptoms occur during night-time.		
The coughing symptoms often occur during exercise, for example, when running or during physical education classes.		
The coughing symptoms occur repeatedly during pollen season.		

The coughing symptoms occur during contact with furry animals.		
The coughing symptoms occur in cold weather.		
The coughing symptoms are linked with mucus excretion from the lungs.		

3. ASTHMA DIAGNOSIS

Please circle the appropriate answer.	**No**	**Yes**
Has your child been diagnosed with asthma by a doctor during the last 12 months? **If "Yes", please provide the name of the health care centre/hospital/private health care organisation:**	0	1
Has your child ever been hospitalised due to asthma or obstructive bronchitis or other wheezing symptoms? **If "Yes", please report the age when hospitalisation occurred:** 6, 7, 8, 9, 10, 11, 12, 13 years of age **If "Yes", please report the name of the hospital:**	0	1

Has your child ever been diagnosed with asthma by a doctor? **If "Yes", please report the age at which your child had asthma requiring medication:** 6, 7, 8, 9, 10, 11, 12, 13 years of age **If "Yes", please provide the name and location of the health care centre/hospital/private health care organisation:**	0	1

4. ASTHMA MEDICATION

Please circle the appropriate answer.	**No**	**Yes**
Has your child had continuous asthma control medication (e.g., Pulmicort, Flixotide, Beclomet , Seretide, Symbicort, Singulair) **during the last 12 months?**	0	1
Has your child ever had continuous asthma control medication? **If "Yes", please report the age when your child had continuous asthma control medication:** 6, 7, 8, 9, 10, 11, 12, 13 years of age	0	1

Please circle the appropriate answer.	**No**	**Yes**
Has your child received inhaled symptom relief medication (e.g., Bricanyl, Serevent, Ventoline, Airomir) **during the last 12 months?** **If "Yes", please report how often by circling the right option:** **daily / weekly / monthly / less than monthly**	0	1
Has your child ever received inhaled symptom relief medication? **If "Yes", please report the age when your child received inhaled symptom relief medication:** 6, 7, 8, 9, 10, 11, 12, 13 years of age	0	1

5. SYMPTOMS OF RHINITIS

Please circle the appropriate answer.	**No**	**Yes**
Has your child suffered from prolonged symptoms of runny nose, sneezing or stuffy nose outside of an infection during the last 12 months?	0	1

Has your child suffered from prolonged symptoms of runny nose, sneezing or stuffy nose outside of an infection before (but after 5–7 years of age)?	0	1

*If you answered **"No"** to both questions, please proceed to item number 6. If you answered **"Yes"** to any question, please answer the following questions.*

Please circle the appropriate answer.	**No**	**Yes**
Have the symptoms of runny nose, sneezing or stuffy nose been linked with a certain time of the year? **If "Yes", please circle the months when:** January, February, March, April, May, June, July, August, September, October, November, December	0	1
Have the symptoms of runny nose, sneezing or stuffy nose been linked with animal contact?	0	1
Has your child been diagnosed with allergic rhinitis by a doctor? **If "Yes", please provide the name and location of the health care centre/hospital/private health care organisation:**	0	1

6. OCULAR SYMPTOMS

Please circle the appropriate answer.	**No**	**Yes**
Has your child suffered from ocular itching, tearing or redness, outside of an infection, during the last 12 months?	0	1
Has your child suffered from ocular itching, tearing or redness, outside of an infection, before (but after 5–7 years of age)?	0	1

*If you answered **"No"** to both questions, please proceed to item number 7. If you answered **"Yes"** to any question, please answer the following questions.*

Please circle the appropriate answer.	**No**	**Yes**
Have the symptoms of ocular irritation been linked with a certain time of the year? If "Yes", please circle the months 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 diagnosed with allergic conjunctivitis by a doctor? If "Yes", please provide the name and location of the health care centre/hospital/private health care organization:	0	1

7. SKIN ECZEMA

Please circle the appropriate answer.	**No**	**Yes**
Has your child suffered from itching eczema during the last 12 months?	0	1
Has your child suffered from itching eczema before (but after 5-7 years of age)?	0	1

*If you answered **"No"** to both questions, please proceed to item number 8. If you answered **"Yes"** to either or both questions, please answer the following question.*

Please circle the appropriate answer.	**No**	**Yes**
Has your child been diagnosed with allergic eczema (atopic eczema) by a doctor? If "Yes", please report where eczema developed, circle the right option: Face, trunk, bends of the arms, bends of the knees, ankles, wrists, other part: **If "Yes", please report the name and location of the health care centre/hospital/private health care organisation:**	0	1

8. FOOD ALLERGY

Please circle the appropriate answer.	**No**	**Yes**
Has your child suffered from eczema or symptoms of rhinitis or wheezing caused by food allergy during the last 12 months?	0	1
Has your child suffered from eczema or symptoms of rhinitis or wheezing caused by food allergy before (but after 5–7 years of age)?	0	1

*If you answered both questions **"No"**, please proceed to item number 9. If you answered **"Yes"**, please answer the following question.*

Please circle the appropriate answer.	**No**	**Yes**
Has your child been diagnosed with food allergy by a doctor? **If "Yes", please describe what allergies have been found and circle the right location:** Milk, egg, grain, other: **If "Yes", please provide the name and location of the health care centre/hospital/private health care organisation:**	0	1

9. ASTHMA IN FAMILY

Please circle the appropriate answer.	**No**	**Yes**
Has mother ever been diagnosed with asthma by a doctor?	0	1
Has father ever been diagnosed with asthma by a doctor?	0	1
Have any of the siblings ever been diagnosed with asthma by a doctor?	0	1
Have any of the siblings ever been hopitalised due to wheezing symptoms?	0	1

10. ALLERGY IN FAMILY

Please circle the appropriate answer .	**No**	**Yes**
Has mother ever been diagnosed with allergic rhinitis or allergic conjunctivitis by a doctor?	0	1
Has father ever been diagnosed with allergic rhinitis or allergic conjunctivitis by a doctor?	0	1
Have any of the siblings ever been diagnosed with allergic rhinitis or allergic conjunctivitis by a doctor?	0	1

Have mother, father or any of the siblings ever been diagnosed with allergic eczema (atopic eczema) by a doctor? **If "Yes", please report which family member(s):**	0	1

11. PETS

Please circle the appropriate answer.	**No**	**Yes**
Have there been any pets in the family during the last 12 months?	0	1
Were there any pets in the family before (but after the child reached 5–7 years of age)?	0	1

*If you answered **"No"** to both questions, please proceed to item number 12. If you answered **"Yes"** to either or both questions, please circle the right options:*

1	Cat	4	Horse
2	Dog	5	Other:
3	Cow		_____

12. EXPOSURE TO TOBACCO SMOKE

Please circle the appropriate answer.	**No**	**Yes**
Did father smoke when the child was under 12 months of age? **If "Yes", which is correct:** indoors only outdoors	0	1
Did father smoke when the child was over 12 months of age? **If "Yes", which is correct:** indoors only outdoors	0	1
Did mother smoke when the 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 the child was over 12 months of age? **If "Yes", which is correct:** indoors only outdoors	0	1

13. LIVING ENVIRONMENT

Please circle the appropriate answer.

Which of the following options best describes the child's living environment during the last 12 months?

1 countryside

2 city

How many children are living in your home at the moment?

0 only one child

1 two children

2 three or more children

Moisture damage

Please circle the appropriate answer.	**No**	**Yes**
Has there <u>ever</u> been <u>established</u> moisture damage (*e.g* by a health inspector) in the child's home?	0	1
Has there <u>ever</u> been a <u>suspicion</u> of moisture damage in the child's home?	0	1
Where and when?		

Has there <u>ever</u> been <u>established</u> moisture damage (*e.g* by a health inspector) in the child's school?	0	1
Has there <u>ever</u> been <u>suspicion</u> of a moisture damage in the child's school?	0	1
Where and when?		

14. HEIGHT AND WEIGHT

Date : ___/____ 2014

Weight	
Height	

THANK YOU!

ORIGINAL PUBLICATIONS

I Törmänen S, Lauhkonen E, Riikonen R, Koponen P, Huhtala H, Helminen M, Korppi M, Nuolivirta K. Risk factors for asthma after infant bronchiolitis. Allergy 2018; 73(4): 916-922.

II Törmänen S, Korppi M, Teräsjärvi J, Vuononvirta J, Koponen P, Helminen M, He Q, Nuolivirta K. Polymorphism in the gene encoding toll-like receptor 10 may be associated with asthma after bronchiolitis. Scientific Reports 2017; 7(1): 2956.

III Törmänen S, Teräsjärvi J, Lauhkonen E, Helminen M, Koponen P, Korppi M, Nuolivirta K, He Q. TLR5 rs5744174 gene polymorphism is associated with the virus etiology of infant bronchiolitis but not with post-bronchiolitis asthma. Health Science Reports, in press.

IV Törmänen S, Korppi M, Lauhkonen E, Koponen P, Teräsjärvi J, Vuononvirta J, Helminen M, He Q, Nuolivirta K. Toll-like receptor 1 and 10 gene polymorphisms are linked to postbronchiolitis asthma in adolescence. Acta Paediatrica 2018; 107(1): 134-139.

Risk factors for asthma after infant bronchiolitis

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Author Contributions

Sari Törmänen has contributed to collecting and analysing the data and writing the paper.

Eero Lauhkonen has contributed to collecting the data and writing the paper.

Riikka Riikonen has contributed to writing the paper.

Petri Koponen has contributed to collecting the data and writing the paper.

Heini Huhtala has contributed to analysing the data.

Merja Helminen has contributed to conceiving and designing the study and writing the paper.

Matti Korppi has contributed to concieving and designing the study, analysing the data and writing the paper.

Kirsi Nuolivirta has contributed to conceiving and designing the study, analysing the data and writing the paper.

Conflicts of Interest Statement

No conflicts of interest.

Short title Asthma risks after infant bronchiolitis

Abstract

Background. Five studies carried out after bronchiolitis at less than 24 months of age, with a follow-up of more than 10 years, reported that atopic dermatitis, family asthma, early-life exposure to tobacco smoke and rhinovirus aetiology were early-life risk factors for later asthma. This study evaluated the long-term outcome at 11-13 years of age of children who were hospitalised for bronchiolitis in early infancy.

Methods. We previously prospectively followed 166 children hospitalised for bronchiolitis at less than 6 months of age until 5-7 years of age. The current study included a structured questionnaire, parental interviews, clinical examinations and bronchodilation test of 138 of those children at 11-13 years of age.

Results. Respiratory syncytial virus caused 66% of the bronchiolitis cases and nearly half of the patients were exposed to tobacco smoke in early life. Doctor-diagnosed asthma was present in 13% of the former bronchiolitis patients at 11-13 years of age. Maternal asthma was the only independently significant risk factor in early life (adjusted OR 3.45, 95% CI 1.07-11.74), as was allergic rhinitis at 5-7 years of age (adjusted OR 4.06, 95% CI 1.35-12.25).

Conclusions. After bronchiolitis at less than 6 months of age, the risk of doctor-diagnosed asthma at 11-13 years was about twice that of the general Finnish population. Maternal asthma was the only independently significant early-life risk factor for current asthma at 11-13 years of age.

Key words: allergic rhinitis, bronchiolitis, childhood asthma, maternal asthma, parental smoking.

Introduction

Bronchiolitis is a lower respiratory infection (LRI) characterised by airway obstruction with tachypnoea and audible wheezes or crackles (1). The age limits used to define bronchiolitis are less than 12 months in Europe and less than 24 months in the United States (2). In a recent study from Finland, the age definition was less than 6 months and the incidence of bronchiolitis admitted to the emergency room was 37 per 1000 per year and 70% of infants were treated in hospital (3).

Respiratory syncytial virus (RSV) is the most common causative agent of bronchiolitis up to 12 months of age (2, 4) and rhinoviruses dominate in older infants (2). Bronchiolitis is a risk factor for wheezing in early childhood (5-7) and for asthma in later childhood (8). In previous postbronchiolitis studies, the asthma risk has been linked to rhinoviruses and, less frequently, to RSV (8, 9). In addition, maternal asthma, passive smoking, atopic dermatitis and the recurrence of wheezing in infancy have been identified as risk factors for postbronchiolitis asthma (10-12).

There have only been 5 prospective postbronchiolitis studies with a follow-up of more than 10 years: 2 from Finland (8, 10), 2 from Sweden (11, 12) and 1 from Norway (13). The 1981-1982 Finnish cohort showed that children hospitalised for bronchiolitis before 24 months of age faced a 2.6 to fivefold risk of asthma at the age of 12-13 years and that the risk factors were repeated wheezing, atopic dermatitis and blood eosinophilia in infancy and allergic rhinitis at school age (10). The 1992-1993 Finnish cohort reported that increased asthma risk after bronchiolitis persisted until teenage and that the risk factors were atopic dermatitis in infancy and sensitisation to inhaled allergens before 3 years of age (8). In that study the asthma risk was fivefold if the causative virus was RSV and 10-fold if it was rhinovirus. The 1984-1985 Swedish cohort showed that children hospitalised for bronchiolitis at less than 24 months of age faced a sixfold risk for asthma at the of age 10 years and that the risk factors were parental smoking and atopy in children (11). The 1989-1990 Swedish cohort focused on children hospitalised for RSV bronchiolitis at less than 12 months

of age and controls matched for sex and age. At the age of 13, the bronchiolitis group had a higher prevalence of asthma and allergy than the control group and the risk factor that predicted asthma was parental asthma (12). In the Norwegian postbronchiolitis cohort that started in 1997-1998, 23% of the children hospitalised for bronchiolitis at less than 12 months of age had asthma at 11 years of age and the predictive factors were parental asthma or atopy and the combination of repeated wheezing and atopic dermatitis in the children (13).

We have prospectively followed 166 children who were hospitalised for bronchiolitis at less than 6 months of age and attended control visits at 1.5 years (14) and 5-7 years (15) of ages. The present clinical follow-up study took place when the children were 11-13 years old and it also included age- and sex-matched controls who had not been hospitalised for bronchiolitis or any other respiratory infection in infancy.

The aim of the present study was to evaluate the long-term outcome at 11-13 years of age of children who were hospitalised for bronchiolitis at less than 6 months of age. In addition, we studied the impact of early-life risk factors on the outcome, such as atopic dermatitis, family allergy or asthma, early-life exposure to tobacco smoke and the viral aetiology of bronchiolitis, as well later risk factors, such as the development of skin test positivity and allergic rhinitis at school age.

Material and methods

Hospitalisation data and follow-up data at 5-7 years of age

The study group comprised 166 children hospitalised for bronchiolitis at less than 6 months of age in 2001- 2004, who were initially prospectively followed until the age of 5-7 years (15). In infancy, bronchiolitis was defined as a LRI characterised by rhinitis, cough and diffuse wheezes or crackles. During hospitalisation for bronchiolitis, nasopharyngeal aspirates were taken to determine the viral

aetiology using antigen detection and polymerase chain reaction. The detected viruses were RSV, rhinoviruses, influenza A virus, metapneumovirus, human bocavirus, adenovirus and parainfluenza type 1, 2 and 3 viruses (15).

At the follow-up visit at the age of 5-7 years, we evaluated the presence of asthma and allergies from the hospitalisation for bronchiolitis to preschool age (15). Bronchial hyper-reactivity was studied by exercise challenge test with impulse oscillometry (16) and skin prick tests (SPT) were performed to assess the children's sensitisation to 8 allergens: birch, timothy grass and mugwort pollens, cat and dog dander, house dust mites (*Dermatophagoides pteronyssimus* and *Dermatophagoides farinae*) and spores of the mould *Alternaria alternate* (15). Current asthma was present in 21/166 (12.7%) children, asthma ever in 45/166 (27.1%) children and SPT positivity in 35/124 (28.2%) children (15).

Follow-up at the age of 11-13 years

The follow-up visit was arranged when the children were 11-13 years of age and took place between 1 June 2014 and 31 January 2015. We invited the 166 former bronchiolitis patients who had attended the previous follow up at 5-7 years of age and for each of the 166 cases we picked 4 controls (n= 664). These were matched for age and sex from the population register of the Pirkanmaa Hospital District, where also the cases lived. Inpatient treatment for any medical reason and inpatient or outpatient treatment for bronchiolitis or LRI in infancy were exclusion criteria.

The follow-up study consisted of medical histories from the last visit at the age of 5-7 years to the latest follow-up visit at 11-13 years. The data were collected using structured questionnaires, which the parent had completed before the visit, and a clinical examination by a doctor, that included an interview, checking the questionnaire data and a bronchodilation test. The questionnaire consisted of questions on doctor-diagnosed atopic dermatitis, allergic rhinitis and food allergies from the last

control visit until the present time and family history of asthma and allergy, exposure to tobacco smoke during pregnancy and infancy, the current use of asthma medication, current symptoms that could suggest asthma, and the presence of a current asthma diagnosis.

The data on the risk factors for asthma were obtained from the structured questionnaire completed when the cases and controls were aged 11-13. This retrospective analysis used the same data collection method for both groups.

When the risk factors for asthma were analysed within the bronchiolitis group, the early-life and preschool age data were obtained from the previous studies performed to this cohort that is, during hospitalisation for bronchiolitis, and at the follow-up visits at 1.5 years (14) and 5-7 years (15) of ages. These data were collected prospectively and included the age on admission, viral findings during hospitalisation, family history of asthma and allergy, exposure to tobacco smoke during pregnancy and infancy, presence of atopic dermatitis, wheezing episodes and asthma treatments before the age of 1.5 years, as well as the presence of asthma and asthma treatments, allergic rhinitis and SPT positivity at 5-7 years of age.

