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Annamari Leino

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To my family

ABSTRACT

Annamari Leino (née Koistinen)

The first wheezing episode and the subsequent risk for asthma

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Background: The rhinovirus etiology of acute wheezing and allergic sensitization are important risk factors for asthma. Early interventions with oral corticosteroids have shown a possible potential to decrease asthma risk in some rhinovirus affected subgroups. However, information about the first wheezing episode overall and the effect of the risk factors diagnosed during the first wheezing episode on asthma development and lung function remain limited.

Aims: The aims of this thesis were to study 1) the virus etiology of the first severe wheezing episode and the associations among the virus etiology, atopic characteristics and vitamin D status; 2) the efficacy of prednisolone during the first severe wheezing episode concerning the time to initiation of asthma control medication and 3) lung function 4 years after the first severe wheezing episode.

Methods: In children aged 3-23 months, virus etiology and patient characteristics of the first wheezing episode were studied using laboratory diagnostics, standard parental questionnaires and patient charts. The efficacy of prednisolone was studied in a randomized placebo-controlled trial. During a 4-year follow-up using impulse oscillometry with exercise and bronchodilation tests, lung function was investigated.

Results: Rhinovirus was the most common etiology (76%) of the first wheezing episode and positively associated with atopic characteristics and prolonged coughing. Vitamin D levels of the children were normal and were not associated with virus etiology or atopic characteristics. Children with a high rhinovirus genome load benefitted from prednisolone in terms of longer time to initiation of asthma control medication. Early allergic sensitization was associated with increased airway reactivity at preschool age.

Conclusions: Rhinovirus is a common etiologic agent in the first severe wheezing episode and linked to atopic characteristics. These findings about the efficacy of prednisolone create a basis for planning the early intervention strategies to secondary prevention of asthma. Diagnosing allergic sensitization early is important for predicting the risk of asthma and compromised lung function development.

Keywords: allergic sensitization, asthma, atopy, child, oral corticosteroid, lung function, rhinovirus, virus, vitamin D, wheezing

TIIVISTELMÄ

LL Annamari Leino

Ensimmäinen uloshengitysvaikeuskohtaus ja sen jälkeinen astmariski

Turun yliopisto, Lääketieteellinen tiedekunta, Lastentautioppi, Turun kliininen tohtoriohjelma, Turun yliopistollinen keskussairaala, Lasten ja nuorten klinikka, Turku, Suomi

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Tausta: Rinoviruksen aiheuttama uloshengitysvaikeuskohtaus ja allerginen herkistyminen ovat merkittäviä astman ennaltaehkäisyssä. Jotkin potilasryhmät saattavat hyötyä varhaisessa vaiheessa suun kautta annetusta kortikosteroidilääkityksestä. Ensimmäistä akuuttia uloshengitysvaikeutta ja sen aikaisten riskitekijöiden vaikutusta keuhkojen toimintaan ja astmaan on kuitenkin tutkittu vasta vähän.

Tavoitteet: Tämän väitöstutkimuksen tavoitteena oli tutkia 1) ensimmäisen vaikean uloshengitysvaikeuskohtauksen virusetiologiaa sekä virusetiologian, atooppisten tekijöiden ja D-vitamiinitason keskinäisiä yhteyksiä; 2) ensimmäisen kohtauksen aikana annetun prednisolonilääkityksen vaikutusta astman kehittymiseen ja 3) keuhkojen toimintaa neljä vuotta ensimmäisen kohtauksen jälkeen.

Menetelmät: Ensimmäisestä uloshengitysvaikeuskohtauksesta kärsivien 3-23 kuukauden ikäisten lasten atooppisia ominaisuuksia ja taudin virusetiologiaa sekä taudin vaikeusastetta selvitettiin laboratoriokokein, kyselykaavakkein ja sairauskertomuksia hyödyntäen. Prednisolonin tehoa tutkittiin satunnaistetulla, kontrolloidulla tutkimuksella. Keuhkojen toimintaa tutkittiin neljän vuoden kuluttua oskillometriatutkimuksella.

Tulokset: Rinovirus oli yleisin virus (76 %) ensimmäisessä uloshengitysvaikeuskohtauksessa ja se oli yhteydessä atooppisiin ominaisuuksiin ja pidentyneeseen yskään. D-vitamiinitaso lapsilla oli normaali eikä se ollut yhteydessä atooppisiin tekijöihin tai virusetiologiaan. Lapset, joilla rinoviruksen määrä hengitysteissä oli suuri, hyötyivät prednisolonihoidosta kun vasteena tarkasteltiin aikaa astmalääkityksen aloitukseen. Varhain diagnosoitu allerginen herkistyminen oli yhteydessä lisääntyneeseen keuhkoputkien reaktiivisuuteen leikki-ikäisenä.

Johtopäätökset: Rinovirus on yleinen löydös ensimmäisestä vaikeasta uloshengitysvaikeuskohtauksesta kärsivillä lapsilla ja se on yhteydessä atooppisiin tekijöihin. Löydökset prednisolonin tehosta luovat pohjaa riskiryhmien astman estämiseen tähtäävien interventiotutkimusten suunnittelulle. Allergisen herkistymisen varhainen diagnosointi on tärkeää, jotta voidaan ennustaa astmariskiä ja keuhkofunktion kehittymistä.