Bronchodilation test

Lung function was measured with flow-volume spirometry using the Vmax V62J Autobox (Becton, Dickinson and Company, NJ, USA), and the results will be published elsewhere. The bronchodilation test was performed as follows: 3 technically acceptable measurements were required before and 15 minutes after the inhalation of 400µg salbutamol (100µg/dos) using a Ventolin Evohaler (GlaxoSmithKline, London, UK), and the best pre-treatment and post-treatment forced expiratory volume in 1 second (FEV1) values were expressed as a percentage of the mean sex-specific and height-adjusted values in the population (predicted FEV1) and used in the analysis. The bronchodilation test was regarded as positive if the predicted FEV1 increased 12% or more

after salbutamol inhalation. A positive finding in the bronchodilation test, which meant reversible airway obstruction, was regarded as experimental, indirect evidence of asthma.

Definition of asthma

Current asthma was considered to be present if the child had used inhaled corticosteroids (ICS) continuously during the last 12 months or if the child had suffered from repeated wheezing or from prolonged cough or night cough for 4 or more weeks during the last 12 months, and, in addition, had a diagnostic increase of FEV1 in the bronchodilation test.

Ethics

The Ethics Committee of Tampere University Hospital approved the study. The parents gave their written and informed consent before enrolling the children. According to the ethical guidelines of our hospital district, the controls were only invited to participate once and reminders were not allowed.

Statistics

The data were analysed by using the SPSS 21.0 package (IBM Corp. Released 2012. IBM SPSS Statistics for Windows, Version 21.0. Armonk, NY: IBM Corp). The statistical significances of differences between the groups were calculated with the unpaired t-test, chi-square test and Fisher's exact test. Logistic regression was used to analyse the association between the early-life and preschool age risk factors and current asthma at the age of 11-13. Those factors, which were

significant in the non-adjusted analyses, were included in the adjusted analyses. The results are expressed as adjusted odds ratios (aOR) and their 95% confidence intervals (95% CI).

Results

In all, 138 children hospitalised for bronchiolitis under the age of 6 months participated in the present follow-up study at the mean age of 11.7 years (range 10.2-13.2). Of those, 89 children attended the clinical follow-up visit and the parents of the other 49 children just completed the questionnaire, which was supplemented by a telephone interview. Current asthma was present in 18 (13.0%) children and 11 (61.1%) of them were boys (Table 1). Fourteen had used ICS during the last 12 months and the other 4 were diagnosed with asthma at the study visit, based on asthma-presumptive symptoms plus a diagnostic response to the bronchodilation test. There were no significant differences in the prevalence of allergic rhinitis, atopic dermatitis, the use of bronchodilators or ICS during the last 12 months, or the occurrence of current asthma or asthma from 5-7 years until 11-13 years of ages between the 89 children who attended the clinical follow-up visit and those 49 who only answered the questionnaire (data not shown).

The number of population-based controls matched for age and sex was 112, due to the refusal rate of 83.1%. Of those, 108 took part in the clinical study and the other 4 parents filled in the questionnaire, supplemented by a telephone interview. Current asthma was present in 12 (10.7%) controls and 9 of them used ICS. This meant that the cases and controls did not differ in terms of current asthma, current allergy or family asthma or family allergy (Table 1). There were no significant differences in the occurrence of allergic rhinitis, atopic dermatitis or asthma after the age of 5-7 years between the 18 asthmatic children in the bronchiolitis group and those 12 in the control group (data not shown). However, smoking exposure was much more common in the cases than in the controls, namely: maternal smoking during pregnancy (13.8% vs 2.7%, p=0.002), maternal

smoking during infancy (21.7% vs 5.4%, p<0.001) and paternal smoking during infancy (36.2% vs 23.2%, p=0.026). Despite this, there were no significant associations between current asthma and early-life exposure to tobacco smoke in the bronchiolitis group (Table 2).

During hospitalisation in infancy, RSV was the causative virus of bronchiolitis in 65.9% of the cases, with rhinovirus in 13.0% and other viruses in 13.8%. No virus was detected in 7.2%, but probably, they were viral cases caused by other viruses than RSV. There was no significant association between the virus aetiology of bronchiolitis and the occurrence of asthma at the mean age 11.7 years (Table 2). The significant risk factors for current asthma in the bronchiolitis group were atopic dermatitis at less than 12 months of age, maternal asthma or allergy and allergic rhinitis or SPT positivity at 5-7 years of age (Table 2).

The presence of doctor-diagnosed asthma at different ages from hospitalisation for bronchiolitis to the follow-up visit at 5-7 years (mean age of 6.3 years), was evaluated in relation to current asthma and current allergic rhinitis at 11-13 years (mean age of 11.7 years). Asthma from the age of 2-3 years onwards revealed significant associations for current asthma. For current rhinitis the association was significant only at the age of 5-7 years (Table 3). Eleven (61.1%) of the 18 children with current asthma also had asthma at the earlier follow-up visit at the age of 5-7 years (p<0.001). All the 11 children who had persistent asthma from 6.3 to 11.7 years of age also reported symptomatic allergic rhinitis.

The factors that predicted current asthma in the univariate analyses were included in the multivariate logistic regression. When atopic dermatitis in infancy, allergy in mothers, asthma in mothers and allergic rhinitis at the age of 5-7 years were included in the analysis, asthma in mothers (aOR 3.5, 95%CI 1.04-11.78) and allergic rhinitis at the age of 5-7 years (aOR 4.06, 95%CI 1.35-12.25) were significant risk factors for current asthma at 11-13 years of age (Table 4). Data on SPT positivity at 5-7 years of age were available for 109 children. When SPT positivity was included in

the analysis instead of allergic rhinitis, SPT positivity at the age of 5-7 years was a significant risk factor for current asthma at 11-13 years of age (aOR 5.01, 95%CI 1.47-17.1), but atopic dermatitis in infancy (aOR 1.34, 95% CI 0.38-4.61), allergy in mothers (aOR 1.99, 95%CI 0.56-7.04) and asthma in mothers (aOR 1.99, 95%CI 0.47-8.38) were not.

Table 1. Baseline data in 138 children hospitalized for bronchiolitis at less than six months of age and in 112 population controls, based on questionnaires completed at the follow-up visits at the age of 11-13 years.

	Bronchiolitis group N=138		Control group N=112		
Baseline characteristics	n	%	n	%	p value
Boys	72	52.2	62	55.4	0.616
Urban environment	84	60.9	68	60.7	0.980
Current allergy					
Atopic dermatitis	35	25.4	26	23.2	0.694
Allergic rhinitis	60	43.5	50	44.6	0.854
Food allergy	13	9.4	14	12.5	0.435
History of family asthma and allergy					
Asthma in mothers	18	13.0	15	13.4	0.953
Asthma in fathers	9	6.5	16	14.3	0.044
Allergy in mothers	49	35.5	46	41.1	0.391
Allergy in fathers	38	27.5	38	33.9	0.291
Asthma in siblings	33	23.9	17	15.2	0.081
Allergy in siblings	45	32.6	33	29.5	0.541

Smoking exposure in infancy					
Maternal smoking (pregnancy)	19	13.8	3	2.7	0.002
Maternal smoking (<12 months of age)	30	21.7	6	5.4	<0.001
Paternal smoking (<12 months of age)	50	36.2	26	23.2	0.026
Current asthma, suggestive symptoms and medications					
Current asthma	18	13.0	12	10.7	0.573
Current use of inhaled corticosteroids	14	10.1	9	8.0	0.566
Wheezing symptoms	31	22.5	27	24.1	0.760
Bronchodilator use	31	22.5	20	17.8	0.369
Prolonged cough (>4 weeks)	16	11.6	7	6.3	0.184
Night cough (apart of infection)	16	11.6	2	1.8	0.003

Only doctor-diagnosed diseases were accepted, and in case of current disease, only those presenting with symptoms or needing treatment during the recent 12 months were registered. The asthma-presumptive symptoms had to be occurred during 12 months preceding the study. Family allergy means presence of atopic dermatitis, allergic rhinitis or allergic conjunctivitis.

Table 2. Viral findings during bronchiolitis, risk factors in infancy and risk factors at 5 to 7 years of age in relation to presence of current asthma at 11 to 13 years of age.

	Current asthma N=18		No current asthma N=120		
Basic characteristics	n	%	n	%	p value
Boys (n=72)	11	61.1	61	50.8	0.420
Age					
≤ 3 months (n=82)	7	38.9	75	62.5	
> 3 months (n=56)	11	61.1	45	37.5	0.057
Viral findings					
RSV (n=91)	10	55.6	81	67.5	
Non-RSV (n=47)*	8	44.4	39	32.5	0.320
Rhinovirus (n=18)	2	11.1	16	13.3	1.000
Early-life risk factors					
Atopic dermatitis (<12 months of age) (n=40)	9	50.0	31	25.8	0.035
Maternal smoking (pregnancy) (n=24)	2	11.1	22	18.3	0.740
Maternal smoking (<12 months of age) (n=37)	5	27.8	32	26.7	1.000
Paternal smoking (<12 months of age) (n=56)	7	38.9	49	40.8	0.880
Asthma in mothers (n=22)	8	44.4	14	11.7	0.002
Asthma in fathers (n=8)	0	0.0	8	6.7	0.600
Allergy in mothers (n=58)	13	72.2	45	37.5	0.005

Allergy in fathers (n=34)	4	22.2	30	25	1.000
Pets at home (n=45)	4	22.2	41	34.2	0.310
Risk factors at age 5-7-years					
Rhinitis at age 5-7 years (n=39)	11	61.1	28	23.3	0.001
SPT positivity at age 5-7 years (n= 32)**	9/14	64.3	23/95	24.2	0.004
Asthma at age 5-7 years (n=20)	11	61.1	9	7.5	<0.001

Data on early-life risk factors were obtained during hospitalisation for bronchiolitis and/or at the 18 months follow-up visit (14). Data on 5-7 years risk factors were obtained at the control visit at that age (15). For different diseases, only the doctor-diagnosed cases were accepted. P values were assessed by chi-square or Fisher's exact tests.

* Non-RSV group consisted of 18 rhinovirus-positive and 10 virus-negative cases and of 19 cases caused by other viruses.

** Skin prick test (SPT) results were available from 109 children (14 of those with present asthma), other data available from 138 children.

Table 3. Current asthma and current allergic rhinitis in relation to doctor-diagnosed asthma at preschool age.

	Current asthma					Current allergic rhinitis				
	No		Yes			No		Yes		
Asthma at different ages	n	%	n	%	p-value	n	%	n	%	p-value
0-1	4	3.3	3	16.7	0.047	4	4.0	3	7.7	0.403
1-2	14	11.7	5	27.8	0.076	12	12.1	7	17.9	0.371
2-3	15	12.5	9	50.0	0.001	14	14.1	10	25.6	0.109
3-4	11	9.2	10	55.6	<0.001	13	13.1	8	20.5	0.277
4-5	8	6.7	9	50.0	<0.001	9	9.1	8	20.5	0.085
5-6	9	7.5	10	55.6	<0.001	10	10.1	9	23.1	0.046
6-7	6	5.0	11	61.1	<0.001	7	7.1	10	25.6	0.007

Table 4. Logistic regression: Risk factors for asthma at age 11-13 years after bronchiolitis in early infancy.

	Univariate		Multivariate	
	OR	95% CI	OR	95% CI
Atopic dermatitis in infancy	2.87	1.05-7.89	2.00	0.65-6.13
Allergy in mother	4.33	1.45-12.96	1.95	0.56-6.80
Asthma in mother	6.06	2.05-17.91	3.50	1.04-11.78
Allergic rhinitis at age 5-7 years	5.16	1.83-14.56	4.06	1.35-12.25

Discussion

There are 4 main results in the present study. First, current doctor-diagnosed asthma was present in 13.0% of former bronchiolitis patients at the age of 11 to 13 years. This figure was rather low, as it was only about twice the prevalence of asthma in the general population of Finnish school children (17-19). On the other hand, the risk of later asthma has been rather low in previous studies if bronchiolitis was caused by RSV and the patients were less than 12 months old (2, 13), and even lower if the patients were less than 6 months old (2), as was the case in the present study. Second, maternal asthma was the only independently significant early-life risk factor for current asthma at 11-13 years. Other factors like atopic dermatitis in infancy and maternal allergy lost their significance in the adjusted analyses. Third, allergic rhinitis and SPT positivity at preschool age were risk factors for current asthma at 11-13 years. Both allergic rhinitis and SPT positivity are markers of atopy, but as they become apparent after infancy they are more useful at preschool age

when predicting the outcome after bronchiolitis. Fourth, there were no significant differences in asthma or allergy between former bronchiolitis patients and population-based controls. An evident reason for this is that the poor participation rates of the controls meant that the children with asthma and allergy were overrepresented.

The prevalence of asthma in school children has ranged from 4 to 7% in Finnish epidemiological studies (17, 19). Thus, the figure of 13% in former bronchiolitis patients in our study means that hospitalisation for bronchiolitis doubled or trebled the risk of asthma, but not any more than that. In many studies, both early age and RSV aetiology of bronchiolitis have been associated with a more beneficial outcome than older age and, for example, rhinovirus aetiology of bronchiolitis (2, 9, 13, 15). Our patients were hospitalised at less than 6 months of age and RSV caused most of the cases, which at least partly explains the low asthma figures. Another explanation is the strict asthma diagnosis we applied. The 10.7% prevalence of asthma in our control group means that the controls who attended the study - 16.9% of those invited - were selected. This kind of selection bias is well-known in clinical studies, since symptomatic subjects are more willing to participate than non-symptomatic ones (20, 21).

Well-known early-life risk factors of asthma include asthma in parents, especially in mothers; exposure to parental smoking in infancy, especially to maternal smoking during pregnancy; atopic dermatitis in infants and blood eosinophilia or merely the lack of an eosinopenic response to viral infection (4, 6, 22, 23). If the causative agent of bronchiolitis was RSV, the risk of childhood asthma was two to three-fold and if it was rhinovirus the risk was as high as 10-fold, compared to the asthma risk in the general paediatric population (24). In line with these earlier observations, maternal asthma was a significant risk factor for post-bronchiolitis asthma at 11-13 years of age in the present study. This finding was robust to adjustments with other early-life risk factors and presence of allergic rhinitis at 5-7 years of age in multivariate analyses. However, in a supplementary analysis in which allergic rhinitis was replaced with SPT positivity in those 109 with

SPT data available, maternal asthma lost its significance. The rhinovirus aetiology of bronchiolitis was not associated with the later asthma risk in this study, probably due to under-powering of the virus-specific analyses.

Asthma, allergic rhinitis and SPT positivity have been closely associated (25) and this was also seen in our cohort at the age of 5-7 years (15) and now at the age of 11-13 years. Allergic rhinitis and SPT positivity at 5-7 years of age were significant risk factors for asthma at 11-13 years of age. Although 5-7 years of age is an appropriate age to study allergy and allergic sensitisation to inhaled seasonal allergens, new allergies still develop after that age (26). In European countries like Finland, the incidence of allergic rhinitis in non-selected child populations has been 5-10% at the ages of 6-7 years and 23-31% at the ages of 13-14 years (27), which is less than in the cases in the present study (43.5%) and also less than in the controls (44.6%) – also this finding speaks for the selection bias. The incidence of allergic sensitisation to inhaled allergens, assessed by SPT positivity, has been 10-20% at 5-6 years of age and 20-40% at 11 years of age (26, 28). These figures were in line with the non-asthmatic cases in the present study.

Early-life exposure to tobacco smoke is known to increase the severity of bronchiolitis (29) and this was reflected by our observation that tobacco exposure was more common in the cases, who were hospitalised for bronchiolitis in infancy, than in controls. Such exposure increases the risk of later lung function reduction and childhood asthma and the effect seems to continue until at least young adulthood (23). The highest risk has been associated with maternal smoking during pregnancy, followed by maternal smoking during infancy and, to a lesser extent, paternal smoking during infancy (23, 30). In the present study, 42.9% of the former bronchiolitis patients were exposed to parental smoking in infancy: 21.7% to maternal and 36.2% to paternal smoking. The figures found in our study were higher than the smoking rate found in young adults in the general population: 28% in men and 25% in women (31). However, early-life exposure to tobacco smoke was not a

significant risk factor for childhood asthma in the bronchiolitis group but the infants of smoking parents are overrepresented among infants hospitalised for bronchiolitis (29, 32).

The limitations of this study were the drop-out rate of 16.8% in the cases, explained by the follow-up time of more than 11 years. Just over one third (35.5%) of the cases did not perform the bronchodilation test, which may have led to asthma under-diagnosis in the bronchiolitis group. The drop-out rate among the controls, who were picked from the regional population register, was 83.1%, and it is clear that allergic and asthmatic children, and maybe non-smoking families, were over-represented. We acknowledge the lack of prospective follow-up data of the control group as a shortcoming of this study.

The main strengths of the study were that it was prospective for bronchiolitis patients and continued for more than 10 years. The cohort is unique, since the patients were hospitalised for bronchiolitis at less than 6 months of age and the causative virus was detected in nearly all cases. In addition, early-life data were carefully collected during hospitalisation and during the first 1.5 years of the study. An identical follow-up study was performed on the same cohort 6 years before this study, which enabled us to compare the findings at preschool age and now in adolescence.

Conclusion

Asthma was present in 13.0% of the former bronchiolitis patients at the age of 11 to 13 years after hospitalisation for bronchiolitis at the age of less than 6 months. This represented an approximate two-fold increased risk compared to Finnish population data. Maternal asthma was the only independently significant early-life risk factor for asthma at 11-13 years of age. Asthma, allergic rhinitis and skin test positivity at age 5-7 years of age were closely linked to asthma at 11-13 years.

References

1. Subcommittee on Diagnosis and Management of Bronchiolitis. Diagnosis and Management of Bronchiolitis. *Pediatrics* 2006;**118**:1774-1793.

2. Jartti T, Lehtinen P, Vuorinen T, Ruuskanen O. Bronchiolitis: age and previous wheezing episodes are linked to viral etiology and atopic characteristics. *Pediatr Infect Dis J* 2009;**28**:311-317.