Avainsanat: allerginen herkistyminen, astma, atopia, D-vitamiini, keuhkofunktio, kortikosteroidi, lapsi, rinovirus, uloshengitysvaikeus, virus

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ABBREVIATIONS

ANOVA	One-way analysis of variance
API	Asthma predictive index
AR	Airway responsiveness
AV	Adenovirus
B-eos	Blood eosinophil count
CDHR3	Cadherin-related family member 3
CI	Confidence interval
COAST	Childhood Origins of ASThma
CV	coronavirus
EV	Enterovirus
FOT	Forced oscillation technique
Flu	Influenza virus
Fres	Resonance frequency
dRrs/df	Frequency dependency of resistance
HBoV	Human bocavirus 1
HR	Hazard ratio
ICAM-1	Intercellular adhesion molecule-1
ICS	Inhaled corticosteroid
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IOS	Impulse oscillometry
IQR	Interquartile range
ISAAC	International Study of Asthma and Allergies in Childhood
LDLR	Low-density lipoprotein receptor
MAS	Multicenter Allergy Study
MPV	Human metapneumovirus
NAEPP	The National Asthma Education and Prevention Program
NPA	Nasopharyngeal aspirate
OCS	Oral corticosteroid
OR	Odds ratio
PCR	Polymerase chain reaction
PIV	parainfluenza virus
RBM	Reticular basement membrane
RCT	Randomized clinical trial
RR	Risk ratio
Rrs	Resistance
RSV	Respiratory syncytial virus

RT	Reverse transcriptase
RV	Rhinovirus
SD	Standard deviation
Th	T helper cell
TLR	Toll like receptor
Xrs	Reactance
Zrs	Total respiratory impedance
25OHD	25-hydroxyvitamin D

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications, which are referred in the text by the Roman numerals I-IV. The original communications have been reproduced with the permission of the copyright holders.

I Turunen R, Koistinen A, Vuorinen T, Arku B, Söderlund-Venermo M, Ruuskanen O, Jartti T. The first wheezing episode: respiratory virus etiology, atopic characteristics, and illness severity. Pediatr Allergy Immunol. 2014;25:796-803.

II Koistinen A, Turunen R, Vuorinen T, Söderlund-Venermo M, Camargo CA Jr, Ruuskanen O, Jartti T. Vitamin D, virus etiology, and atopy in first-time wheezing children in Finland. Pediatr Allergy Immunol. 2014;25:834-7.

III Koistinen A, Lukkarinen M, Turunen R, Vuorinen T, Vahlberg T, Camargo CA Jr, Gern J, Ruuskanen O, Jartti T. Prednisolone for the first rhinovirus-induced wheezing and 4-year asthma risk: A randomized trial. Pediatr Allergy Immunol. 2017;28:557-63.

IV Leino A, Lukkarinen M, Turunen R, Vuorinen T, Vahlberg T, Camargo CA Jr, Bochkov, Y, Gern, J, Jartti T. Pulmonary function and bronchial reactivity 4 years after the first virus-induced wheezing. Submitted.

Leino (née Koistinen)

1 INTRODUCTION

Before the age of three years, approximately a third of all children suffer from an acute wheezing illness (Taussig et al. 2003, Meissner 2016) and a third of these develop recurrent wheezing (Martinez et al. 1995, Taussig et al. 2003, Kurukulaaratchy et al. 2003, Matricardi et al. 2008). Being diagnosed in 4-9% of children, asthma is one of the most common chronic illnesses. In high risk children with early wheezing, asthma prevalence during later childhood can be 40% (Jackson et al. 2008, Garcia-Garcia et al. 2017a).

Early wheezing, especially induced by rhinovirus (RV) is known to be an important risk factor for recurrent wheezing and asthma (Jartti & Gern 2017). Furthermore, atopic sensitization is also connected to asthma development (Illi et al. 2006, Rubner et al. 2017). The virus etiology and atopic characteristics, as well as other patient characteristics of the first wheezing episode and their effect on later asthma risk and lung function development are however poorly understood. Vitamin D is known to play an important role in immunologic mechanisms, but its association with asthma predicting factors during early childhood is less known (Jones et al. 2015, Jiao & Castro 2015).

Corticosteroid treatment is an important part of asthma control medication, but its efficacy on early wheezing episodes is unclear. In early interventions for preventing asthma, oral corticosteroid treatment has turned out to be promising if concentrated on wheezing that is induced by RV, especially in children with a high RV genome load (Lehtinen et al. 2007, Lukkarinen et al. 2013, Jartti et al. 2015), but these results are inconsistent (Collins & Beigelman 2014). However, concerning the first severe RV-induced wheezing episode and the efficacy of prednisolone on long-term follow-up, data are limited.

Identifying the children who may benefit from interventions in order to retard or prevent asthma is important. Better understanding about the risk factors of recurrent wheezing and asthma may be achieved by determining the virus etiology of the first wheezing episode and its association with the patient characteristics. In this study also the efficacy of prednisolone in the 4-year follow-up was studied. Furthermore, we assessed the lung function four years after the first wheezing episode and compared it with the early patient characteristics.

2 REVIEW OF LITERATURE

2.1 Definitions

2.1.1 Wheezing

Wheezing is defined as a continuous high-pitched sound from airways during expiration accompanied by dyspnea (National Asthma Education and Preventing Program [NAEPP] guidelines 2007). Wheezing can be diagnosed if a reversible expiratory airway obstruction exists, and if the illness cannot be defined as bronchiolitis or asthma. Wheezing indicates narrowing of the airways and a limitation in the expiratory flow. Airway narrowing is caused by bronchospasm, inflammation, mucus secretion and/or tightening of the smooth muscles in the airway wall (de Benedictis & Bush 2017).

According to the clinical picture, wheezing is divided into different phenotypes, such as “transient early”, “recurrent” and “late-onset”. Wheezing is defined as recurrent if it occurs more than once. Earlier, recurrent wheezing episodes were thought to be induced also by other causes such as exposure to allergens or exercise, but later on it has been noticed that viral infection is present in almost all of the wheezing episodes (NAEPP 2007). Sixty percent of children wheeze at least once before the age of six years, but not all of these children develop asthma. Thus, solving the problem of which phenotypes of recurrent wheezing have a higher risk of asthma and who would benefit from early interventions is a challenge (NAEPP 2007, Reddel et al. 2015, Reddy & Covar 2016).