3. Pruikkonen H, Uhari M, Dunder T, Pokka T, Renko M. Infants under 6 months with bronchiolitis are most likely to need major medical interventions in the 5 days after onset. *Acta Paediatr* 2014;**103**:1089-1093.

4. Korppi M, Kotaniemi-Syrjanen A, Waris M, Vainionpaa R, Reijonen TM. Rhinovirus-associated wheezing in infancy: comparison with respiratory syncytial virus bronchiolitis. *Pediatr Infect Dis J* 2004;**23**:995-999.

5. Fjaerli H, Farstad T, Rod G, Ufert G, Gulbrandsen P, Nakstad B. Acute bronchiolitis in infancy as risk factor for wheezing and reduced pulmonary function by seven years in Akershus County, Norway. *BMC Pediatr* 2005;**5**:31.

6. Kotaniemi-Syrjänen A, Reijonen TM, Korhonen K, Korppi M. Wheezing requiring hospitalization in early childhood: Predictive factors for asthma in a six-year follow-up. *Pediatr Allergy Immunol* 2002;**13**:418-425.

7. Valkonen H, Waris M, Ruohola A, Ruuskanen O, Heikkinen T. Recurrent wheezing after respiratory syncytial virus or non-respiratory syncytial virus bronchiolitis in infancy: a 3-year follow-up. *Allergy* 2009;**64**:1359-1365.

8. Hyvarinen MK, Kotaniemi-Syrjanen A, Reijonen TM, Korhonen K, Korppi MO. Teenage asthma after severe early childhood wheezing: an 11-year prospective follow-up. *Pediatr Pulmonol* 2005;**40**:316-323.

9. Jartti T, Korppi M. Rhinovirus-induced bronchiolitis and asthma development. *Pediatr Allergy Immunol* 2011;**22**:350-355.

10. Hyvärinen M, Piippo-Savolainen E, Korhonen K, Korppi M. Teenage asthma after severe infantile bronchiolitis or pneumonia. *Acta Paediatr* 2005;**94**:1378-1383.

11. Wennergren G, Amark M, Amark K, Oskarsdottir S, Sten G, Redfors S. Wheezing bronchitis reinvestigated at the age of 10 years. *Acta Paediatr* 1997;**86**:351-355.

12. Sigurs N, Gustafsson PM, Bjarnason R, Lundberg F, Schmidt S, Sigurbergsson F, et al. Severe Respiratory Syncytial Virus Bronchiolitis in Infancy and Asthma and Allergy at Age 13. *Am J Respir Crit Care Med* 2005;**171**:137-141.

13. Mikalsen IB, Halvorsen T, Eide GE, Øymar K. Severe bronchiolitis in infancy: Can asthma in adolescence be predicted? *Pediatr Pulmonol* 2013;**48**:538-344.

14. Nuolivirta K, Hurme M, Halkosalo A, Koponen P, Korppi M, Vesikari T, et al. Gene polymorphism of IFNG +874 T/A and TLR4 +896 A/G and recurrent infections and wheezing in toddlers with history of bronchiolitis. *Pediatr Infect Dis J* 2009;**28**:1121-1123.

15. Koponen P, Helminen M, Paassilta M, Luukkaala T, Korppi M. Preschool Asthma after Bronchiolitis in Infancy. *Eur Respir J* 2012;**39**:76-80.

16. Lauhkonen E, Koponen P, Nuolivirta K, Paassilta M, Toikka J, Korppi M. Lung function by impulse oscillometry at age 5-7 years after bronchiolitis at age 0-6 months. *Pediatr Pulmonol* 2015; **50**:389-395.

17. Hugg T, Ruotsalainen R, Jaakkola M, Pushkarev V, Jaakkola JK. Comparison of allergic diseases, symptoms and respiratory infections between Finnish and Russian school children. *Eur J Epidemiol* 2008;**23**:123-133.

18. Harju M, Keski-Nisula L, Georgiadis L, Raatikainen K, Räisänen S, Heinonen S. Maternal socioeconomic status and the risk of asthma among offspring. *BMC Public Health* 2015;**15**:27.

19. Pekkanen J, Remes ST, Husman T, Lindberg M, Kajosaari M, Koivikko A, et al. Prevalence of asthma symptoms in video and written questionnaires among children in four regions of Finland. *Eur Respir J* 1997;**10**:1787-1794.

20. Bornehag C, Moniruzzaman S, Larsson M, Lindström C, Hasselgren M, Bodin A, et al. The SELMA Study: A Birth Cohort Study in Sweden Following More Than 2000 Mother-Child Pairs. *Paediatr Perinat Epidemiol* 2012;**26**:456-467.

21. Joseph CLM, Saltzgaber J, Havstad SL, Johnson CC, Johnson D, Peterson EL, et al. Comparison of early-, late-, and non-participants in a school-based asthma management program for urban high school students. *Trials* 2011;**12**:141.

22. Grabenhenrich LB, Gough H, Reich A, Eckers N, Zepp F, Nitsche O, et al. Early-life determinants of asthma from birth to age 20 years: A German birth cohort study. *J Allergy Clin Immunol* 2014;**133**:979-988.

23. Goksör E, Åmark M, Alm B, Gustafsson PM, Wennergren G. The impact of pre- and post-natal smoke exposure on future asthma and bronchial hyper-responsiveness. *Acta Paediatr* 2007;**96**:1030-1035.

24. Kotaniemi-Syrjänen A, Vainionpää R, Reijonen TM, Waris M, Korhonen K, Korppi M. Rhinovirus-induced wheezing in infancy—the first sign of childhood asthma? *J Allergy Clin Immunol* 2003;**111**:66-71.

25. Ruokonen M, Kaila M, Haataja R, Korppi M, Paassilta M. Allergic rhinitis in school-aged children with asthma - still under-diagnosed and under-treated? A retrospective study in a children's hospital. *Pediatr Allergy Immunol* 2010;**21**:e149-54.

26. Pesonen M, Kallio MJT, Siimes MA, Ranki A. Allergen Skin Prick Testing in Early Childhood: Reproducibility and Prediction of Allergic Symptoms into Early Adulthood. *J Pediatr* 2015;**166**:401-406.

27. Strachan D, Sibbald B, Weiland S, Ait-Khaled N, Anabwani G, Anderson HR, et al. Worldwide variations in prevalence of symptoms of allergic rhinoconjunctivitis in children: the International Study of Asthma and Allergies in Childhood (ISAAC). *Pediatr Allergy Immunol* 1997;**8**:161-168.

28. Kulig M, Bergmann R, Klettke U, Wahn V, Tacke U, Wahn U. Natural course of sensitization to food and inhalant allergens during the first 6 years of life. *J Allergy Clin Immunol* 1999;**103**:1173-1179.

29. Bradley JP, Bacharier LB, Bonfiglio J, Schechtman KB, Strunk R, Storch G, et al. Severity of Respiratory Syncytial Virus Bronchiolitis Is Affected by Cigarette Smoke Exposure and Atopy. *Pediatrics* 2005;**115**:e7-e14.

30. Stevenson MD, Mansbach JM, Mowad E, Dunn M, Clark S, Piedra PA, et al. Prenatal Versus Postnatal Tobacco Smoke Exposure and Intensive Care Use in Children Hospitalized With Bronchiolitis. *Acad Pediatr* 2016;**16**:446-452.

31. Patja K, Hakala SM, Bostrom G, Nordgren P, Haglund M. Trends of tobacco use in Sweden and Finland: do differences in tobacco policy relate to tobacco use? *Scand J Public Health* 2009;**37**:153-160.

32. Simoes EA, Carbonell-Estrany X. Impact of severe disease caused by respiratory syncytial virus in children living in developed countries. *Pediatr Infect Dis J* 2003;**22**:S13-18,discussion S18-20.

Polymorphism in the gene encoding toll-like receptor 10 may be associated with asthma after bronchiolitis

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Abstract

Toll-like receptors (TLRs) recognise microbes that contribute to the severity of bronchiolitis and the subsequent risk of asthma. We evaluated whether post-bronchiolitis asthma was associated with polymorphisms in the *TLR3* rs3775291, *TLR4* rs4986790, *TLR7* rs179008, *TLR8* rs2407992, *TLR9* rs187084, and *TLR10* rs4129009 genes. The gene polymorphisms were studied at the age of 6.4 years (mean) in 135 children hospitalised for bronchiolitis in infancy. The outcome measure was current or previous asthma. Current asthma was more common (30%) in children with the variant AG or GG genotype in the *TLR10* rs4129009 gene versus those who were homozygous for the major allele A (11%) (p=0.03). The adjusted odds ratio (aOR) was 4.30 (95% CI 1.30–14.29). Asthma ever was more common (34.6%) in girls with the *TLR7* variant AT or TT genotype versus those who were homozygous for the major allele A (12.5%) (p=0.03). The adjusted OR was 3.93 (95% CI 1.06–14.58). Corresponding associations were not seen in boys. There were no significant associations between *TLR3*, *TLR4*, *TLR8*, or *TLR9* polymorphisms and post-bronchiolitis asthma. Polymorphism in the *TLR10* gene increases and in the *TLR7* gene may increase the risk of asthma in preschool-aged children after infant bronchiolitis.

Introduction

Bronchiolitis in infancy increases the risk of subsequent wheezing and childhood asthma.[1] Although many asthma risk factors, such as asthma in parents, atopy or eosinophilia in children, and rhinovirus aetiology of bronchiolitis,[2] are well documented, predicting the outcome of an individual patient is not possible. Innate immunity, which is highly regulated by genes, plays a crucial role in both infection and inflammation.[3]The development of asthma is a complicated and multifactorial process in which genes interact with the environment.[4] In early life, the Th2-dominated immune responses shift towards Th1-dominated responses,[5] but among genetically susceptible individuals, environmental factors like viruses may lead to the persistence of Th2-dominated immunity and to subsequent atopy and asthma.[6]

Toll-like receptors (TLRs) are pattern-recognising proteins that, after recognising foreign material like microbes, are able to trigger the production of mediators of innate immunity and, subsequently, after complex signalling processes, the development of adaptive immune responses.[7, 8] TLRs 1, 2, 4, 5, 6, and 10 are located on the cell surface, whereas TLRs 3, 7, 8, and 9 are located inside the cells,[9] recognising microbial components after endocytosis. TLR1, TLR2, TLR6, and TLR10 comprise the TLR2 subfamily, and *TLR1*, *TLR2*, *TLR6*, and *TLR10* gene polymorphisms seem to play a role in susceptibility to asthma, atopic eczema, and allergic rhinitis.[10-12] TLR3 recognises double-stranded viral ribonucleic acid (RNA), and, in mice, TLR3 activation by viruses combined with allergen inhalation resulted in allergic airway disease.[13] TLR4 recognises bacterial lipopolysaccharides and the F glycoprotein of the respiratory syncytial virus (RSV).[14] TLR7 and TLR8, which are regulated by genes located in the X chromosome, recognise single-stranded viral RNA.[15] An American study found that TLR7 contributed to human airway relaxation via the production of nitric oxide.[16] There is evidence that polymorphisms in the *TLR7* and *TLR8* genes are associated with susceptibility to asthma and related atopic disorders[15] and to susceptibility to respiratory viral infections.[17] Signalling via TLR7 and TLR9 affects the function of eosinophils, engendering a link between viral

infection and allergic exacerbations.[18] Although TLR10 is a pattern-recognition receptor without known ligand specificity, it has shown to be a modulatory receptor with mainly inhibitory properties.[19]

We have prospectively followed 166 children who were hospitalised for bronchiolitis at less than 6 months of age.[2] At 5 to 7 years of age, 127 of the children attended a clinical control visit, and questionnaire data were available for another 39 children.[2] We have previously studied the *TLR1* rs5743618, *TLR2* rs5743708, and *TLR6* rs5743810 polymorphisms and reported their associations with post-bronchiolitis asthma at preschool age.[10] The present study was carried out to complement this exploratory study series by evaluating whether the *TLR3* rs3775291, *TLR4* rs4986790, *TLR7* rs179008, *TLR8* rs2407992, *TLR9* rs187084, and *TLR10* rs4129009 polymorphisms are associated with post-bronchiolitis asthma. The aim of this study was to compare these polymorphisms between children with and without current asthma, current atopic dermatitis, or current allergic rhinitis at preschool age, or with and without asthma ever combining current and previous asthma in children hospitalised for bronchiolitis in infancy.

Results

The mean age of the 135 patients was 6.4 years at the control visit, and 51% were males. Asthma ever was present in 37 patients (27.4%), current asthma in 18 (13.3%), atopic dermatitis in 46 (34.1%), and allergic rhinitis in 39 (28.9%). The genotypes and minor allele frequencies (MAF) and population data on the MAFs are listed in Table 1. The MAFs of the cases and the Finnish population MAF data[20] did not differ substantially in terms of *TLR3* rs3775291, *TLR4* rs4986790, *TLR7* rs179008, *TLR8* rs2407992, *TLR9* rs187084, or *TLR10* rs4219009 genes.

The *TLR3* genotype was wild (CC) in 45.9% and variant (TC or TT) in 54.1% of the cases. The *TLR4* genotype was wild (AA) in 83.7% and variant (AG) in 16.3% of the cases. The *TLR9* genotype was wild (TT) in 32.1% and variant (TC or CC) in 67.9% of the cases. There were no significant associations between the *TLR3*, *TLR4*, or *TLR9* genotypes and asthma ever, current asthma, current atopic dermatitis, or current allergic rhinitis (Table 2).

In females, the *TLR7* genotype was wild (AA) in 60.6% and variant (AT or TT) in 39.4% of the cases. In males, allele A was present in 79.4% and allele T in 20.6%. Asthma ever was present in 34.6% of the girls who had the variant AT or TT genotype compared to 12.5% of those who were homozygous for the major allele A (p=0.03) (Table 2). The odds ratio (OR) adjusted for age was 3.71 (95% confidence intervals [CI] 1.08–12.77). This association was significant in logistic regression adjusted first for early-life risk factors, and then separately for current confounders (data not shown). The association remained significant in logistic regression adjusted for age, early-life risk factors, and current confounders in the same model (OR 3.93, 95% CI 1.06–14.58). The corresponding figures in boys were 33.3% (allele A present) and 37.5% (allele T present) (p=1.00). There were no significant associations between the *TLR7* genotypes and current asthma, current atopic dermatitis, or current allergic rhinitis in either girls or boys (Table 3).

In females, the *TLR8* genotype was wild (GG) in 34.8% and variant (GC or CC) in 65.2% of the cases. In males, allele G was present in 50.7% and allele C in 49.3%. There were no significant associations between the *TLR8* genotypes and asthma ever, current asthma, current atopic dermatitis, or current allergic rhinitis in either girls or boys (Table 3).

The *TLR10* genotype was wild (AA) in 84.3% and variant (AG or GG) in 16.7% of the cases. Current asthma was present in 30.0% of the children who had the variant AG or GG genotype compared to 10.6% of those who were homozygous for the major allele A (p=0.03) (Table 2). The OR adjusted for age and gender was 3.74 (95% CI 1.19–11.78). This association was significant in

logistic regression adjusted first for early-life risk factors, and then separately for current confounders (data not shown). The association remained significant in logistic regression adjusted for age, gender, early-life risk factors, and current confounders in the same model (OR 4.30, 95% CI 1.30–14.29). There were no statistically significant associations between *TLR10* gene polymorphisms and asthma ever, current atopic dermatitis, or current allergic rhinitis (Table 2).

Discussion

There were three main results in our study on the association of TLRs with asthma at 5 to 7 years of age after hospitalisation for bronchiolitis at less than 6 months of age. Firstly, current asthma was more common in children who had the variant *TLR10* rs4129009 genotype. Secondly, asthma ever was more common in girls who had the variant *TLR7* rs179008 genotype. Both findings were robust to adjustments with known early-life risk factors for asthma as well as with current confounders at the age of 5 to 7 years. However, *TLR10* and *TLR7* gene polymorphisms had no significant associations with current allergy. And thirdly, there were no significant associations between *TLR3* rs3775291, *TLR4* rs4986790, *TLR8* rs2407992, or *TLR9* rs187084 polymorphisms and earlier or current asthma or allergy.

TLRs play a pivotal role in promoting and controlling innate immune responses. Functional gene polymorphism alters the amino acid structure of the receptor, as has been shown in the cases of *TLR3* rs3775291 (Leu>Phe), *TLR4* rs4986790 (Asp>Gly), *TLR7* rs179008 (Glu>Leu), *TLR9* rs187084 (Arg>Trp), and *TLR10* rs4219009 (Ile>Leu) gene polymorphisms.[21, 22] The consequence of the mutation is dependent on its location. Mutation in the extracellular domain of the receptor may further lead to an altered binding affinity and subsequent immune response,[23] whereas mutation in the cytoplasmic TIR (toll/interleukine-1 receptor) domain, as in the case of *TLR10*

rs4219009, may result in an altered downstream signalling, despite normal binding.[12, 24] Although polymorphism in the *TLR8* rs2407992 (2040 C/G) does not change the amino acid (651Leu>Leu), it can potentially affect TLR8 splicing.[15]

TLR10 is a modulatory pattern-recognition receptor with mainly inhibitory properties, and it is able to reduce TLR2 responses by increasing the production of anti-inflammatory IL-1Ra.[19] Further, a recent meta-analysis revealed that polymorphisms of the IL-1Ra encoding genes were associated with asthma, especially in Caucasian populations.[25] Our finding that the *TLR10* gene polymorphism was associated with current asthma is in accordance with these observations. The genetic variation in *TLR10* rs4129009, which was also determined in the present study, was associated with asthma risk in two independent samples from the USA.[26] In addition, in a Canadian–Australian study, a weak association was observed between another *TLR10* polymorphism (rs11096957) and atopic asthma.[27]

In a large German study, a protective effect of genetic variants on atopic asthma was identified in the TLR2-associated heterodimer network consisting of TLR1, TLR6, and TLR10.[12] Corresponding findings in the genes encoding TLR1, TLR2, and TLR6 were also seen in the present post-bronchiolitis cohort, but the direction of the effect was opposite.[10] The variant genotype in the *TLR1* gene was associated with asthma during the first 6 years of life, and asthma was present in only two children with the wild genotype in all three polymorphisms.[10] In the most recent study from this cohort,[28] polymorphism of *TLR6* was associated with bronchial hyper-reactivity, and if all of the four genes including *TLR10* presented with the wild genotype, exercise-induced responses in resistance at 5HZ by impulse oscillometry were significantly smaller than in those with one or more variant genotypes. These findings are in accordance with our current observations stressing the role of the variant *TLR10* genotypes in the emergence of post-bronchiolitis asthma. The differences

between the German[12] and Finnish cohorts may be due to different asthma phenotypes, allergic asthma in the German study, and post-bronchiolitis asthma in the current study.