2.1.2 Bronchiolitis

An acute virus-induced infection in bronchioles is called bronchiolitis. In Europe, bronchiolitis diagnosis is usually limited to infants suffering from their first acute wheezing episode before the age of 12 months (Scottish Intercollegiate Guidelines Network [SIGN] 2006, Smyth & Openshaw 2006, Ralston et al. 2014, Tapiainen et al. 2016, Meissner 2016), whereas USA and Canada use an older age limit of 24 months (American Academy of Pediatrics [AAP] 2006, Ralston et al. 2014, Hancock et al. 2017). This variability in definition leads to contradictions in terminology.

Bronchiolitis is a clinical diagnosis and begins with clinical symptoms of an upper respiratory tract infection such as rhinitis, and low-grade fever, which develops in approximately 3 to 5 days into a breathing difficulty with cough, dyspnoea and tachypnoea. Auscultatory findings may include fine crepitation with wide inspiratory crackles and/or expiratory wheezing (Smyth & Openshaw 2006, Hancock et al. 2017). The infection causes inflammation and oedema also in the surrounding tissue (Smyth & Openshaw 2006). Respiratory syncytial virus (RSV) is the most common viral agent in bronchiolitis followed by RV (Marguet et al. 2009, Midulla et al. 2010, Meissner 2016).

2.1.3 Asthma

Asthma is a chronic inflammatory disorder that has airway obstruction variability, increased bronchial responsiveness and increased mucus secretion. These changes lead to recurrent episodes of expiratory wheezing, cough and shortness of breath (SIGN 2006, NAEPP 2007, Papadopoulos et al. 2012, Global Initiative for Asthma [GINA] 2016). At the beginning, the airway obstruction is reversible and is relieved by itself or with medication. Proceeding disease and prolonging inflammation can cause edema, which together with increased mucus secretion, further limits airflow. Inappropriate smooth muscle tissue contraction after an exposure to a stimulus leads to bronchoconstriction. Remodeling changes can also be seen, such as thickening of the basement membrane, smooth muscle growth, revascularization, innervation and disturbances in the epithelial-mesenchymal trophic unit (NAEPP 2007, Papadopoulos et al. 2012).

In young children, diagnosing asthma is difficult. Diagnosis is based on the pattern, frequency and severity of symptoms as well as clinical findings and by eliminating the possibility of other diagnoses (NAEPP 2007, Brand et al. 2014, GINA 2016). Symptoms and findings typical of asthma include expiratory wheezing, prolonged expiration, use of accessory muscles and, in more severe or prolonged cases, chest deformity. Since the disease is variable and can be asymptomatic between episodes, the absence of the findings during the physical examination does not rule out asthma. Signs in the patient history that refer to asthma include prolonged cough and recurrent wheezing caused by viral infections, exercise, exposure to inhaled allergens, cold air or tobacco smoke (NAEPP 2007, Papadopoulos et al. 2012). Starting from preschool age, diagnosis can be ensured by non-invasive lung function tests such as impulse oscillometry. Reversible obstruction typical of asthma can also be diagnosed by a medical treatment test with a course of inhaled or systemic corticosteroid (Beydon et al. 2007).

In small children (<5 years of age), the diagnosis and initiation of asthma control therapy are mainly based on an asthma predictive index (API), which pays attention to symptoms and risk factors, such as atopic eczema, sensitization, parental history of asthma and blood eosinophilia. In very young children aged less than two years even lung function tests are possible but their availability is not common. Medication with inhaled corticosteroid (ICS) treatment is recommended if the child has had asthma-like symptoms that are reduced by the bronchodilator use. Additionally, the child should have had prolonged symptoms requiring symptomatic medication more than two times a week and at least for four weeks, or two episodes requiring systematic corticosteroid treatment during six months. Medication is also recommended if the child has had at least three to four wheezing episodes during the last 12 months lasting at least one day and that has affected sleep in addition to one major risk factor (doctor diagnosed eczema, aeroallergen sensitization or parental asthma) or 2 minor risk factors (wheezing apart from colds, blood eosinophil count [B-eos] >0.4 $x10^9$/L or food sensitization) (NAEPP 2007).

2.2 Epidemiology

2.2.1 Wheezing

Before the age of three years, approximately 30% of children are diagnosed with bronchiolitis or acute wheezing (Martinez et al. 1995, Taussig et al. 2003, Meissner 2016). Of these children, before school-age, 30-40% suffer from recurrent wheezing (Martinez et al. 1995, Taussig et al. 2003, Kurukulaaratchy et al. 2003, Matricardi et al. 2008).

In up to 95% of the cases, virus etiology is involved in bronchiolitis and early wheezing illnesses (Jackson et al. 2008, Marguet et al. 2009, Jartti et al. 2009). At the age of less than 6 months, the most common pathogen is RSV (Hall et al. 2009), at the age of 6-12 months RSV and RV are found equally (Kotaniemi-Syrjänen et al 2003, Kusel et al. 2006, Jartti et al. 2009, Midulla et al. 2010) and, at the age over 12 months, RV is the most common pathogen (Rakes et al. 1999, Kotaniemi-Syrjänen et al 2003, Jackson et al. 2008, Jartti et al. 2009). The overall virus detection rates tend to decrease by age, as in older children, the virus is involved in 80% to 90% of the infections (Jartti et al. 2009).

2.2.2 Asthma

Asthma is one of the most common chronic illnesses in children. Globally, according to the International Study of Asthma and Allergy in Childhood (ISAAC), at the age of 6-7 years, asthma prevalence in children is 11.7% and, at the age of 13-14 years, the prevalence is 14.1% (Mallol et al. 2013). This prevalence differs regionally. In Finland, the overall prevalence of asthma in children is 4-9% (Pekkanen et al. 1997, Hugg et al. 2008, Lai et al. 2009). Asthma is more common in boys (9.3%) than girls (3.8%) (Hugg et al 2008).