The German study reported that primary cells derived from carriers of protective *TLR1*, *TLR6*, and *TLR10* variants showed augmented inflammatory responses, increased Th1 cytokine expression, and reduced Th2-associated IL-4 production after specific stimulation.[12] The suppressed secretion of allergy-related cytokines, like IL-4, IL-15, and IL-13, seems to be associated with asthma phenotypes not related to allergy.[29]

We found preliminary evidence that the role of the *TLR7* gene in asthma may differ between girls and boys, which may be explained by the situation of the *TLR7* rs179008 in the X chromosome.[30] A recent experimental study reported that a *TLR7* gene defect and early pneumovirus infection in mice interacted with each other, first leading to a severe bronchiolitis-like disease, then to Th2-dominated immunity, and finally to an asthma-like pathology.[31] A Danish study revealed that *TLR7* rs179008 polymorphism was associated with asthma.[15] An American study found that TLR7 was expressed in human airway nerves and contributed to relaxation of the airways via the production of nitric oxide.[16] Therefore, normal TLR7 function may be protective against airway hyper-reactivity and asthma, whereas *TLR7* polymorphism may predispose to asthma, and our observations suggest that the influence is greater in girls than in boys.

Polymorphism in the *TLR8* rs2407992 gene had no association with current or earlier asthma, atopic dermatitis, or allergic rhinitis. This finding contradicts the results of the Danish study,[15] in which the same *TLR8* polymorphism was associated with asthma, atopic dermatitis, allergic rhinitis, and elevated allergen-specific immunoglobulin E. A Swedish case-control study found an association between *TLR8* gene variation and allergic rhinitis in adults.[9]

Polymorphisms in the *TLR3* rs3775291, *TLR4* rs4986790, or *TLR9* rs187084 genes were not associated with current or earlier asthma or allergies. A previous study from the present cohort offered preliminary evidence that the wild *TLR3* rs3775291 genotype increased the risk for repeated post-bronchiolitis wheezing.[32] The negative result of the present study at the preschool age was due, at least partly, to the more beneficial outcome of subjects hospitalised for RSV bronchiolitis compared to subjects hospitalised for rhinovirus bronchiolitis. Herein, subsequent asthma was more common after rhinovirus bronchiolitis than after RSV bronchiolitis.[2, 33] Recent meta-analyses have failed to find any associations between *TLR4* rs4986790 polymorphism and asthma,[34] and between *TLR9* rs187084 polymorphisms and asthma.[35]

There were certain limitations in the present study. The number of patients was relatively small for genetic studies. In addition, blood samples for genetic studies were not available from all cases with sufficient follow-up data available, although we consider the 81% proportion as acceptable. The small number of patients means a risk of type-2 statistical error. On the other hand, we carried out multiple analyses for polymorphisms of six different TLR-encoding genes, which means a risk of type-1 statistical error. We considered our study as an exploratory study aimed at finding preliminary evidence for associations, if present, which needs to be confirmed or rejected in future confirmatory studies. Therefore, and because only two polymorphisms were associated with asthma risk, we did not regard any multiplicity adjustments as necessary.[36, 37] Multivariate logistic regression was used, allowing adjustments with early-life risk factors and current confounding factors, but the power of the study was not sufficient for incorporation of all six polymorphisms in the same model.

The strengths of the present study are the prospective design, relatively long follow-up period of six years, extensive virological test panel available during hospitalisation for bronchiolitis, and careful data collection during bronchiolitis and subsequent control visits. The homogeneity of study populations, as in the present study, is a clear benefit for genetic studies. We consider the revealed

significant association between *TLR10* polymorphism and current asthma at preschool age a reliable finding, since the MAF figures were the same, 0.08, in both cases and population-based controls from the FIN data.[20] Since *TLR7* and *TLR8* genes are located in the X chromosome, the analyses were carried out separately for both sexes, and indeed, the findings seemed to be different in girls and boys. RSV caused over 70% of the bronchiolitis cases, but subsequent asthma was more common after rhinovirus bronchiolitis than after RSV bronchiolitis.[2] Therefore, the final analyses on the role of *TLR10* rs4129009 were performed with multivariate logistic regression adjusted for age, sex, early-life risk factors, and current risk factors for asthma, including the RSV aetiology of early-life bronchiolitis, and the conclusions did not change.

In conclusion, polymorphism in the *TLR10* gene seems to increase the risk of post-bronchiolitis asthma in preschool-aged children, and polymorphism in the *TLR7* gene seems to increase the risk of post-bronchiolitis asthma in preschool-aged girls. This result was rather strong, since the associations were robust to adjustments for early-life and current risk factors for asthma.

Methods

The study was conducted at the Department of Paediatrics, Tampere University Hospital, Finland, and the design was previously described.[2] In brief, 166 previously healthy, full-term infants hospitalised for bronchiolitis at less than 6 months of age in 2002–2004 attended a follow-up study in 2008–2010, when the children were 5 to 7 years of age. In infancy, bronchiolitis was defined as an acute lower respiratory illness characterised by rhinorrhea, cough, and diffuse wheezes or crackles.[38] Early-life data were collected by interviewing the parents during hospitalisation using structured questionnaires.[2] This showed that 14.5% of the mothers and 6% of the fathers had asthma, and 29.5% of the children had atopic dermatitis or food allergy. Data on the viral aetiology

of bronchiolitis were studied on admission by antigen detection and polymerase chain reaction (PCR), and a viral infection was identified in most cases: RSV in 70.5% and rhinovirus in 12.7%.[2] During the follow-up study, 127 children attended the clinical control visit, and an additional 39 children returned the study questionnaire. The structured questionnaire completed by the parents of both groups consisted of separate questions on wheezing episodes, asthma diagnoses, and use of bronchodilators and inhaled corticosteroids (ICS) for the preceding 12 months. The presence of atopic dermatitis and allergic rhinitis, night cough in the absence of infections, and prolonged cough for more than four weeks were also charted for the preceding 12 months. The subjects of the present study consisted of those 135 children for whom samples for genetic studies are available.

Definition of asthma

Current asthma was defined as the current use of continuous maintenance medication with ICS for asthma, or suffering from doctor-diagnosed episodes of wheezing, with a prolonged or night cough during the preceding 12 months and with a diagnostic finding in the exercise challenge test (ECT).[2] The ECT consisted of free running outdoors for 8 minutes and measurements of pre- and post-exercise airway resistance by impulse oscillometry (Jaeger, Master Screen IOS, Höchberg, Germany). An increase in resistance of 35% or more at 5Hz was considered to be pathological.[2, 39] Previous asthma before the control visit was defined as the previous use of inhaled ICS as continuous or intermittent maintenance medication for asthma.[2] If the child had either previous or current asthma, the term *asthma ever* was used.

Allergic rhinitis was defined as episodes of watery nasal discharge not accompanied by fever or by other symptoms of respiratory tract infection.[2] Atopic dermatitis was defined by doctor-diagnosed eczema and atopy.[2]

Genotyping

Polymorphisms of *TLR3* rs3775291 (1234 C/T), *TLR4* rs4986790 (1194 A/G), *TLR7* rs179008 **(**171 A/T), *TLR8* rs2407992 (2040 C/G), *TLR9* rs187084 (1486 T/C), and *TLR10* rs4129009 (2322 A/G) were selected due to their evident functional properties. The genotyping of *TLR3* rs3775291 (1234 C/T) was performed by pyrosequencing (PSQ™96MA Pyrosequencer, Biotage, Uppsala, Sweden) using a PSQ™96 Pyro Gold Q96 reagent kit according to the manufacturer's protocol.[32] The genotyping of TLR4 rs4986790 (299 A/G) was performed by the ABIPRISM 7000 Sequence Detection System (Applied Biosystems, CA)[40] supplemented later with pyrosequencing (PSQ™96MA Pyrosequencer, Biotage, Uppsala, Sweden) using a PSQ™96 Pyro Gold Q96 reagent kit.[41, 42] The genotyping of *TLR8* rs2407992 (2040 C/G) was performed in the same manner as described for *TLR3* rs3775291 (1234 C/T). For *TLR7* rs179008 (171 A/T), the PCR products were first purified using the QIA quick PCR Purification Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. PCR products with low deoxyribonucleic acid (DNA) content were eluted to 30µl of elution buffer. After purification, the PCR products were pipetted to a 96-well plate (5µl) together with *TLR7* rs179008 **(**171 A/T) forward primer (1.6µl), and the 96-well plate was sent to the Institute for Molecular Medicine laboratory in Helsinki, Finland, for sequencing, as described recently.[28]

TLR9 rs187084 (1486 T/C) genotyping was performed using BspTI restriction enzyme (ThermoFhisher Scientific, Waltham, USA) for digestion of the PCR product.[28] High-resolution melting analysis (HMR) (Roche Diagnostics Light Cycler 480, Basel, Switzerland) was used for genotyping of *TLR10* rs4129009 (2322 A/G), as described previously.[28] There were 135 samples available for genotyping of the *TLR3*, *TLR4*, *TLR7*, and *TLR8*. For the genotyping of the *TLR9* and *TLR10*, 134 samples were available.

Ethics

The study was carried out in accordance with the approved guidelines of the WMA Declaration of Helsinki. The protocol of the study was approved by the Ethics Committee of the Tampere University Hospital District, Tampere, Finland. Before we enrolled the children, we obtained informed parental consent, including the use of samples for genetic studies on bronchiolitis and asthma risk, during both hospitalisation and the control visit. The personal data of the study subjects were not given to the two laboratories that performed the genetic studies, the National Institute of Health and Welfare, Turku, Finland, or the Institute for Molecular Medicine, Helsinki, Finland.

Statistics

Statistical analyses were carried out with SPSS version 23.0 software (IBM Corp, NY, USA). The chi-square test and Fisher`s exact test were used, when appropriate, to analyse genotype frequencies between those with and without current asthma, asthma ever, current allergic rhinitis, or current atopic dermatitis. Multivariate analyses were conducted if univariate analyses revealed statistically significant ($p<0.05$) associations. Logistic regression was first adjusted for gender and age, and then for gender, age, maternal asthma, and RSV aetiology of bronchiolitis (early-life risk factors), and further for gender, age, and current atopic dermatitis (current confounders). Finally, age, gender, early-life risk factors, and current confounders were included in the same model. The results were expressed as OR and 95% CI. The FINETTI programme was used to evaluate the Hardy–Weinberg equilibrium (HWE) of the studied *TLR3*, *TLR4*, *TLR9*, and *TLR10* alleles, and they were in the HWE. Since *TLR7* and *TLR8* genes are located in the X chromosome, the HWE was not studied, and males and females were analysed separately.

References

1. Piippo-Savolainen, E. & Korppi, M. Wheezy babies — wheezy adults? Review on long-term outcome until adulthood after early childhood wheezing. *Acta. Paediatr.* **97,** 5-11 (2008).

2. Koponen, P., Helminen, M., Paassilta, M., Luukkaala, T. & Korppi, M. Preschool asthma after bronchiolitis in infancy. *Eur. Respir. J.* **39,** 76-80 (2012).

3. Message, S. D. & Johnston, S. L. Host defense function of the airway epithelium in health and disease: Clinical background. *J. Leukoc. Biol.* **75,** 5-17 (2004).

4. Reijmerink, N. E. *et al*. Toll-like receptors and microbial exposure: Gene-gene and gene-environment interaction in the development of atopy. *Eur. Respir. J.* **38,** 833-840 (2011).

5. Daley, D. *et al*. Associations and interactions of genetic polymorphisms in innate immunity genes with early viral infections and susceptibility to asthma and asthma-related phenotypes. *J. Allergy Clin. Immunol.* **130,** 1284-1293 (2012).

6. Koponen, P. *et al*. Polymorphism of the rs 1800896 IL 10 promoter gene protects children from post-bronchiolitis asthma. *Pediatr. Pulmonol.* **49,** 800-806 (2014).

7. Rämet, M., Korppi, M. & Hallman, M. Pattern recognition receptors and genetic risk for RSV infection: Value for clinical decision-making? *Pediatr. Pulmonol.* **46,** 101-110 (2011).

8. Hewson, C. A., Jardine, A., Edwards, M. R., Laza-Stanca, V. & Johnston, S. L. Toll-like receptor 3 is induced by and mediates antiviral activity against rhinovirus infection of human bronchial epithelial cells. *J. Virol.* **79,** 12273-12279 (2005).

9. Nilsson, D. *et al*. Toll-like receptor gene polymorphisms are associated with allergic rhinitis: A case control study. *BMC Medic. Genet.* **13,** 66 (2012).

10. Koponen, P. *et al*. The association of genetic variants in toll-like receptor 2 subfamily with allergy and asthma after hospitalization for bronchiolitis in infancy. *Pediatr. Infect. Dis. J.* **33,** 463-466 (2014).

11. Qian, F. *et al*. Polymorphisms in the *Toll-like receptor 2* subfamily and risk of asthma: a case-control analysis in a Chinese population. *J. Investi. Allergol. Clin. Immunol.* **20,** 340-346 (2010).

12. Kormann, M. *et al*. Toll-like receptor heterodimer variants protect from childhood asthma. *J. Allergy Clin. Immunol.* **122,** 86-92 (2008).

13. Reuter, S. *et al*. TLR3 but not TLR7/8 ligand induces allergic sensitization to inhaled allergen. *J. Immunol.* **188,** 5123-5131 (2012).

14. Rallabhandi, P. *et al*. Respiratory syncytial virus fusion protein-induced toll-like receptor 4 (TLR4) signaling is inhibited by the TLR4 antagonists rhodobacter sphaeroides lipopolysaccharide and eritoran (E5564) and require direct interaction with MD-2. *MBIO* **3,** e00218 (2012).

15. Moller-Larsen, S. *et al*. Association analysis identifies TLR7 and TLR8 as novel risk genes in asthma and related disorders. *Thorax* **63,** 1064-1069 (2008).

16. Drake, M. G. *et al*. Toll-like receptor 7 rapidly relaxes human airways. *American Journal of Respiratory and Critical Care Medicine* **188,** 664-672 (2013).

17. Roponen, M. *et al*. Toll-like receptor 7 function is reduced in adolescents with asthma. *Eur. Respir. J.* **35,** 64-71 (2010).

18. Mansson, A. & Cardell, L. Role of atopic status in Toll-like receptor (TLR)7- and TLR9-mediated activation of human eosinophils. *J. Leukoc. Biol.* **85,** 719-727 (2009).

19. Oosting, M. *et al*. Human TLR10 is an anti-inflammatory pattern-recognition receptor. *Proc. Natl. Acad. Sci. USA* **111,** E4478-E4484 (2014).

20. 1000 Genomes Project Consortium *et al*. An integrated map of genetic variation from 1,092 human genomes. *Nature* **491,** 56-65 (2012).

21. Misch, E. & Hawn, T. Toll-like receptor polymorphisms and susceptibility to human disease. *Clin. Sci. (Lond)* **114,** 347-360 (2008).

22. Lin, Y., Verma, A. & Hodgkinson, C. Toll-like receptors and human disease: lessons from single nucleotide polymorphisms. *Curr. Genomics* **13,** 633-645 (2012).

23. Lee, W. *et al*. Stronger toll-like receptor 1/2,4, and 7/8 but less 9 responses in peripheral blood mononuclear cells in non-infectious exacerbated asthmatic children. *Immunobiol.* **218,** 192-200 (2013).

24. Guan, Y. *et al*. Human TLRs 10 and 1 share common mechanisms of innate immune sensing but not signaling. *J. Immunol.* **184,** 5094-5103 (2010).

25. He, Y., Peng, S., Xiong, W., Xu, Y. & Liu, J. Association between polymorphism of interleukin-1beta and interleukin-1 receptor antagonist gene and asthma risk: a meta-analysis. *Scientific World Journal* **2015,** 685684 (2015).

26. Lazarus, R. *et al*. TOLL-like receptor 10 genetic variation is associated with asthma in two independent samples. *Am. J. Respir. Crit. Care Med.* **170,** 594-600 (2004).

27. Daley, D. *et al*. Analyses of associations with asthma in four asthma population samples from Canada and Australia. *Hum. Genet.* **125,** 445-459 (2009).

28. Lauhkonen, E. *et al*. Gene polymorphism of toll-like receptors and lung function at five to seven years of age after infant bronchiolitis. *PloS ONE* **11,** e0146526 (2016).

29. Dunican, E. M. & Fahy, J. V. The role of type 2 inflammation in the pathogenesis of asthma exacerbations. *Ann. Am. Thorac. Soc.* **12 Suppl,** 144-149 (2015).

30. Shen, N. *et al*. Sex-specific association of X-linked Toll-like receptor 7 (TLR7) with male systemic lupus erythematosus. *Proc. Natl. Acad. Sci. USA* **107,** 15838-15843 (2010).

31. Kaiko, G. E. *et al*. Toll-like receptor 7 gene deficiency and early-life pneumovirus infection interact to predispose toward the development of asthma-like pathology in mice. *J. Allergy Clin. Immunol.* **131,** 1331-1339 (2013).

32. Nuolivirta, K. *et al*. Toll-like receptor 3 L412F polymorphisms in infants with bronchiolitis and postbronchiolitis wheezing. *Pediatr. Infect. Dis. J.* **31,** 920-923 (2012).