Children with early wheezing illnesses have an increased risk of developing asthma. In high risk children who suffer from recurrent wheezing, at the age of 6 to 8 years, asthma prevalence is 30-40% and is associated with virus etiology and atopic tendency (Jackson et al. 2008, Garcia-Garcia et al. 2017a). Because prospective birth cohort studies show that 75% of children who developed asthma had started wheezing by the age of 3 years, persistent wheezing seems to develop at an early age (Martinez et al. 1995, Lau et al. 2003).

2.3 The virus etiology of wheezing

2.3.1 Rhinovirus

Rhinoviruses are a heterogenous group of small, positive-stranded, non-enveloped RNA viruses, which belong to the *Enterovirus* genus in the *Picornaviridae* family. RV was first found in the 1950s (Andrewes et al. 1953) and to date more than 160 RV-types have been detected (Simmonds et al. 2010, McIntyre et al. 2013, Bochkov et al. 2014). First, the groups of rhinovirus-A (RV-A, currently 80 serotypes) and rhinovirus-B (RV-B, currently 32 serotypes) were discovered (Horsnell et al. 1995, Savolainen et al. 2002a, Savolainen et al. 2002b). Until the 21st century, RV diagnostics were mainly based on virus culture. However, the third rhinovirus group, rhinovirus C (RV-C) does not grow in conventional cell culture (Bochkov et al. 2011), which delayed its discovery until 2006 (Lamson et al. 2006). Due to the improvement of the reverse transcriptase polymerase chain reaction (RT-PCR) method, RV-C (currently 65 genotypes) was discovered (Palmenberg et al. 2009, Simmonds et al. 2010, McIntyre et al. 2013, Bochkov & Gern 2016).

Multiple coexisting RV genotypes are widely spread and circulate year-round (Jartti et al. 2008, Rollinger & Schmidtke 2011, van der Zalm et al. 2011a). In the Northern hemisphere, peaks in prevalence are seen in autumn and in late

spring (Jartti et al. 2004, Malmström et al. 2006, Rollinger & Schmidtke 2011). The broad variability of RVs has caused challenges to vaccine development; however, several clinical trials are ongoing (Edwards et al. 2018). The circulation of RV-A and RV-C among individuals is more common than the circulation of RV-B, which makes RV-A and RV-C more common (Lee et al. 2012, Marcone et al. 2014, Turunen et al. 2017). RV spreads through contact (i.e. most commonly in hands) or through aerosol particles. A virus survives on surfaces several days and on healthy skin for a few hours (Winther et al. 2011, L'Huillier et al. 2015). The incubation time of the RV infection is 2-3 days (Lessler et al. 2009). The method of choice in diagnosing RVs is PCR. Serological tests are used for seroepidemiological studies, but they do not have a role in the diagnosis of acute infections. Currently, no rapid antigen detection tests are available for clinical use (Jartti & Gern 2017).

The clinical picture of the RV infection varies from asymptomatic infections to the common cold to otitis media, and also to lower respiratory tract infections such as pneumonia, bronchiolitis, wheezing and asthma exacerbations (Miller et al. 2007, Kieninger et al. 2013, Toivonen et al. 2016). RV causes respiratory symptoms by the slight destruction of airway tissue due to the direct effects of the virus, pro-inflammatory immune responses and upregulation of cellular receptors (Jacobs et al. 2013, Blaas & Fuchs 2016).

The first line defense against RV is the airway epithelium, which is a relatively resistant barrier when healthy and undamaged. RV by itself can disrupt the barrier function (Blaas & Fuchs 2016). To enter cells, RV-A and RV-B use the intercellular adhesion molecule-1 (ICAM-1) or the low-density lipoprotein receptor (LDLR). These receptors are expressed in ciliated and non-ciliated epithelium cells of the airway, and RV further induces ICAM-1 expression in the lower airways (Greve et al. 1989, Jacobs et al. 2013, Blaas & Fuchs 2016). Recent studies show that RV-C may exploit the cadherin-related family member 3 (CDHR3) to enter the cells (Figure 1) (Bochkov et al. 2015, Bønnelykke et al. 2018).

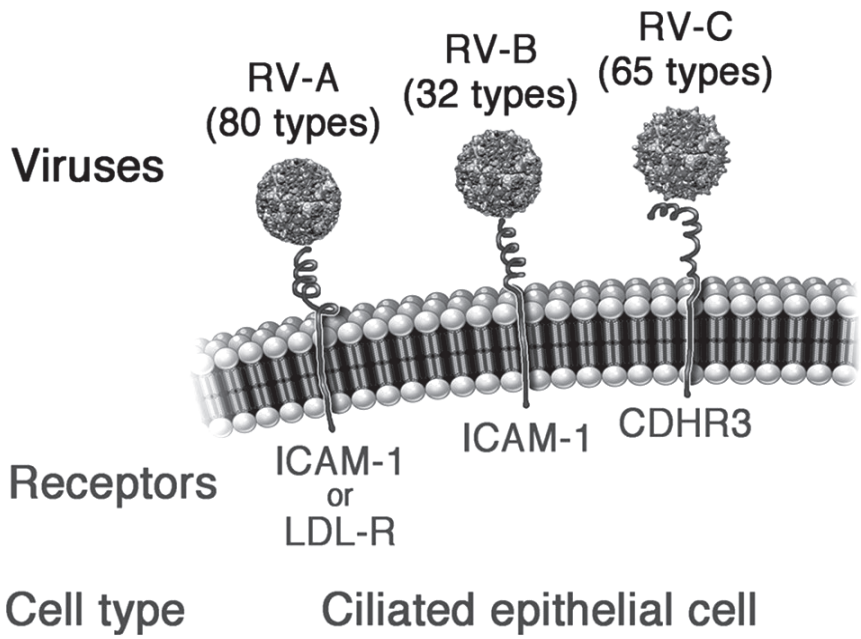

Figure 1 RV interactions with airway epithelial cells. ICAM-1, Intercellular adhesion molecule 1; LDL-R, low-density lipoprotein receptor; CDHR3, cadherin-related family member 3 (Modified from Jartti & Gern 2017).