33. Lodge, C. J. *et al*. Early-life risk factors for childhood wheeze phenotypes in a high-risk birth cohort. *J. Pediatr.* **164,** 289-294 (2014).

34. Chen, S. Association between the TLR4 +896A>G (Asp299Gly) polymorphism and asthma: A systematic review and meta-analysis. *J. Asthma.* **49,** 999-1003 (2012).

35. Tizaoui, K., Kaabachi, W., Hamzaoui, K. & Hamzaoui, A. Association of single nucleotide polymorphisms in toll-like receptor genes with asthma risk: a systematic review and meta-analysis. *Allergy Asthma Immunol. Res.* **7,** 130-140 (2015).

36. Bender, R. & Lange, S. Adjusting for multiple testing—when and how? *J. Clin. Epidemiol.* **54,** 343-349 (2001).

37. Streiner, D. L. Best (but oft-forgotten) practices: the multiple problems of multiplicity — whether and how to correct for many statistical tests. *Am. J. Clin. Nutr.* **doi:10.3945/ajcn.115.113548** (2015).

38. AAP, American Academy of Pediatrics. & Subcommittee on Diagnosis and Management of Bronchiolitis. Diagnosis and management of bronchiolitis. *Pediatrics* **118,** 1774-1793 (2006).

39. Malmberg, L. P., Mäkelä, M. J., Mattila, P. S., Hammaren-Malmi, S. & Pelkonen, A. S. Exercise-induced changes in respiratory impedance in young wheezy children and nonatopic controls. *Pediatr. Pulmonol.* **43,** 538-544 (2008).

40. Helminen, M. *et al*. IL-10 gene polymorphism at -1082 A/G is associated with severe rhinovirus bronchiolitis in infants. *Pediatr. Pulmonol.* **43,** 391-395 (2008).

41. Gröndahl-Yli-Hannuksela, K. *et al*. Gene polymorphisms in toll-like receptor 4: Effect on antibody production and persistence after acellular pertussis vaccination during adolescence. *J. Inf. Dis.* **205,** 1214-1219 (2012).

42. Vuononvirta, J. *et al*. Nasopharyngeal bacterial colonization and gene polymorphisms of mannose-binding lectin and toll-like receptors 2 and 4 in infants. *PloS ONE* **6,** e26198 (2011).

Authors' contributions

ST and PK had responsibility for patient screening, data analysis, and writing the manuscript.

JV, JT, and QH had responsibility for the genetic analysis and participated in writing the manuscript.

MH participated in the protocol development and in writing the manuscript.

MK and KN were responsible for protocol development, planning, and interpretation of the analyses, and they participated in writing the manuscript.

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SNP (Major>Minor)	Major/ Major	Major/ Minor	Minor/ Minor	MAF	FIN
TLR3 rs3775291 (C>T)	0.46	0.41	0.13	0.33	0.33
TLR4 rs4986790 (A>G)	0.84	0.16	0	0.08	0.12
TLR7 rs179008 (A>T)	0.61 (girls)	0.35 (girls)	0.04 (girls)	0.22 (girls and boys)	0.31 (girls and boys)
	0.79 (boys)	0	0.21 (boys)		
TLR8 rs2407992 (G>C)	0.35 (girls)	0.48 (girls)	0.17 (girls)	0.45 (girls and boys)	0.36 (girls and boys)
	0.79 (boys)	0	0.49 (boys)		
TLR9 rs187084 (T>C)	0.32	0.43	0.25	0.46	0.45
TLR10 rs4129009 (A>G)	0.84	0.15	0.01	0.08	0.08

Table 1. Genotypes and minor allele frequencies of genes encoding toll-like receptors 3, 4, 7, 8, 9, and 10 in 135 children hospitalised for bronchiolitis and in the Finnish population. MAF=minor allele frequency, FIN=Finnish MAFs as in [20]. N=135 for *TLR3*, *TLR4*, *TLR7*, and *TLR8*; N=134 for *TLR9* and *TLR10*.

	Asthma ever	Current asthma	Current atopic dermatitis	Current allergic rhinitis
***TLR3* rs3775291** N=135	N=37	N=18	N=46	N=39
Wild CC N=62 (%)	19 (30.6)	8 (12.9)	20 (32.3)	16 (25.8)
Variant CT, TT N=73 (%)	18 (24.7) p=0.45	10 (13.7) p=0.55	26 (35.6) p=0.72	23 (31.5) p=0.57
***TLR4* rs4986790** N=135	N=37	N=18	N=46	N=39
Wild AA N=113 (%)	31 (27.4)	16 (14.2)	39 (34.5)	34 (30.1)
Variant AG, GG N=22 (%)	6 (27.3) p=1.00	2 (9.1) p=0.74	7 (31.8) p=1.00	5 (22.7) p=0.61
***TLR9* rs187084** N=134	N=37	N=18	N=46	N=39
Wild TT N=43 (%)	11 (25.6)	4 (9.3)	10 (23.3)	13 (30.2)
Variant TC, CC N=91	26 (28.0) p=0.44	14 (15.4) p=0.25	36 (40.0) p=0.06	26 (28.6) p=0.50
***TLR10* rs4129009** N=134	N=37	N=18	N=46	N=39
Wild AA N=113 (%)	28 (24.8)	12 (10.6)	39 (34.5)	31 (27.4)
Variant AG, GG N=21 (%)	9 (42.9) p=0.08	6 (28.6) p=0.03	7 (33.3) p=0.57	8 (38.1) p=0.23

Table 2. Genotypes of *TLR3* rs3775291, *TLR4* rs4986790, *TLR9* rs187084, and *TLR10* rs4129009 encoding genes in relation to asthma and allergy at preschool age in 135 former bronchiolitis patients

	Asthma ever	Current asthma	Current atopic dermatitis	Current allergic rhinitis
***TLR7* rs179008**	N=14	N=8	N=23	N=17
Females N= 66				
Wild AA	5 (12.5)	3 (7.5)	15 (37.5)	10 (25.0)
N=40 (%)				
Variant AT, TT	9 (34.6)	5 (19.2)	8 (30.8)	7 (27.0)
N=26 (%)	p=0.03	p=0.25	p=0.61	p=1.00
***TLR8* rs2407992**	N=14	N=8	N=23	N=17
Females N=66				
Wild GG	5 (21.7)	2 (8.7)	8 (34.8)	5 (21.7)
N=23 (%)				
Variant GC, CC	9 (20.9)	6 (14.0)	15 (32.6)	12 (26.1)
N=43 (%)	p=1.00	p=0.70	p=1.00	p=0.77
***TLR7* rs179008**	N=23	N=10	N=23	N=22
Males N=68				
Wild, allele A present	18 (33.3)	7 (13.0)	19 (35.2)	19 (35.2)
N= 54 (%)				
Variant, allele T present	5 (37.5)	3 (21.4)	3(21.4)	3 (21.4)
N= 14 (%)	p=1.00	p=0.68	p=0.76	p=0.36
***TLR8* rs2407992**	N=23	N=10	N=23	N=22
Males N=69				
Wild, allele G present	11 (31.4)	6 (17.1)	12 (34.3)	13 (37.1)
N=35 (%)				
Variant, allele C present	12 (35.3)	4 (11.8)	11 (32.4)	9 (26.5)
N=34 (%)	p=0.80	p=0.73	p=1.00	p=0.44

Table 3. Genotypes of *TLR7* rs179008 and *TLR8* rs2407992 encoding genes in relation to asthma and allergy at preschool age in 135 former bronchiolitis patients. *TLR7* and *TLR8* are presented separately for females and males. One test result of a *TLR7* male presented AT heterozygote and was deleted from the analyses.

TLR5 rs5744174 gene polymorphism is associated with the virus etiology of infant bronchiolitis but not with post-bronchiolitis asthma

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Key words: innate immunity, respiratory syncytial virus, single nucleotide polymorphism, Toll-like receptor 5

Abbreviated title: *TLR5* gene polymorphism in infant bronchiolitis

Abstract

Background and aim: Bronchiolitis is a leading cause of hospitalization in infants and is associated with a risk of subsequent asthma. The innate immunity genes, such as those encoding toll-like receptors (TLRs), are likely to play a role in bronchiolitis and post-bronchiolitis outcome. Thus far, only one study has considered *TLR5* genes in respiratory syncytial virus (RSV) bronchiolitis. The aim of this study was to investigate the association of *TLR5* gene polymorphism with virus etiology and severity of bronchiolitis, and with post-bronchiolitis asthma.

Methods: We recruited 164 infants (age < 6 months) hospitalized for bronchiolitis in this study, and determined *TLR5* rs5744174 (C>T) single nucleotide polymorphism, virus etiology and severity markers of bronchiolitis, and presence of post-bronchiolitis asthma until age 11-13 years.

Results: RSV was detected in 113 (68.9%), rhinovirus in 19 (11.6%) and some other virus in 20 (12.2%) cases. Non-RSV etiology was more common among infants with the variant CT or TT genotype in the *TLR5* rs5744174 gene than in those with the CC genotype (89.7% vs. 71.7%, p=0.03). *TLR5* rs5744174 polymorphism was not associated with the need of supplementary oxygen or feeding support, with the length of hospital stay, or with post-bronchiolitis asthma at any age.

Conclusion: The *TLR5* rs5744174 variant genotype may increase the susceptibility to bronchiolitis not caused by RSV.

Introduction

Asthma is a chronic inflammatory disease of the airways, usually presenting with the dominance of Th2-type cytokines [1]. Both genetic susceptibility and environmental factors, such as early-life virus infections, contribute to the development of asthma. Still, it remains unclear which abnormalities in the innate immunity-based host defense, such as cytokine misproduction, are hereditary, and which develop later due to environmental stress factors, such as infections [2].

Bronchiolitis is the most common lower respiratory tract infection (LRTI) requiring hospitalization in young children [3]. Among the various respiratory viruses causing bronchiolitis, respiratory syncytial virus (RSV) is the single most important one, especially in the youngest children [4,5]. The clinical course of primary RSV infection is highly variable, and genetic variations in genes regulating the immune response certainly influence disease severity [6]. Bronchiolitis in infancy is associated with subsequent wheezing in early childhood and asthma in later childhood [7-9]. The link between bronchiolitis and subsequent asthma remains unclear, but it has been suggested that the causative virus modifies the immature immunity towards a Th2-oriented cytokine profile [10].

Toll-like receptors (TLRs) are innate immune molecules that recognize conserved structures of microbial pathogens [11]. They activate immediate and early mechanisms of innate host defense as well as initiate and orchestrate adaptive immune responses [11]. Several single-nucleotide polymorphisms (SNPs) within the *TLR* genes have been associated with altered susceptibility to infectious, inflammatory, and allergic diseases [11,12].

TLRs 1, 2, 4, 5, 6 and 10 are located on the cell surface, directly recognizing microbial components, whereas TLRs 3, 7, 8 and 9 are located inside the cell [13], recognizing microbial components after

endocytosis. TLR5 recognizes bacterial flagellin, which is an important virulence factor of many bacteria [14] and is also found in house dust [15]. The effect of bacterial products in house dust are mainly protective for allergy and asthma [16]. In prior studies, activation of TLR5 by flagellin has been associated with atopic eczema [17] and also with asthma [15], but there are studies with contradictory results reporting that asthmatic patients have decreased expression[18,19] or impaired function of TLR5 [19]. Moreover, polymorphisms in the *TLR5* gene have been described to be associated with both acute and chronic lung diseases, *e.g* pneumonia caused by *Legionella pneumophila* [20] and bronchopulmonary dysplasia in preterm infants [21].

TLR5, as a receptor of bacterial flagellin, is known to be involved in allergy development and subsequent asthma [15]. A Dutch study found preliminary evidence that the association of *TLR5* rs5744174 (Phe616Leu) polymorphism with bronchiolitis risk may be divergent in preterm and term infants [22]. We have previously investigated the associations of SNPs of nine *TLR* genes, not including the *TLR5* gene, with bronchiolitis and post-bronchiolitis outcome [23-27]. The aim of this study was to complete our previous exploratory studies by evaluating the association of *TLR5* rs5744174 gene polymorphism with bronchiolitis in infancy, with post-bronchiolitis wheezing until the age of 18 months, with preschool asthma at the age of five to 7 years, and with childhood asthma at the age of 11 to 13 years.

Materials and methods

Design

The study was conducted at the Department of Pediatrics, Tampere University Hospital, Finland, and the design has previously been described [9]. In brief, 187 previously healthy, full-term infants hospitalized for bronchiolitis at less than 6 months of age in 2001-2004 were eligible for the study.

Of them, 139 attended a follow-up visit in 2002-2004 when the mean age of the children was 18 months. The second follow-up visit was arranged in 2008-2010, at 5 to 7 years of age, to which 166 attended, and the third took place in 2014-2015, at the age of 11-13 years, to which 138 attended. Whole blood samples were obtained for genetic studies in infancy, supplemented at age 5-6 years, if needed. Overall, data on the *TLR5* rs5744174 (C>T) gene polymorphism were available from 164 infants. In infancy, bronchiolitis was defined as an acute lower respiratory illness characterized by rhinorrhea, cough, and diffuse wheezes or crackles [28]. The viral etiology of bronchiolitis was studied by antigen detection and polymerase chain reaction (PCR) in nasopharyngeal aspirates (NPA), as described previously [29]. The studied viruses were RSV, rhinovirus, human metapneumovirus, influenza A and B virus, parainfluenza type 1, 2 and 3 viruses, bocavirus, and adenovirus. Data on disease severity, such as the need for supplementary oxygen and feeding support, and the length of hospital stay (LOS), were recorded during the inpatient care [27].

After hospitalization for bronchiolitis, the children were invited to a follow-up visit at, on average, 1.5 years of age. At the follow-up visit, the parents were interviewed using a structured questionnaire on the occurrence of atopic eczema, wheezing episodes, and the use of corticosteroids for wheezing after hospitalization for bronchiolitis [30]. The parents had recorded all infections and wheezing periods diagnosed by a family doctor or a pediatrician and all given treatments in a diary during the 1.5 years post-bronchiolitis follow-up period. Only doctor-diagnosed episodes were included in the analyses. Repeated wheezing was defined as two or more wheezing episodes during the 1.5-year post-bronchiolitis follow-up period [30].

The follow-up study when the children were 5-7 years old, consisted of medical histories since the last visit at the age of 1.5 years. The data were collected using structured questionnaires, which the parents had completed before the visit, and a clinical examination by a doctor that included an interview, checking the questionnaire data, and an exercise challenge test (ECT) with impulse

oscillometry (IOS) for bronchial hyper-reactivity [9]. Current asthma was defined as the current use of continuous maintenance medication with inhaled corticosteroids (ICS) for asthma, or suffering from doctor-diagnosed episodes of wheezing after the age of 1.5 years, or from prolonged or night cough during the preceding 12 months, with a diagnostic finding in the ECT with IOS [9]. Previous asthma before the control visit was defined as the previous use of ICSs as continuous or intermittent maintenance medication for asthma [9].

The follow-up study, when the children were 11-13 years old, consisted of medical histories since the last visit at the age of 5-7 years. As in previous visits, data were collected using structured questionnaires, which the parents had completed before the visit, and a clinical examination by a doctor that included an interview and checking the questionnaire data. Lung function was measured with flow-volume spirometer Vmax™ Carefusion (Becton, Dickinson and Company, NJ, USA), and a bronchodilation test was performed. Forced expiratory volume in one second (FEV1) was measured before and 15 minutes after salbutamol inhalation (Ventolin Evohaler 0.1mg/dos, GlaxoSmithKline, London, UK) with a spacer and in both times, the best value out of three technically acceptable measurements was considered into the analyses. An increase of 12% in FEV1 was considered as diagnostic for asthma. Current asthma was considered to be present if the child had used ICSs continuously during the last 12 months. It was also considered to be present if the child had suffered from repeated wheezing or from a prolonged cough or night cough for four or more weeks during the last 12 months, and, in addition, had a diagnostic increase of FEV1 in the bronchodilation test. Persistent asthma was considered to be present if the child with current asthma had also had asthma at the last control visit at the age of 5-7 years.

At both the 5-7-year and the 11-13-year visit, the questionnaire consisted of questions concerning doctor-diagnosed atopic eczema, allergic rhinitis, and food allergies at different ages since the last

control visit. It also covered the current use of asthma medication, current symptoms that could suggest asthma, and the current asthma diagnosis, if present.

Genetic studies

High resolution melting analysis (HRMA) (Roche Diagnostics Light Cycler 480, Basel, Switzerland) was used for genotyping of *TLR5* rs5744174 (C>T) polymorphism, as published recently [31]. HRMA is a post-PCR melt analysis method which is based on the detection of changes in fluorescence due to the binding of a double stranded –specific intercalacting fluorescent dye, at different temperatures. Primers (forward 5´-ACCTTCCGTGGAAAGAGAGAA-3´ and reverse 5´-TGCAGACATATATTGTGTGTACCCT-3) were designed with Primer-Blast design tool. Amplicon size was only 70 bp which is small enough to maximize the difference between melting peaks (T_m) in variant genotypes and to avoid the other SNPs [32]. Three samples with known genotypes were used to determine the proper concentration of $MgCl_2$ and annealing temperature (T_a°C) for assay.

Each run reaction (20µl total) consisted of 3µl genomic DNA (~8,0ng/µl) and 17µl of master mix, which includes 10 µl melting master dye (LightCycler 480 High Resolution Melting Master, Product No.04909631001, Roche, Basel, Switzerland), 2.4 µl of $MgCl_2$ with a final concentration of 3 mM, 1 µl of each primer with a final concentration of 0.2µM, and 2.6µl of water. The Master mix provides a final concentration of 3 mM of $MgCl_2$. HRMA reactions were run at 95°C for 10 min followed by 45 cycles amplification at 95°C for 10 s, at 60°C for 10 s and at 72°C for 15 s. After the PCR run, final melting cycle conditions were as outlined by Roche: first heating to 95°C and hold for 1 min and cooling to pre-hold temperature (40°C). Melting interval for collecting

fluorescence from 60°C -95° at ramp rate 0.02°C per second. In each run, known *TLR5* rs5744174 standards (wild type, heterozygote and homozygote) were used.