After binding to the receptor, the virus is internalized into an endosome, which leads to a drop in the pH level and the uncoating of the viral RNA. Translation of the viral proteins and assembly of new viral particles begin. The early innate immune response is started when uncoated viral RNA is recognized by toll-like receptor (TLR) 3 and TLR 7/8. TLR3 activation leads to induction of melanoma differentiation-associated gene-5 and retinoic acid-inducible gene-1. This causes an increase in the expression of type I interferon (IFN)-β and type III IFN-λ which improves antiviral activity. Epithelial cells start to secrete proinflammatory cytokines (e.g. interleukin [IL] 6 and tumor necrosis factor [TNF]-α), chemokines (e.g. IL-8) and growth factors, which induce neutrophils, lymphocytes and eosinophils. This inflammation causes epithelial edema, increased mucus production and leads to airway obstruction and wheezing (Jacobs et al. 2013, Royston & Tapparel 2016). As a sign of the adaptive immune response serotype-specific serum immunoglobulin (Ig) G and IgA antibodies can be detected 1-2 weeks after the incubation (Jacobs et al. 2013).

2.3.2 Respiratory syncytial virus

Respiratory syncytial virus (RSV) is an enveloped, single-stranded RNA virus, which belongs to the *Pneumovirinae subfamily* in the *Paramyxoviridae* family. RSV has two major antigenic groups, A and B (Pangesti et al. 2018). RSV appears seasonally in the Northern hemisphere and peaks in prevalence take place between late autumn and early spring (Rossi & Colin 2015, Obando-Pacheco et al. 2018). In Finland RSV follows regular biannual double-humped pattern (Waris 1991, Gunell et al. 2016).

RSV is the causing agent in almost 80% of the bronchiolitis cases. The incidence of RSV is highest in the age group of <6 months (Mansbach et al. 2012, Meissner 2016, Jartti & Gern 2017). Most of the RSV infections are asymptomatic, but the clinical severity varies (Mansbach et al. 2012). About 20% of the children suffer from RSV bronchiolitis before the age of 1 year. Two to three percent of the children need hospitalization because they have severe symptoms (Smyth & Openshaw 2006, Meissner 2016). Severe infection is more likely to occur in children <3 months of age, born prematurely, and those with immunodeficiency or neuromuscular disorders (Ralston et al. 2014, Meissner 2016, Jartti & Gern 2017). During the first acute wheezing episode, RSV is present in 41-71% of the children (Bosis et al. 2008, Jartti et al. 2009, Midulla et al. 2010). PCR is applicable for RSV detection, albeit rapid antigen detection most often based on fluorescence or enzyme immunoassay is still more commonly used for clinical decision making (Griffiths et al. 2017, Jartti & Gern 2017).

2.3.3 Other viruses

Any virus has been present in the airways of up to 95% of the children suffering from a wheezing episode during their first 3 years of life (Jartti et al. 2004, Jackson et al. 2008, Jartti et al. 2009, Marguet et al. 2009). Besides the two most common virus agents, RV and RSV, human bocavirus 1 (HBoV) is an important pathogen, as it is present in up to 25% of the cases (Jartti et al. 2004, Bosis et al. 2008, Söderlund-Venermo et al. 2009, Deng et al. 2012). Most of the findings have been coinfections. Other noteworthy viruses include metapneumovirus, parainfluenza viruses 1-4, influenza virus A and B, adenoviruses, human coronaviruses 229E, OC43, NL63, HKU1 and enteroviruses. These viruses are present in the airways of 3-21% of the wheezing children (Jartti et al. 2002a, Kotaniemi-Syrjänen et al. 2003, Jartti et al. 2004, Kusel et al. 2006, Jackson et al. 2008, Jartti et al. 2009, Marguet et al. 2009, Midulla et al. 2010).

2.4 The predictive factors for recurrent wheezing and asthma

2.4.1 Virus etiology of the early life wheezing

RV-induced wheezing is an important predicting factor for recurrent wheezing and asthma (Lemanske et al. 2005, Kusel et al. 2007, Jackson et al. 2008, Gern 2009, Lukkarinen et al. 2013, Ruotsalainen et al. 2013, Bergroth et al. 2016, Rubner et al. 2017, Lukkarinen et al. 2017, Backman et al. 2018) and when asthma is diagnosed, RV has been found to be the most common causing agent of exacerbations especially in children (Arden et al. 2010, Jartti & Gern 2017). The Childhood Origins of Asthma (COAST), which is an American birth cohort study of high risk children, demonstrated that children who had outpatient RV-induced wheezing before the age of 3 years had almost a 10-fold risk of developing asthma before the age of 6 years when compared to children who did not wheeze with RV or RSV (odds ratio [OR] 9.8). In the same study, the children who had RSV-induced wheezing during infancy had not an elevated risk of asthma during later childhood when compared to children with no RV- or RSV-induced wheezing (Jackson et al. 2008). Later they extended their findings by demonstrating that the increase in asthma risk remained until the age of 13 years after RV induced wheezing (OR 3.3) but not after RSV-induced wheezing (OR 1.0) (Rubner et al. 2017).