The 1000 Genome Project FIN data on *TLR5* rs5744174 gene polymorphism was obtained from 99 Finnish subjects [33], and the minor allele frequencies (MAF) were compared between our cases and that FIN data.

Ethics

The study was carried out in accordance with the approved guidelines of the WMA Declaration of Helsinki. The protocol of the study was approved by the Ethics Committee of the Tampere University Hospital District, Tampere, Finland. Before enrolling the children, we obtained informed parental consent, including the use of samples for genetic studies on bronchiolitis and asthma risk, both during hospitalization and at the control visit. The personal data of the study subjects were not given to the laboratory that performed the genetic studies, the Department of Medical Microbiology and Immunology, University of Turku, Finland.

Statistics

Statistical analyses were carried out with SPSS version 23.0 software (IBM Corp, NY, USA). The Chi-square test and Fisher`s exact test were used, as appropriate, for categorized variables. Logistic regression with adjustments for sex and age was used to analyze the association between the *TLR5* rs5744174 genotypes and virus etiology of bronchiolitis. The results were expressed as odds ratios (OR) and 95% confidence intervals (95% CI).

The FINETTI program, version 3.0.5 (http://ihg.gsf.de/cgi-bin/hw/hwa1.pl) was used to evaluate the Hardy-Weinberg equilibrium of the studied *TLR5* alleles, and they were in the Hardy-Weinberg equilibrium.

Results

Hospitalization data

There were 164 (87.7%) patients with available genetic and clinical data during hospitalization. Eighty-three (50.6%) of them were boys. Fifty-seven (34.8%) children needed feeding support and 31 (18.9%), oxygen supplementation during the hospitalization. The mean age of the children was 10.6 weeks (range 1-25 weeks, SD 6.77). The mean LOS in hospital was 4.49 days (range 0-22 days, SD 3.21). The causative virus was RSV in 113 (68.9%) cases (RSV A in 61 and RSV B in 52 cases), rhinovirus in 19 (11.6%), and some other virus in 20 (12.2%) (human metapneumovirus in 6, Influenza A virus in 9, parainfluenza type-3 virus in 4 and adenovirus in 1 case). Thus, non-RSV etiology of bronchiolitis was present in 39 (23.8%) cases. The virus was not identified in 12 (7.3%) cases.

The *TLR5* rs5744174 genotype was CC in 39 (23.8%) cases, variant CT in 84 (51.2%) cases and variant TT in 41 (25%) cases. There was no significant association between sex and the genotypes. Variant genotypes (CT or TT) were more common in infants with bronchiolitis caused by some other virus (non-RSV group) than by RSV (89.7% vs. 71.7%, p=0.03) (Table 1.). The OR was 3.46 (95% CI 1.14-10.52) and when adjusted by age and sex, the aOR was 3.17 (95% CI 1.03-9.74). When RSV A and RSV B were included separately in supplementary analyses, the difference was still clear. The variant genotypes (CT or TT) were more common in infants with bronchiolitis caused by non-RSV (89.7%) than by RSV A (70.5%, p=0.02) or by non-RSV than by RSV B

(73.1%, p=0.04). Concerning RSV A, the OR was 3.66 (95% CI 1.14-11.82) and the aOR was 3.22 (95% CI 0.98-10.62), and concerning RSV B, the OR was 3.22 (95% CI 0.97-10.73) and the aOR 3.01 (95% CI 0.90-10.13).

There were no significant associations between the *TLR5* rs5744174 genotypes and the severity markers of bronchiolitis, such as the need of feeding support, oxygen supplementation, or the LOS (Table 2.).

Comparison with FIN data

The MAF (allele T) in this study population was 0.51 and did not differ from the general Finnish population according to FIN data of the 1000 Genomes Project [33], where the MAF of the *TLR5* rs5744174 (C>T) was 0.50. In the RSV group, the MAF was 0.47 (p=0.47 *vs*. the FIN data) and in the non-RSV group, the MAF was significantly higher, at 0.64 (p=0.03 *vs.* the FIN data) (Table 1).

1.5 years follow-up visit

There were 112 children with clinical data available from the 1.5 years follow-up visit. The *TLR5* rs5744174 genotype was CC in 22 (19.6%) cases and CT or TT (variant) in 90 (80.4%) cases. There were no significant associations between the *TLR5* rs5744174 genotype and post-bronchiolitis wheezing, presence of atopic eczema, or the use of ICS medication (Table 3).

5-7 years follow-up visit

There were 139 children with clinical data available from the 5-7 years follow-up visit. The *TLR5* rs5744174 genotype was CC in 36 (25.9%) cases and CT or TT (variant) in 103 (74.1%) cases.

There were no significant associations between the *TLR5* rs5744174 genotype and prolonged cough, ICS use, current asthma, or presence of atopic eczema or allergic rhinitis (Table 4).

11-13 years follow-up visit

There were 123 children with clinical data available from the 11-13 years follow-up visit. The *TLR5* rs5744174 genotype was CC in 32 (26.0%) cases and CT or TT (variant) in 91 (74.0%) cases. There were no significant associations between the *TLR5* rs5744174 genotypes and prolonged cough, ICS use, current asthma, persistent asthma continuing from preschool age until the latest follow-up visit, or presence of atopic eczema or allergic rhinitis (Table 5.).

Discussion

There are three main results in this study concerning the *TLR5* rs5744174 (C>T) gene polymorphism in bronchiolitis and post-bronchiolitis outcome. First, the *TLR5* rs5744174 variant genotype was associated with non-RSV etiology of bronchiolitis. Second, *TLR5* rs5744174 polymorphism was not associated with severity of bronchiolitis. Third, *TLR5* rs5744174 polymorphism was not associated with post-bronchiolitis wheezing, preschool asthma or current asthma in 11-13 years old children after bronchiolitis in infancy.

Approximately 2-3% of children are hospitalized for bronchiolitis before the age of 12 months [34]. In a recent Finnish study, the figure was 2.6% in infants under 6 months of age [35]. RSV is the most common cause for bronchiolitis, especially among infants less than 12 months, whereas other viruses, especially rhinovirus, become more frequent after that age [4]. Consistent with this, RSV

caused 68.9% of bronchiolitis cases (74.3% of the virus-positive cases) in the present study in infants hospitalized for bronchiolitis at less than 6 months of age. However, bronchiolitis caused by some other virus than RSV was significantly more common among children with the variant genotype in the *TLR5* rs5744174 gene. This result was confirmed with adjusted analyses when infants with non-RSV bronchiolitis were compared to all RSV cases, but the significance was marginally lost when compared with RSV A and RSV B cases separately, though the risk was more than 3-fold in all analyses. Further, the MAF was significantly higher in the non-RSV group (0.64) than in the Finnish population (0.50), according to the FIN data of the 1000 Genome Project [33].

There is some evidence that polymorphisms in the *TLR5* gene are associated with bacterial infections [20,36], but their role in viral infections is less studied. So far, there has been only one study on TLR5 in infant bronchiolitis, where *TLR5* rs5744174, the same SNP as in the present study, did not have any significant effect on the risk of RSV bronchiolitis [22]. In stratified analyses, however, preliminary evidence was found that the influence may be divergent in term and preterm infants with bronchiolitis. No studies are available on the association between *TLR5* genes and bronchiolitis caused by other viruses, or between *TLR5* genes and the post-bronchiolitis outcome.

The *TLR5* rs5744174 site is located in the coding region of the gene, and the SNP results in a missense mutation, where the 616th amino acid of the TLR5 protein, phenylalanine (Phe), is substituted by a leucine (Leu) [37]. Depending on its location, polymorphisms in the encoding gene of TLRs may affect the level of expression or alternatively, lead to an altered binding affinity or to an altered downstream signaling [38]. Subsequently, these changes in the primary defense may lead to attenuated immune responses, and further, to increased susceptibility to infections. In primary human cell cultures, *TLR5* rs5744174 polymorphism was found to attenuate TLR5 signaling in response to bacterial flagellin [39]. In addition to recognizing flagellin, there is preliminary evidence that TLR5 might have immune modulating effects in virus infections. A recent study found that

flagellin-induced activation of TLR5 prevented rotavirus infection in mice through activating innate immunity [40]. Moreover, flagellin has been shown to be effective as an adjuvant in influenza vaccines by triggering TLR5 activity and boosting immune responses [41,42]. In the present study, the *TLR5* rs5744174 variant genotype was associated with an increase in susceptibility to non-RSV bronchiolitis, which may take place via an attenuated function of TLR5.

The data on the role of TLR5 in asthma is inconsistent, and even less is known about the effect of the genetic variants of the *TLR5* rs5744174 gene on asthma susceptibility. In the present study, *TLR5* rs5744174 polymorphism had no significant association with recurrent wheezing or asthma during the longer than 10 years follow-up after bronchiolitis in infancy. This is in agreement with a study of a German population in which no significant associations between *TLR5* rs5744174 polymorphism (the polymorphism studied here), or two other *TLR5* gene polymorphisms, rs5744168 and rs2072493, and childhood asthma, was found [38].

It has been suggested, that in healthy subjects, the activation of TLR5 induces Th1-type immune responses, but in asthmatic patients, the expression and activation of TLR5 and subsequent release of Th1-type and anti-inflammatory cytokines are decreased, and the normal function of TLR5 probably is protective from asthma [19]. In another study, TLR5 expression was decreased only in severe asthmatics [18]. A different study reported contradictory results, proposing that the activation of TLR5 leads to Th2-oriented responses in mice [43]. Further, a few studies have implicated that the effect of contact with bacterial flagellin may be dose-dependent: a small amount of flagellin may promote asthma by priming allergic responses [15], whereas higher amounts may suppress allergic asthma [44].

Compared to other viruses, RSV is associated with more severe symptoms of bronchiolitis, whereas rhinovirus is more commonly associated with post-bronchiolitis asthma than is RSV [45]. In the present study, *TLR5* rs5744174 genotypes did not associate with severity markers of bronchiolitis

during hospitalization, although non-RSV bronchiolitis was more common among children with variant genotypes of the *TLR5* rs5744174 gene. This may be due to the small sample size, as only 39 children had bronchiolitis caused by some other virus than RSV. It is also notable, that a loss-of-function mutation in the ligand-binding domain of *TLR5*, 392STOP, was associated with defective responses to flagellin and increased susceptibility to Legionnaire's disease [20]. This polymorphism is present in 5-10% of Europeans and in 23% of some other populations, and when present in both alleles, leads to a complete TLR5 defect without any general immunodeficiency [46]. Thus, TLR5 seems not to be crucial for host defense, and in case of attenuated or even complete loss of function, other molecules of innate immunity are able to compensate for its lacking function [46].

The strengths of this study are the prospective design and carefully collected data during both the hospitalization and repeated follow-up visits, and a long follow-up time of over 10 years. The homogeneous ethnic background of the study children is also a benefit in genetic studies. One clear limitation of this study, however, is the fact that we were not able to measure the expression of TLR5 or the functionality of the *TLR5* rs5744174 gene. However, the functionality of this polymorphism has been proven in previous studies [39]. The small sample size is a limitation for genetic studies and, therefore, the findings need to be repeated in larger study populations.

In conclusion, the *TLR5* rs5744174 variant genotypes were associated with non-RSV etiology of bronchiolitis, but not with the severity of bronchiolitis or with the incidence of post-bronchiolitis asthma. Thus, the findings of this exploratory study suggest that TLR5 may have a modulating role in virus bronchiolitis but larger confirmatory studies in other populations are needed.

Conflicts of Interest

The authors declare no conflicts of interest.

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References

1. Message SD, Johnston SL. The immunology of virus infection in asthma. *Eur Respir J*. 2001;18(6):1013.

2. Thomsen SF, van der Sluis S, Stensballe LG, et al. Exploring the association between severe respiratory syncytial virus infection and asthma: A registry-based twin study. *Am J Respir Crit Care Med*. 2009;179:1091-1097.

3. Nair H, Nokes DJ, Gessner BD, et al. Global burden of acute lower respiratory infections due to respiratory syncytial virus in young children: A systematic review and meta-analysis. *The Lancet*. 2010;375(9725):1545-1555.

4. Jartti T, Lehtinen P, Vuorinen T, Ruuskanen O. Bronchiolitis: Age and previous wheezing episodes are linked to viral etiology and atopic characteristics. *Pediatr Infect Dis J*. 2009;28(4):311-317.

5. Mansbach JM, Piedra PA, Teach SJ, et al. Prospective multicenter study of viral etiology and hospital length of stay in children with severe bronchiolitis. *Arch Pediatr Adolesc Med*. 2012(166):700-706.

6. Janssen R, Bont L, Siezen CLE, et al. Genetic susceptibility to respiratory syncytial virus bronchiolitis is predominantly associated with innate immune genes. *Journal of Infectious Diseases*. 2007;196(6):826-834.

7. Wennergren G, Amark M, Amark K, Oskarsdottir S, Sten G, Redfors S. Wheezing bronchitis reinvestigated at the age of 10 years. *Acta Paediatr*. 1997;86(4):351-355.

8. Hyvarinen MK, Kotaniemi-Syrjanen A, Reijonen TM, Korhonen K, Korppi MO. Teenage asthma after severe early childhood wheezing: An 11-year prospective follow-up. *Pediatr Pulmonol*. 2005;40(4):316-323.

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

10. Gern JE, Busse WW. The role of viral infections in the natural history of asthma. *J Allergy Clin Immunol*. 2000;106(2):201-212.

11. Medvedev AE. Toll-like receptor polymorphisms, inflammatory and infectious diseases, allergies, and cancer. *J Interferon Cytokine Res*. 2013;33:467-484.

12. Tizaoui K, Kaabachi W, Hamzaoui K, Hamzaoui A. Association of single nucleotide polymorphisms in toll-like receptor genes with asthma risk: A systematic review and meta-analysis. *Allergy Asthma Immunol Res*. 2015;7:130-140.

13. Lin YT, Verma A, Hodgkinson CP. Toll-like receptors and human disease: Lessons from single nucleotide polymorphisms. *Curr Genomics*. 2012;13:633-645.

14. Hayashi F, Smith KD, Ozinsky A, et al. The innate immune response to bacterial flagellin is mediated by toll-like receptor 5. *Nature*. 2001;410:1099-1103.

15. Wilson RH, Maruoka S, Whitehead GS, et al. The TLR5 ligand flagellin promotes asthma by priming allergic responses to indoor allergens. *Nat Med*. 2012;18(11):1705-1710.

16. Valkonen M, Taubel M, Pekkanen J, et al. Microbial characteristics in homes of asthmatic and non-asthmatic adults in the ECRHS cohort. *Indoor Air*. 2018;28(1):16-27.

17. Le TA, Takai T, Vu AT, et al. Flagellin induces the expression of thymic stromal lymphopoietin in human keratinocytes via toll-like receptor 5. *Int Arch Allergy Immunol*. 2011;155:31-37.

18. Shikhagaie MM, Andersson CK, Mori M, et al. Mapping of TLR5 and TLR7 in central and distal human airways and identification of reduced TLR expression in severe asthma. *Clin Exp Allergy*. 2013;44:184-196.

19. Lun SWM, Wong CK, Ko FWS, Hui DSC, Lam CWK. Expression and functional analysis of toll-like receptors of peripheral blood cells in asthmatic patients: Implication for immunopathological mechanism in asthma. *J Clin Immunol*. 2009;29:330-342.

20. Hawn TR, Verbon A, Lettinga KD, et al. A common dominant TLR5 stop codon polymorphism abolishes flagellin signaling and is associated with susceptibility to legionnaires' disease. *J Exp Med*. 2003;198:1563-1572.

21. Sampath V, Garland JS, Le M, et al. A TLR5 (g.1174C > T) variant that encodes a stop codon (R392X) is associated with bronchopulmonary dysplasia. *Pediatr Pulmonol*. 2011;47:460-468.

22. Siezen CLE, Bont L, Hodemaekers HM, et al. Genetic susceptibility to respiratory syncytial virus bronchiolitis in preterm children is associated with airway remodeling genes and innate immune genes. *Pediatr Infect Dis J*. 2009;28:333-334.

23. Tormanen S, Korppi M, Terasjarvi J, et al. Polymorphism in the gene encoding toll-like receptor 10 may be associated with asthma after bronchiolitis. *Sci Rep*. 2017;7(1):2956.

24. Koponen P, Vuononvirta J, Nuolivirta K, Helminen M, He Q, Korppi M. The association of genetic variants in toll-like receptor 2 subfamily with allergy and asthma after hospitalization for bronchiolitis in infancy. *Pediatr Infect Dis J*. 2014;33(5):463-466.

25. Nuolivirta K, Tormanen S, Terasjarvi J, et al. Post-bronchiolitis wheezing is associated with toll-like receptor 9 rs187084 gene polymorphism. *Sci Rep*. 2016;6:31165.

26. Nuolivirta K, Vuononvirta J, Peltola V, et al. Toll-like receptor 2 subfamily genotypes are not associated with severity of bronchiolitis or postbronchiolitis wheezing in infants. *Acta Paediatrica*. 2013;102(12):1160-1164.

27. Nuolivirta K, He Q, Vuononvirta J, Koponen P, Helminen M, Korppi M. Toll-like receptor 3 L412F polymorphisms in infants with bronchiolitis and postbronchiolitis wheezing. *Pediatr Infect Dis J*. 2012;31(9):920-923.

28. Subcommittee on Diagnosis and Management of Bronchiolitis. Diagnosis and management of bronchiolitis. *Pediatrics*. 2006;118:1774-1793.

29. Helminen M, Nuolivirta K, Virta M, et al. IL-10 gene polymorphism at -1082 A/G is associated with severe rhinovirus bronchiolitis in infants. *Pediatr Pulmonol*. 2008;43(4):391-395.

30. Nuolivirta K, Hurme M, Halkosalo A, et al. Gene polymorphism of IFNG +874 T/A and TLR4 +896 A/G and recurrent infections and wheezing in toddlers with history of bronchiolitis. *Pediatr Infect Dis J*. 2009;28:1121-1123.

31. Terasjarvi J, Hakanen A, Korppi M, et al. Rapid detection of functional gene polymorphisms of TLRs and IL-17 using high resolution melting analysis. *Sci Rep*. 2017;7:41522.

32. Gundry CN, Dobrowolski SF, Martin YR, et al. Base-pair neutral homozygotes can be discriminated by calibrated high-resolution melting of small amplicons. *Nucleic Acids Res*. 2008;36(10):3401-3408.

33. The 1000 Genomes Project Consortium. Abecasis, G R, Altshuler D, Auton A, et al. A map of human genome variation from population scale sequencing. *Nature*. 2010;467(7319):1061-1073.

34. Smyth RL, Openshaw PJ. Bronchiolitis. *The Lancet*. 2006;368(9532):312-322.

35. Pruikkonen H, Uhari M, Dunder T, Pokka T, Renko M. Infants under 6 months with bronchiolitis are most likely to need major medical interventions in the 5 days after onset. *Acta Paediatr*. 2014;103(10):1089-1093.

36. Hawn TR, Scholes D, Li SS, et al. Toll-like receptor polymorphisms and susceptibility to urinary tract infections in adult women. *PLoS ONE*. 2009;4:e5990.

37. Sheridan J, Mack DR, Amre DK, et al. A non-synonymous coding variant (L616F) in the TLR5 gene is potentially associated with crohn's disease and influences responses to bacterial flagellin. *PLoS ONE*. 2013;8:e61326.

38. Kormann MSD, Depner M, Hartl D, et al. Toll-like receptor heterodimer variants protect from childhood asthma. *J Allergy Clin Immunol*. 2008;122(1):92.e8.

39. Klimosch SN, Forsti A, Eckert J, et al. Functional TLR5 genetic variants affect human colorectal cancer survival. *Cancer Res*. 2013;73:7232-7242.

40. Zhang B, Chassaing B, Shi Z, et al. Prevention and cure of rotavirus infection via TLR5/NLRC4-mediated production of IL-22 and IL-18. *Science*. 2014;346:861-865.

41. Huleatt JW, Nakaar V, Desai P, et al. Potent immunogenicity and efficacy of a universal influenza vaccine candidate comprising a recombinant fusion protein linking influenza M2e to the TLR5 ligand flagellin. *Vaccine*. 2008;26(2):201-214.

42. Song L, Liu G, Umlauf S, et al. A rationally designed form of the TLR5 agonist, flagellin, supports superior immunogenicity of influenza B globular head vaccines. *Vaccine*. 2014;32:4317-4323.

43. Didierlaurent A, Ferrero I, Otten LA, et al. Flagellin promotes myeloid differentiation factor 88-dependent development of Th2-type response. *The Journal of Immunology*. 2004;172(11):6922-6930.

44. Shim J, Lee SE, Hwang W, et al. Flagellin suppresses experimental asthma by generating regulatory dendritic cells and T cells. *J Allergy Clin Immunol*. 2016;137(2):426-435.

45. Mansbach JM, Clark S, Teach SJ, et al. Children hospitalized with rhinovirus bronchiolitis have asthma-Like Characteristics. *J Pediatr*. 2016;172:204.e1.

46. Wlasiuk G, Khan S, Switzer W, Nachman MW. A history of recurrent positive selection at the toll-like receptor 5 in primates. *Mol Biol Evol*. 2009;26:937-949.

Table 1. *TLR5* rs5744174 genotypes and minor allele T frequencies in relation to virus etiology of bronchiolitis, calculated by Chi-square test or Fisher's exact test.

	RSV	Non-RSV		Rhinovirus		Other virus than RSV or rhinovirus	
	n=113	n=39		n=19		n=20	
Genotype	n (%)	n (%)	p *	n (%)	p *	n (%)	p*
CC n=39	32 (28.3)	4 (10.3)	---	2 (10.5)	---	2 (10.0)	---
CT (variant) n=84	57 (50.4)	20 (51.3)	0.08	10 (52.6)	0.33	10 (50.0)	0.33
TT (variant) n=41	24 (21.2)	15 (38.4)	0.008	7 (36.8)	0.07	8 (40.0)	**0.04**
CT or TT (variant) n=125	81 (71.7)	35 (89.7)	**0.03**	17 (89.5)	0.15	18 (90.0)	0.10
Minor allele T frequency n= 164/328	105/226 (46.5) p=0.47 vs. the FIN data **	50/78 (64.1) **p=0.03** vs. the FIN data		24/38 (63.2) p=0.14 vs. the FIN data		26/40 (65.0) p=0.08 vs. the FIN data	

* *vs.* the genotype CC

** FIN data from the 1000 genomes Project[33]

Table 2. *TLR5* rs5744174 genotypes in relation to the severity markers of bronchiolitis, including the need of feeding support or oxygen supplementation during hospitalization, and the length of hospital stay, calculated by Chi-square test or Fisher's exact test.

	Genotype CC		Genotype CT or TT (variant)		
	n=39		n=125		
Clinical finding	n	%	n	%	P-value
Feeding support n=57	13	33.3	44	35.2	0.83
Oxygen supplementation n=31	78	17.9	24	19.2	0.86
Length of hospital stay (mean, SD)	5.21 (SD 3.88)		4.27 (SD 2.95)		0.11

Table 3. *TLR5* rs5744174 genotypes in relation to clinical outcome at 1.5 years of age after bronchiolitis in infancy, calculated by Chi-square test or Fisher's exact test.

	Wild genotype (CC)		Variant genotype (CT or TT)		
	n=22		n=90		
Clinical finding	n	%	n	%	P-value
Atopic eczema n=15	1	4.5	14	15.6	0.16
Repeated wheezing n=21	4	18.2	17	18.9	0.60
ICS use n=16	3	13.6	13	14.4	0.59

Table 4. *TLR5* rs5744174 genotypes in relation to clinical outcome at 5-7 years of age after bronchiolitis in infancy, calculated by Chi-square test or Fisher's exact test.

	Wild genotype (CC)		Variant genotype (CT or TT)		
	n=36		n=103		
Clinical finding	n	%	n	%	P-value
ICS use n=17	7	19.4	10	9.7	0.13
Current asthma n=15	6	16.7	9	8.7	0.19
Allergic rhinitis n=40	13	36.1	27	26.2	0.26
Atopic eczema n=40	8	22.2	32	31.2	0.31

Table 5. *TLR5* rs5744174 genotypes in relation to clinical outcome at 11-13 years of age after bronchiolitis in infancy.

	Wild genotype (CC)		Variant genotype (CT or TT)		
	n=32		n=91		
Clinical finding	n	%	n	%	P-value
ICS use n=10	3	9.4	7	7.7	0.72
Current asthma n=14	6	18.8	8	8.8	0.13
Persistent asthma n=8	3	9.4	5	5.5	0.43
Allergic rhinitis n=53	11	34.4	42	46.2	0.25

Toll-like receptor 1 and *10* gene polymorphisms are linked to postbronchiolitis asthma in adolescence

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Abbreviated title: Gene polymorphisms in post-bronchiolitis asthma

Abstract

Aim: Toll-like receptors (TLR) are innate immunity molecules and our previous studies found that *TLR1* gene polymorphism was associated with post-bronchiolitis asthma at one to six years of age, as was *TLR10* at five to seven years of age. This study examined any associations at 11-13 years of age.

Methods: This prospective follow-up study was part of an ongoing evaluation of children admitted to Tampere University Hospital Finland for bronchiolitis in 2001-2004 at less than six months of age. We evaluated the association of *TLR1* rs5743618 and *TLR10* rs4129009 polymorphisms with asthma and asthma medication in 125 children aged 11-13 years.

Results: Associations were measured as adjusted odd ratios (aOR) with 95% confidence intervals (95% CI). The variant *TLR1* rs5743618 (aOR 4.04, 95% CI 0-99-13.01) and *TLR10* rs4129009 (aOR 7.02, 95% CI 1.56-31.53) genotypes increased the risk of needing inhaled corticosteroids (ICSs) at 11-13 years of age. The variant *TLR10* genotype (aOR 7.69, 95% CI 1.35-43.95) increased the risk of persistent asthma continuing from five to seven years of age until 11-13 years of age. The results were similar when the combined genotypes were analysed.

Conclusion: Polymorphisms in both the *TLR1* and *TLR10* genes may increase the risk of asthma at 11-13 years after infant bronchiolitis.

Key notes

- Our previous studies showed that *TLR1* and *TLR10* gene polymorphisms were associated with asthma at one to six and five to seven years of age, respectively, following infant bronchiolitis.
- This study followed up 125 cohort members, hospitalised for bronchiolitis at less than six months of age, when they were 11-13.

- We found that variant *TLR1* rs5743618 and *TLR10* rs4129009 genotypes were associated with asthma medication and the variant *TLR10* rs4129009 genotype with persistent asthma.

INTRODUCTION

Toll-like receptors (TLRs) are cell membrane proteins that recognise microbial pattern molecules and play a significant role in regulating innate immune responses and further activating adaptive immune responses (1-3). So far, 10 functional TLRs have been identified in humans (4) and TLRs 1-10 are expressed in cells at all interfaces of the body, as well as in macrophages, neutrophils, dendritic cells and natural killer cells. TLR1, TLR2, TLR6 and TLR10 are considered to form a subfamily because of their close interaction (5). The susceptibility of an individual to infectious, inflammatory, autoimmune and allergic diseases may be changed by alterations in their molecular structure or in the expression of TLRs due to mutations in the encoding genes (6).

Microbial exposure in early childhood is an essential process in shaping adaptive immunity (7) and normal TLR activation could be expected to protect an individual from chronic diseases like asthma. Certain early-life virus infections, especially infant bronchiolitis, are risk factors for subsequent asthma, probably because they modify the balance between Th1-dominated and Th2-dominated immune responses (8). The role of putative genetic factors in the development of asthma, especially those regulating innate immunity, has been the subject of increased research interest in recent years.

Genes encoding the TLR2 subfamily are located on chromosome 4 in close proximity to each other (3) and TLR2 forms heterodimers with TLR1, TLR6 and TLR10 (5). However, unlike other receptors in the subfamily, TLR10 also seems to act as a homodimer (9) and weakens the responses triggered by TLR2 heterodimers (3). Thus, TLR10 is a primarily anti-inflammatory receptor, which

also presents with certain pro-inflammatory effects. Evidence has been published to support an association between polymorphisms in TLR2 subfamily genes and increased asthma risk (5,10,11).

We have prospectively followed 166 Finnish children since they were hospitalised for bronchiolitis when they were less than six months of age (12) and by the age of 11-13, 138 children were still taking part in the study. Our explanatory studies of the polymorphisms of all 10 TLR genes in this cohort showed that *TLR1* rs5743618 polymorphism was associated with asthma at one to six years of age (12) and *TLR10* rs4129009 polymorphism was associated with asthma at five to seven years of age (13). In both polymorphisms, the variant genotype, including the minor allele, was associated with a greater asthma risk. However, asthma may still be transient at these ages or some non-symptomatic cases may relapse (14), which is why it is important to carry out follow-up studies to confirm these findings as patients get older.

Our hypothesis for the present confirmatory study was that the variant genotypes of *TLR1* and *TLR10* gene polymorphisms would be associated with post-bronchiolitis asthma at 11-13 years of age. The aim of this study was to examine the associations between *TLR1* rs5743618 and *TLR10* rs4129009 polymorphisms and asthma at that age in our prospectively followed post-bronchiolitis cohort.

MATERIAL AND METHODS

A total of 166 children have been prospectively followed up after hospitalisation in 2001-2004 for bronchiolitis at less than six months of age in the Department of Pediatrics, Tampere University Hospital, Finland. The detailed study design has previously been described (12). During the subjects' hospitalisation, bronchiolitis was defined as the first episode of lower respiratory infection with rhinitis, cough and audible diffuse wheezes or crackles (15,16). The causative virus was

determined in nasopharyngeal aspirates by antigen detection and polymerase chain reaction. This showed that the respiratory syncytial virus (RSV) caused 117 (70.5%) of the 166 bronchiolitis cases and rhinovirus caused 21 (12.7%) (15,16). Whole blood samples were obtained for genetic studies during hospitalisation and supplemented at later follow-up visits.

Earlier follow-up visits were arranged at the ages of 1.5 years (15) and five to seven years (16) to evaluate the presence of recurrent wheezing, asthma and atopy. At the 1.5-year follow-up visit, data were collected on recurrent wheezing and atopic eczema during infancy. The follow-up study at five to seven years consisted of a structured questionnaire, interview with the doctor and assessment of bronchial reactivity with an exercise challenge test using impulse oscillometry, and in addition, early-life data were supplemented if needed.

The current follow-up assessments took place between 1 June 2014 and 31 January 2015 when the children were 11-13 years old. Before the follow-up visit, the parents completed a structured questionnaire that evaluated the occurrence of doctor-diagnosed atopic eczema, allergic rhinitis and asthma, self-reported symptoms suggestive of asthma and the use of asthma medication since the control visit at 5-7 years of age. The follow-up visit included a medical examination and interview, the questionnaire data was checked and the bronchodilation test was carried out. In all, 138 of 166 (83.1%) children took part in the follow-up visit and blood samples for genetic studies were available from 125 children, who formed the subjects of the present study. Of these, 89 attended the clinical follow-up visit and performed the bronchodilation test and 36 of the parents returned the completed questionnaire.

The outcome events in this study at 11-13 years of age were continuous ICS medication during the preceding 12 months, current asthma and persistent asthma. Asthma was considered to be present if

the child had been on continuous inhaled corticosteroid (ICS) medication or alternatively, had suffered from repeated wheezing and, or, a prolonged cough and, or, night cough for four weeks without a respiratory infection during the preceding 12 months, and in addition, had a diagnostic increase in forced expiratory volume in one second (FEV_1) in the bronchodilation test. Persistent asthma was considered if the child with current asthma also presented with asthma at the last control visit at five to seven years of age.

Bronchodilation test

Lung function was measured with the Vmax Carefusion flow-volume spirometer (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). The best FEV_1 of three technically acceptable measurements were taken into account in the analyses, before and 15 minutes after the inhalation of 400µg salbutamol using a Ventolin Evohaler 0.1mg/dos (GlaxoSmithKline, London, UK). FEV_1 was expressed as the percentage of the mean sex-specific, height-adjusted values in the population namely predicted FEV_1. An increase of 12% or more in the bronchodilation test after inhaling salbutamol was regarded as a positive indicator of reversible airway obstruction.

***TLR1* and *TLR10* genes**

Polymorphisms of the *TLR1* rs5743618 (1805 G>T) and *TLR10* rs4129009 (2322 A>G) genes were respectively determined by the National Institute of Health and Welfare, Turku (*TLR1*), and the Department of Medical Microbiology and Immunology, University of Turku (*TLR10*), Finland, as previously described (17,18). These single nucleotide polymorphisms were selected due to their previously reported functional properties (5,19) and the previous exploratory findings from this cohort (12,13).

Ethics

The study was carried out in accordance with the Declaration of Helsinki and the Ethics Committee of Tampere University Hospital District approved the study. We obtained a written, informed parental consent for all subjects during hospitalisation and at the control visits, which included the use of samples for genetic studies on bronchiolitis and asthma risk. The personal data of the study subjects were not given to the laboratories that performed the genetic studies.

Statistics

The statistical analyses were performed by using SPSS for Windows version 21.0 (IBM Corp, Armonk, North Castle, NY, USA). Logistic regression was used to analyse the genotypes and genotype combinations between those with and without current asthma, persistent asthma, current ICS treatment for asthma, current allergic rhinitis and current atopic eczema. These were initially carried out as univariate analyses and then as multivariate analyses, adjusted for sex, age and early-life risk factors, namely non-RSV aetiology of bronchiolitis and atopic eczema at less than 12 months of age. The results were expressed as odds ratios (OR) and 95% confidence intervals (95% CI).

RESULTS

Basic data

The mean age of the 125 (51.2% boys) subjects in this study was 11.7 years, with a range of 10.31-13.19 years and a standard deviation (SD) of 0.95. We found that 11 (8.8%) children were on continuous ICS medication for asthma and another four (3.2%) had symptoms that suggested asthma and a positive bronchodilation test. Thus, 15 (12.0%) children had current asthma and, of these, nine (7.2%) had also presented with asthma at the control visit at the age five to seven years and were considered to have persistent asthma. In addition, 55 (44.0%) children had doctor-

diagnosed allergic rhinitis with symptoms during the preceding 12 months, 31 (24.8%) children were reported to have atopic eczema and 12 (9.6%) had continued to have food allergies. Only doctor-diagnosed cases were included.

Table 1 presents the genotypes and minor allele frequencies of the *TLR1* rs5743618 and *TLR10* rs4129009 genes in this cohort, and the minor allele frequencies in the Finnish subjects from the 1000 Genomes Project (20). There were no significant differences in the minor allele frequencies between the subjects of the present study and the Finnish controls.

Univariate analyses

Children with the variant genotype GT or TT in *TLR1* rs5743618 had an increased risk of needing continuous ICS medication during the preceding 12 months (OR 3.70, 95% CI 1.04-13.01) compared to the children with the wild genotype GG (Table 2). The presence of the variant *TLR1* genotype increased the risk of current asthma 2.0-fold and the risk of persistent asthma 3.7-fold, but the differences between those with variant and wild genotypes were not statistically significant (Table 2).