Moreover, an elevated asthma risk after RV-induced wheezing occurs also in population-based studies of children hospitalized for acute wheezing (Kotaniemi-Syrjänen et al. 2003, Midulla et al. 2012, Ruotsalainen et al. 2013, Backman et al. 2018). Recently, Backman et al. reported that the children hospitalized with an RV- and RSV-induced early wheezing episode had an increased risk of asthma even during adulthood (OR 17.0 and 6.1, respectively) when compared to population controls (Backman et al. 2018). This finding is in line with the study of Ruotsalainen et al. with no virus specific analyses, which reported that asthma was currently present in 20% of subjects with the history of bronchiolitis in infancy, whereas the prevalence in controls at the age of 27 years was 5% (Ruotsalainen et al. 2010) (Table 1). However, information focusing on the first RV-induced wheezing episode remains limited.

Table 1 Prospective studies about wheezing illnesses during infancy and subsequent risk of recurrent wheezing and asthma*

Study site (name)	Inclusion criteria	Detected viruses	First author, yr	N	Outcome, age (y)
Borås, Sweden	RSV bronchiolitis, <1 yr, hospitalized	RSV	Sigurs, 1995	47 bronchiolitis, 93 controls	Asthma, 3
			Sigurs, 2000	47 bronchiolitis, 89 controls	Asthma, 7.5
			Sigurs, 2005	46 bronchiolitis, 92 controls	Asthma, 13.4
			Sigurs 2010	46 bronchiolitis, 92 controls	Asthma, 18
Kuopio, Finland	Bronchiolitis, 1-23 mo, hospitalized	RV, RSV, AV, CV, EV, Flu, PIV	Kotaniemi-Syrjänen, 2003	82	Asthma, 7.2
			Hyvärinen, 2005	81	Asthma, 12.3
			Ruotsalainen, 2013	67 bronchiolitis, 155 controls	Asthma, 16.5
			Backman, 2018	49 bronchiolitis, 60 controls	Asthma, 18.8
Kuopio, Finland	Bronchiolitis or pneumonia, ≤ 23 mo, hospitalized	RSV	Korppi, 2004	36 bronchiolitis or pneumonia, 45 controls	Asthma, 18-20
			Ruotsalainen, 2010	59 bronchiolitis, 121 controls	Asthma, 27.3
Avon, United Kingdom (ALSPAC)	RSV bronchiolitis, <12 mo, hospitalization, birth cohort	RSV	Henderson, 2005	73 bronchiolitis, 8039 controls	Asthma, 7.6
Madison, Wisconsin, USA (COAST)	Wheezing, <12 mo, outpatients, high atopy risk, birth cohort	RV, RSV, AV, Flu A and B, PIV, non-RV picornaviruses	Lemanske, 2005	275	Recurrent wheezing, 3-4
			Jackson, 2008	259	Asthma, 6
			Rubner, 2017	217	Asthma, 13
Turku, Finland (Vinku)	First wheezing, 3-23 mo, hospitalized	RV, RSV, AV, CV, EV, Flu, MPV, PIV	Lehtinen, 2007	118	Recurrent wheezing, 2.1
			Lukkarinen, 2013	111	Recurrent wheezing, 8
			Lukkarinen, 2017	127	Atopic/nonatopic asthma, 7.7
Perth, Australia	Wheezing, <12 mo, outpatients, high atopy risk	RV, RSV, AV, CV, MPV, Flu, PIV, non-RV picornaviruses	Kusel, 2007	198	Recurrent wheezing, 5
			Kusel, 2012	147	Asthma, 10
Rome, Italy	First bronchiolitis, <12 mo, hospitalized	RV, RSV, AV, CV, Flu, MPV, PIV	Midulla, 2012	262 with bronchiolitis, 39 controls	Recurrent wheezing, 14 mo
			Midulla, 2014	230	Recurrent wheezing, 3.2
Three centers, Finland (MARC-30)	Bronchiolitis, <24 mo, hospitalized	RV, RSV, AV, CV, Flu, HBoV, MPV, PIV	Bergroth, 2016	365	Asthma, 1.7

AV, adenovirus; CV, coronavirus; EV, enterovirus; Flu, influenza virus; HBoV, human bocavirus; mo, months; MPV, human metapneumovirus, PIV, parainfluenza virus; RSV, respiratory syncytial virus; RV, rhinovirus; yr, year. * Including prospective studies that have used any virus detection. Modified from Jartti & Gern al. 2017.

Wheezing episodes caused by RSV have also been noticed to be associated with recurrent wheezing and asthma development in young children (Sigurs et al. 1995, Korppi et al. 2004, Sigurs et al. 2010, Jartti & Gern 2017, Backman et al. 2018). Tucson study, which is a population-based birth cohort study with healthy infants, reported the association between RSV-induced lower respiratory tract infection before the age of three years and recurrent wheezing (OR 4.3) but not with sensitization (Stein et al. 1999). In line with this study, a British birth cohort study has reported an increased risk of asthma (OR 2.5) at the age of 7.6 years after hospitalization during the first year of life due to RSV bronchiolitis, but they did not find association with the development of sensitization (Henderson et al. 2005). A Swedish prospective study, which included hospitalized children and matched controls, reported that the risk of asthma (risk ratio [RR] 8.7) and allergic rhinitis (RR 2.6) at the age of 13 years was increased also in children who needed treatment in the hospital due to RSV-induced wheezing during their first year of life when compared to healthy controls (Sigurs et al. 2005). This suggests that RSV-associated risk may be stronger depending on illness severity. The same study group reported that the risk remained increased at least until the age of 18 years (Sigurs et al. 2010) (Table 1).

However, the causality of the association between RSV-bronchiolitis and asthma development is uncertain. A large Danish registry-based study with more than 18,000 twins showed a positive association with RSV induced hospitalization and asthma, but modeling the direction of causation showed that RSV infection is more likely an indicator of the genetic predisposition of asthma than a causative agent (Stensballe et al. 2009, Thomsen et al. 2009). Moreover, two recent studies with preterm or high-risk children showed that immunoprophylaxis of RSV with palivizumab decreased the risk of recurrent wheezing but not the risk of atopic asthma (Carroll et al. 2017, Mochizuki et al. 2017).

2.4.2 Atopic characteristics

Diagnosed during early childhood, atopic characteristics are linked to an increased risk for recurrent wheezing and asthma (Hyvärinen et al. 2005, Illi et al. 2006, NAEPP 2007, Lehtinen et al. 2007, Matricardi et al. 2008, Jackson et al. 2008, Just et al 2010, Pescatore et al. 2014, Rubner et al. 2017). The Multicenter Allergy Study (MAS) cohort, a birth cohort of 1,314 children with a follow-up until the age of 13 years, reports that the family history of atopy (OR 2.5 for non-wheezing children and OR 8.3 for wheezing children) and wheezing with sensitization (OR 4.7) before the age of 3 years are associated with persistent wheezing during the ages of 11-13 years (Matricardi et al. 2008). Many subsequent studies

have affirmed these findings by showing the association between early aeroallergen and/or food sensitization and persistent wheezing or asthma at the age of 5 to 13 years (Caudri et al. 2010, Just et al. 2010, Amat et al. 2011, van der Mark et al. 2014, Pescatore et al. 2014, Lukkarinen et al. 2017, Rubner et al. 2017).

Other earlier studies show that the effect of early sensitization as a risk marker for asthma development is the highest if sensitization appears during the first three years of life (Kusel et al. 2007, Sly et al. 2008, Simpson et al. 2010, Stoltz et al. 2013). In the MAS study, they report that asthma risk is 5.5-fold higher in children who still had food sensitization at the age of 2 years when compared to the children whose sensitization disappeared earlier (Kulig et al. 1998). Moreover, the COAST study reports a synergistic effect between allergic inflammation and RV-induced wheezing. They demonstrated that children who had RV infection together with aeroallergen sensitization during the first 3 years of life had the highest risk of developing asthma during later childhood when compared to children with one or none of these characteristics (Jackson et al. 2008).

One possible mechanism underlying the association between allergic sensitization and the risk of asthma is related to immune regulation and type 2 - inflammation. Both, atopic characteristics in children and increased airway reactivity in asthmatic patients are linked to increased interleukin (IL)-13 production (Ingram & Kraft 2012, Lee et al. 2016). Moreover, IL-4 and IL-13 play an important role both in allergic inflammation and airway remodeling (Richter et al. 2001, Cui et al. 2012, Maes et al. 2012, Nie et al. 2013). A T helper cell (Th)1-mediated mechanism may also play a role since reduced IFN-λ production has been linked to a reduction in lung function (Contoli et al. 2006) and, further, Baraldo et al. reports an association between reduced IFN-λ production and elevated serum IgE levels (Baraldo et al. 2012). Increased expression of the high-affinity IgE receptor before RV infection reduces IFN-α and IFN-λ secretion, which strengthens the link between atopic tendency and asthma development (Durrani et al. 2012). Allergic inflammation also causes increased reactivity and mucus secretion in the airways (Kloepfer & Gern 2010).

The synergistic effect of allergic sensitization and RV infection on the increased risk of asthma is partly explained by a weakened epithelial barrier. Allergic inflammation damages the epithelium, which allows increased virus replication. This predisposes to inflammation, and thus may lead to a prolonged and more severe infection (Lopez-Souza et al. 2004, Jakiela et al. 2008, Lachowicz-Scroggins et al. 2010). RV stimulates cytokine secretion, such as thymic stromal lymphopoietin secretion, which further enhances allergic inflammation (Kato et al. 2007, Perez et al. 2014, Mehta et al. 2016, Garcia-Garcia et al. 2017b). RV infection and allergens increase the epithelial production of IL-33, which pro-

motes Th2-type inflammation and decreases Th1-type cytokine production (Mehta et al. 2016, Garcia-Garcia et al. 2017b).

Other atopic characteristics besides the allergic sensitization are associated with the risk of recurrent wheezing and asthma. Eczema is known to be the earliest sign of the classic atopic march, followed by food allergy, allergic rhinitis and finally asthma. Martinez et al. identified eczema as an independent predictor of persistent wheezing (Martinez et al. 1995). Many following studies have confirmed the finding (van der Hulst et al. 2007, Caudri et al. 2009, Pescatore et al. 2014, Neuman et al. 2014). Eosinophilic inflammation together with RV infection have been found to increase the risk for acute wheezing episodes (Midulla et al. 2014, Nicolai et al. 2017). Miller et al. reported maternal atopy to be associated with more severe HRV-associated illness (Miller et al. 2011) and, moreover, parental asthma has been found to be independently associated with recurrent wheezing (Martinez et al. 1995, Kurukulaaratchy et al. 2003, Matricardi et al. 2008, Caudri et al. 2013).

However, also non-atopic asthmatics exist. Earlier studies show that atopic asthma is associated with parental asthma, early eczema and RV-induced wheezing (NAEPP 2007), whereas non-atopic asthma is associated with parental smoking (Rönmark et al. 1999, Goksör et al. 2007, Civelek et al. 2011, Lukkarinen et al. 2017). Therefore it is important to accurately define the phenotype of the early wheezing children so that the possible interventions will be properly chosen.

2.4.3 The role of Vitamin D

Vitamin D is one of the four fat-soluble vitamins and well-known for its role in bone metabolism. Moreover, it also has important immunomodulatory properties (Mullins & Camargo 2012). The recommended minimum level of serum 25-hydroxyvitamin D varies between 50 and 75 nmol/l (Ross et al. 2011, Mullins & Camargo 2012). This level is mostly based on the effects of vitamin D on calcium metabolism and bone mineralization. The recommended supplementary intake level also varies between nationalities. In Finland, the National Nutrition Council recommends to give 10 µg (400 IU) of vitamin D supplement per day year-round for children <2 years of age and 7.5 µg (300 IU) per day for older children. Nevertheless, the serum vitamin D level appears to be inadequate in even up to 30% of the Finnish children (Jartti et al. 2010a, Viljakainen et al. 2011, Määttä et al. 2017). In other Western populations, vitamin D deficiency is diagnosed in 10-15% of the children (Mullins & Camargo 2012). Whether the used cut-off levels of the serum are applicable to other physiological actions than bone metabolism remains unknown (Jiao & Castro 2015).