Children with the variant genotype AG or GG in *TLR10* rs4129009 had an increased risk for needing continuous ICS medication during the preceding 12 months (OR 5.05, 95% CI 1.38-18.53) compared to the children with the wild genotype AA (Table 2). Likewise, the variant *TLR10* genotype was associated with an increased risk of persistent asthma (OR 4.61, 95% CI 1.12-18.93) (Table 2). The presence of the variant *TLR10* genotype increased the risk of current asthma 2.9-fold, but the difference between those with variant and wild genotypes was not statistically significant (Table 2).

There were no significant associations between the determined polymorphisms and atopic eczema or allergic rhinitis at 11-13 years of age in the present study (data not shown).

Multivariate analyses

Non-RSV aetiology of bronchiolitis in infancy and atopic eczema before the age of 12 months were significantly associated with the outcome of the patients at 1.5 years and five to seven years (16,17). Therefore, we re-analysed the data using multivariate logistic regression for the associations between the *TLR1* variant genotype, the *TLR10* variant genotype, and the combination of both these variant genotypes and the outcomes of ICS use, current asthma and persistent asthma at 11-13 years of age.

When we included age, sex, non-RSV aetiology of bronchiolitis and atopic eczema at less than 12 months of age in the model, the *TLR1* variant genotype marginally lost statistical significance as a risk factor for current ICS use, but the *TLR10* variant genotype retained statistical significance as a risk factor for persistent asthma and current ICS use (Table 3). The combination of the two variant genotypes was significantly associated with persistent asthma (aOR 7.1, 95% CI 1.58-31.9) and current ICS use (aOR 7.7, 95% CI 1.36-44.3). In these adjusted analyses, non-RSV aetiology of bronchiolitis was a significant risk factor for persistent asthma and current ICS use and atopic eczema at age less than 12 months was a significant risk factor for persistent asthma (data not shown).

Ad hoc analyses

We constructed a combined variable of wild (TLR1) + wild (TLR10) + RSV bronchiolitis and 64 (51.2%) cases fell into this group. None of them had persistent asthma, compared to 14.1% of the children who did not fall into this group (p=0.003) and none of them had used an ICS in the last 12

months, compared to 17.2% of the other children (p=0.001). Furthermore, we constructed a combined variable of variant (TLR1) + variant (TLR10) + non-RSV bronchiolitis and found that seven (5.6%) cases belonged to this group. Of these seven cases, three (42.9%) had current asthma, versus 10.2% of the cases who were not in this group (p=0.037), three had persistent asthma (versus 5.1% of the others, p=0.008) and three had used an ICS in the last 12 months (versus 6.8% of the others, p=0.015).

DISCUSSION

Three main results emerged from this study on *TLR1* rs5743618 and *TLR10* rs4129009 gene polymorphisms as predictors of asthma at 11-13 years of age in children who were hospitalised for bronchiolitis at under six months of age. First, having the variant *TLR1* rs5743618 genotype resulted in a 4.0-fold increase for ICS use for asthma during the 12 months before the follow-up visit at 11-13 years and having the variant *TLR10* rs4129009 genotype resulted in a 7.0-fold increase. The use of ICS was a surrogate for severe asthma requiring continuous maintenance medication. Second, having the variant *TLR10* rs4129009 genotype resulted in a 7.7-fold increase for persistent post-bronchiolitis asthma continuing from preschool age until early adolescence. Third, the findings were robust when the analyses were adjusted for early-life risk factors and the aetiology of bronchiolitis and the findings did not change when the combined genotypes were analysed.

TLR1 rs5743618 and *TLR10* rs4129009 are both non-synonymous polymorphisms resulting in amino acid changes in TLR1 and TLR10 proteins, respectively, and further changes in their functions (11,18). TLRs play an important role as gatekeepers of innate immunity, especially in

infants, because of the premature adaptive immunity at that age. TLR1/2 heterodimers were originally described in connection with the recognition of bacteria-derived ligands, but increasing evidence has also emerged about their role in virus infections (21). The specific ligand for TLR10 is not known, but it seems to compete for the same ligands as TLR1 (22). TLR10 is the only receptor in the TLR2 subfamily that is capable of homodimerisation but it also seems to form heterodimers with TLR1 (9). The function of TLR10 is mainly inhibitory, via the production of the Interleukin-1 receptor antagonist, which is a natural inhibitor of cytokine excretion (3,9).

In the present study, 17.6% of the children with variant genotypes in the *TLR1* rs5743618 gene needed continuous maintenance medication for asthma in early adolescence, compared with 5.5% of those with the wild genotype. The same was true for 23.8% of the children with variant genotypes in the *TLR10* rs4129009 gene, compared to 5.8% of those with the wild genotype. The 5.5-5.8% prevalence of asthma in the children with wild *TLR1* or *TLR10* genotypes was about the same as the 7-9% reported for children of the same age in the general population in Finland (23,24). *TLR1* rs5743618 and *TLR10* rs4129009 gene polymorphisms did not differ between bronchiolitis cases and controls from the Finnish data in the 1000 Genomes Project (20). Thus, the *TLR1* and *TLR10* genes do not play a role in bronchiolitis, but they have a specific impact on the development of post-bronchiolitis asthma.

Only a few previous studies have been published on TLR1 in asthma or atopy. In a German study, two polymorphisms in *TLR1* - rs5743595 and rs4833095 - were found to protect from atopic asthma in childhood (5). A 2017 Taiwanese study did not find an association between *TLR1* rs4833095 and asthma or other atopic diseases, but the variant genotype was associated with lower total immunoglobulin E levels in allergic children (25). Another *TLR1* polymorphism, namely

rs4543123, was also associated with a decreased risk of atopy in a study that analysed the data from four independent cohort studies. Interestingly, in the polymorphism and virus interaction analyses the same polymorphism seemed to increase the risk for atopy and atopic asthma after an RSV infection before 12 months of age. This interaction with the RSV infection has also been reported in the case of several other polymorphisms in the *TLR1*, *TLR6* and *TLR10* genes, respectively (8). Our *ad hoc* analyses highlighted the fact that *TLR1* and *TLR10* gene polymorphisms are risk factors for asthma in adolescence when they are associated with infant bronchiolitis not caused by RSV.

In line with the findings of our study, a case-control study from the US reported that the variant *TLR10* rs4129009 genotype was associated with asthma and the results were repeated in an independent cohort study (10). However, the German study mentioned above reported opposite results and found the same polymorphism (rs4129009) in *TLR10* to be protective for atopic asthma (5). A large Canadian-Australian study aimed to replicate the findings from four genetic association studies on asthma and atopy candidate genes (8). It found a weak association between two *TLR10* polymorphism (rs10776483 and rs11096957*)* and a decreased risk for atopic asthma in one of the studies, but could not replicate the associations in the three others. The rs11096957 polymorphism also retained its statistical significance in the analysis when all four studies were combined (8).

Earlier follow-up studies in this prospective post-bronchiolitis cohort documented the association between *TLR1* rs5743618 polymorphism and asthma at one to six years of age (12), and the association between *TLR10* rs4129009 polymorphism and asthma at five to seven years of age (13). There have not been any other previous studies with regard to *TLR1* rs5743618 and asthma. In fact, we evaluated all 10 TLRs that have been identified to date in these previous exploratory studies (12,13), but the rest of them did not show any associations with post-bronchiolitis asthma at any

age. Therefore, we just included *TLR1* rs5743618 and *TLR10* rs4129009 polymorphisms in this confirmatory study at 11-13 years of age.

It is interesting to note that there were no significant associations between the determined *TLR1* or *TLR10* polymorphisms and atopic eczema or allergic rhinitis in early adolescence in the present study, even though the same *TLR1* polymorphism was associated with atopic eczema at one to six years of age in this cohort (12). At that age, the prevalence of atopic eczema was 31.9% (12), while in the current study it was only 24.8% at the age of 11-13. Thus, the natural decrease in atopic eczema may at least partly explain the current negative result.

The strengths of our study were the prospective design, the homogeneous study population of Finnish origin and the relatively long follow-up time of more than ten years, which enabled us to compare current and earlier findings. In fact, *TLR1* rs5743618 and *TLR10* rs4129009 polymorphisms were selected based on the observations of our previous exploratory studies in this same post-bronchiolitis cohort (12,13). The small sample size was a clear limitation of our study and it may have led to us underestimating the associations between the determined polymorphisms and asthma. On the other hand, we were able to confirm the significant findings of crude analyses by carrying out multivariable analyses adjusted for sex, age and early childhood risk factors. Another limitation was that we did not study the functionality of the single nucleotide polymorphisms that we included by measuring, for example messenger ribonucleic acid expression or protein production. However, it is notable that the *TLR1* rs5743618 and *TLR10* rs4129009 polymorphisms have been shown to be functional in other studies (5,9,19,22).

CONCLUSION

The results of our study suggest that polymorphisms in the *TLR1* and *TLR10* gene may increase the risk of asthma at the age of 11-13 after bronchiolitis in early infancy through modified immune responses. Our patients represented severe bronchiolitis cases, since all were hospitalised at less than six months of age. Better understanding of innate immunity and the genetics behind it may help to identify those children who are at risk of asthma and in need of scheduled follow up after infant bronchiolitis.

ABBREVIATIONS

ICS, inhaled corticosteroid; TLR, toll-like receptors; FEV_1, forced expiratory volume in one second.

REFERENCES

1. Akira S, Takeda K, Kaisho T. Toll-like receptors: Critical proteins linking innate and acquired immunity. *Nat Immunol* 2001;2:675-680.

2. Medzhitov R, Preston-Hurlburt P, Janeway CAJ. A human homologue of the drosophila toll protein signals activation of adaptive immunity. *Nature* 1997;388:394-397.

3. Oosting M, Cheng S, Bolscher JM, Vestering-Stenger R, Plantinga TS, Verschueren IC, et al. Human TLR10 is an anti-inflammatory pattern-recognition receptor. *Proc Natl Acad Sci U S A* 2014;111:E4478-E4484

4. Skevaki C, Pararas M, Kostelidou K, Tsakris A, Routsias J.G. Single nucleotide polymorphisms of toll-like receptors and susceptibility to infectious diseases. *Clin Exp Immunol* 2015;180:165-177.

5. Kormann MSD, Depner M, Hartl D, Klopp N, Illig T, Adamski J, et al. Toll-like receptor heterodimer variants protect from childhood asthma. *J Allergy Clin Immunol* 2008;122:86-92.e8.
6. Medvedev AE. Toll-like receptor polymorphisms, inflammatory and infectious diseases, allergies, and cancer. *J Interferon Cytokine Res* 2013;33:467-484.
7. Strachan DP. Hay fever, hygiene, and household size. *Brit Med J* 1989;299:1259-1260.
8. Daley D, Park JE, He J, Yan J, Akhabir L, Stefanowicz D, et al. Associations and interactions of genetic polymorphisms in innate immunity genes with early viral infections and susceptibility to asthma and asthma-related phenotypes. *J Allergy Clin Immunol* 2012;130:1284-1293.
9. Hasan U, Chaffois C, Gaillard C, Saulnier V, Merck E, Tancredi S, et al. Human TLR10 is a functional receptor, expressed by B cells and plasmacytoid dendritic cells, which activates gene transcription through MyD88. *J Immunol* 2005;174:2942-2950.
10. Qian F, Zhang Q, Zhou L, Jin G, Bai J, Yin K. Polymorphisms in the toll-like receptor 2 subfamily and risk of asthma: A case-control analysis in a Chinese population. *J Invest Allerg Clin Immunol* 2010;20:340-346.
11. Lazarus R, Raby B, Lange C, Silverman EK, Kwiatkowski DJ, Vercelli D, et al. Toll-like receptor 10 genetic variation is associated with asthma in two independent samples. *Am J Respir Crit Care Med* 2004:594-600
12. Koponen P, Vuononvirta J, Nuolivirta K, Helminen M, He Q, Korppi M. The association of genetic variants in toll-like receptor 2 subfamily with allergy and asthma after hospitalization for bronchiolitis in infancy. *Pediatr Infect Dis J* 2014;33:463-466.

13. Törmänen S, Korppi M, Teräsjärvi J, Vuononvirta J, Koponen P, Helminen M, et al. Polymorphism in the gene encoding toll-like receptor 10 may be associated with asthma after bronchiolitis. *Sci Rep* 2017 (in press).

14. .Piippo-Savolainen E, Korppi M. Wheezy babies--wheezy adults? Review on long-term outcome until adulthood after early childhood wheezing. *Acta Paediatr* 2008;97:5-11

15. Nuolivirta K, Hurme M, Halkosalo A, Koponen P, Korppi M, Vesikari T, et al. Gene polymorphism of IFNG +874 T/A and TLR4 +896 A/G and recurrent infections and wheezing in toddlers with history of bronchiolitis. *Pediatr Infect Dis J* 2009;28:1121-1123

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

17. Nuolivirta K, Vuononvirta J, Peltola V, Koponen P, Helminen M, He Q, et al. Toll-like receptor 2 subfamily genotypes are not associated with severity of bronchiolitis or postbronchiolitis wheezing in infants. *Acta Paediatr* 2013;102:1160-1164

18. Lauhkonen E, Koponen P, Vuononvirta J, Teräsjärvi J, Nuolivirta K, Toikka JO, et al. Gene polymorphism of toll-like receptors and lung function at five to seven years of age after infant bronchiolitis. *PLoS ONE* 2016;11:e0146526

19. Hawn TR, Misch EA, Dunstan SJ, et al. A common human TLR1 polymorphism regulates the innate immune response to lipopeptides. *Eur J Immunol* 2007;37:2280-2289

20. The 1000 Genomes Project Consortium. Abecasis,G.R., Altshuler D, Auton A, DePristo MA, Durbin RM, Handsaker RE, et al. A map of human genome variation from population scale sequencing. *Nature* 2010;467:1061-1073

21. Vaidya SA, Cheng G. Toll-like receptors and innate antiviral responses. *Curr Opin Immunol* 2003;15:402-407.

22. Guan Y, Ranoa DRE, Jiang S, Mutha SK, Li X, Baudry J, et al. Human TLRs 10 and 1 share common mechanisms of innate immune sensing but not signaling. *J Immunol* 2010;184:5094-5103.

23. Harju M, Keski-Nisula L, Georgiadis L, Raatikainen K, Räisänen S, Heinonen S. Maternal socioeconomic status and the risk of asthma among offspring. *BMC Public Health*. 2015;15:27

24. Hugg T, Ruotsalainen R, Jaakkola M, Pushkarev V, Jaakkola JK. Comparison of allergic diseases, symptoms and respiratory infections between Finnish and Russian school children. *Eur J Epidemiol*. 2008;23:123-133.

25. Yang CA, Chiang BL. Toll-like receptor 1 N248S polymorphism affects T helper 1 cytokine production and is associated with serum immunoglobulin E levels in Taiwanese allergic patients. *J Microbiol Immunol Infect* 2017;50:112-117.

Table 1. *TLR1* and *TLR10* genotypes and respective minor allele frequencies in 125 children hospitalised for bronchiolitis under the age of six months, compared to Finnish population data.

Gene	SNP[a]	Genotype frequencies		MAF[b] in the study population	MAF[b] in Finnish population[c]
		wild	variant		
TLR1	rs5743618	GG[d] 0.73	GT 0.22 TT 0.05	0.16	0.17
TLR10	rs4129009	AA 0.83	AG 0.16 GG 0.01	0.09	0.09

[a] SNP = single nucleotide polymorphism

[b] MAF = minor allele frequency

[c] Finnish data in the 1000 Genomes Project (20)

[d] In Europe, the *TLR1* rs5743618 wild genotype is GG and worldwide it is TT

Table 2. Univariate logistic regression: wild and variant *TLR1* and *TLR10* genotypes as risk factors for inhaled corticosteroid (ICS) use, current asthma and persistent asthma at 11-13 years of age in 125 children hospitalised for bronchiolitis under the age of six months.

TLR1 rs5743618	**ICS use during last 12 months (n=11)**				**Current asthma (n=15)**				**Persistent asthma (n=9)**			
	n	%	OR	95% CI	n	%	OR	95% CI	n	%	OR	95% CI
GG (wild) n=91	5	5.5			9	9.9			4	4.4		
GT/TT (variant) n=34	6	17.6	**3.69**	**1.04-13.01**	6	17.6	1.95	0.64-6.00	5	14.7	3.75	0.94-14.91

TLR10 rs4129009	**ICS use during previous 12 months (n=11)**				**Current asthma (n=15)**				**Persistent asthma (n=9)**			
	n	%	OR	95% CI	n	%	OR	95% CI	n	%	OR	95% CI
AA (wild) n=103	6	5.8			10	9.7			5	4.9		
AG/GG (variant) n=21	5	23.8	**5.05**	**1.38-18.53**	5	23.8	2.91	0.88-9.63	4	19.0	**4.61**	**1.12-18.93**

Statistically significant ORs are expressed as bolded.

Table 3. Multivariate logistic regression: current inhaled corticosteroid (ICS) use, current asthma, and persistent asthma at 11-13 years of age in relation to *TLR1* and *TLR10* genotypes, adjusted for age, gender and early-life risk factors of non-RSV aetiology of bronchiolitis and atopic eczema under 12 months of age.

***TLR1* and *TLR10* variant genotypes**	**ICS use during last 12 months (n=11)**		**Current asthma (n=15)**		**Persistent asthma (n=9)**	
	OR	95% CI	OR	95% CI	OR	95% CI
TLR1 variant genotype (n=34)	4.04	0.99-16.54	1.95	0.64-6.00	3.75	0.94-14.91
TLR10 variant genotype (n=21)	**7.02**	**1.56-31.53**	3.47	0.95-12.64	**7.69**	**1.35-43.95**
TLR1 and *TLR10* variant genotypes (n=21)	**7.09**	**1.58-31.89**	3.52	0.97-12.80	**7.76**	**1.36-44.37**

Non-RSV aetiology of bronchiolitis at less than six months of age was present in 8/15, 8/11 and 7/9 cases and atopic eczema was present at less than 12 months of age in 8/15, 6/11 and 6/9 cases. Statistically significant ORs are expressed as bolded.