Vitamin D is known to have significant effects on immune function (Cantorna et al. 2004, Jones et al. 2015) but the results on the role that vitamin D plays in the risk of recurrent wheezing and asthma are inconsistent (Mak & Hanania 2011, Jiao & Castro 2015). Earlier studies have found lower vitamin D levels from children with recurrent wheezing when compared to healthy controls (Demirel et al. 2014, Uysalol et al. 2014, Özdemir et al. 2016, Dogru & Seren 2017). Dogru et al. reported a mean vitamin D level of 54 nmol/l in children with recurrent wheezing, which was significantly lower when compared to healthy controls (mean of 63 nmol/l). Furthermore, low vitamin D levels are linked to decreased lung function (Chinellato et al. 2011, Brehm et al. 2012, Yao et al. 2014) or to increased airway reactivity (Chinellato et al. 2011, Määttä et al. 2017) in children with or without asthma. However, the results on this association are contradictory. In a study with asthmatic children aged 6-18 years, Dabbah et al. (Dabbah et al. 2015) found no association between vitamin D levels and airway reactivity.

Vitamin D deficiency is associated with a higher rate of exacerbations in children with recurrent wheezing or asthma (Brehm et al. 2012, Dogru et al. 2014, Beigelman et al. 2014). Brehm et al. reported a 2.6-fold risk of asthma exacerbation in children aged 6-14 years with vitamin D serum level ≤75 nmol/l (Brehm et al. 2012). However, several clinical trials have not shown consistently the protective effect of vitamin D supplementation of 500-2000 IU/day on asthma control (Urashima et al. 2010, Majak et al. 2011, Bar Yoseph et al. 2015). Interestingly, a Canadian study with children aged 6–12 years showed that both low (≤49 nmol/l) and high (≥75 nmol/l) levels of serum vitamin D were related to an increased risk of current wheezing (OR 3.3 and OR 2.1, respectively) and decreased lung function, suggesting a nonlinear association of vitamin D level with immune response and respiratory disease (Niruban et al. 2014).

2.4.4 Exposure to smoking

Exposure to tobacco smoke during infancy and especially maternal smoking during pregnancy are known to affect the growth of airway structures, lung function and the risk of asthma and airway reactivity during childhood (Le Souef 2000, Goksör et al. 2007, Carlsen & Carlsen 2008, Kalliola et al. 2013, GINA 2016). Maternal smoking during pregnancy is an independent risk factor for recurrent wheezing and asthma (den Dekker et al 2015, Vardavas et al. 2016). Continued maternal smoking, but not only first trimester smoking, during pregnancy was associated with early (OR 1.2) and persistent wheezing (OR 1.5) and asthma (OR 1.7) in a population based prospective study (den Dekker et al. 2015). In addition, in another prospective study which included 1,737 pregnant women, the

children, whose mothers continued smoking beyond the first trimester, had reduced lung function and an increased need for asthma therapy at the age of 5 years (OR 2.2) (Prabhu et al. 2010). These findings underline the importance of smoking cessation during the first trimester considering the lung development of the fetus. Thus, it is noteworthy that 25-50% of women who smoke continue smoking throughout their pregnancy despite the known risks (Smedberg et al. 2014, Alshaarawy & Anthony 2015, Cooper et al. 2017).

Tobacco smoke exposure during early childhood has also been linked to an increased risk of wheezing, decreased lung function and asthma (Martinez et al. 1995, Burke et al. 2012, den Dekker et al. 2015, Vardavas et al. 2016). Furthermore, children suffering from wheezing have been shown to have poorer lung function when maternal smoking was present (Kalliola et al. 2013). Thus, motivating the parents for cessation of smoking pre- and post-natally is an essential part of comprehensive prevention strategy of wheezing illnesses and asthma.

2.4.5 Genetics

Ten years ago Moffatt et al. (Moffatt et al. 2007) reported the first genome-wide association study of asthma and made a groundbreaking finding of an asthma-related locus on chromosome 17q21. Subsequent studies have clarified that variations at this locus are specifically associated with early-onset asthma (Bouzigon et al. 2008, Bisgaard et al. 2009, Halapi et al. 2010, Smit et al. 2010). The effects of the different 17q21 genotypes on asthma risk are modified by early life exposures such as environmental tobacco smoke as a risk increasing factor (Bouzigon et al. 2008, Smit et al. 2010, van der Valk et al. 2012, Blekic et al. 2013) or owning a hairy pet as a protecting factor (Bräuner et al. 2012, Blekic et al. 2013, Stokholm et al. 2018). The prime candidates for asthma genes at this locus include ORM1-like 3 (ORMDL3) and gasdermin B (GSDMB) (Moffatt et al. 2007, Galanter et al. 2008, Halapi et al. 2010). Further, Çalişkan et al. reported an association between increased expression levels of these genes and asthma, especially in children with a history of RV-induced wheezing (Çalişkan et al. 2013). However, the exact functions and mechanisms of these genes in asthma development remain unclear (Stein et al. 2018).

A transmembrane protein from the cadherin family, CDHR3, has recently been identified as a RV-C receptor (Bochkov et al. 2015, Bønnelykke et al. 2018). Increased expression of CDHR3 in the airway epithelial cells and a specific rs6967330 mutation of CDHR3 gene have been reported to be associated with increased RV-C binding and replication suggesting that this mutation is a risk factor for RV-C wheezing illnesses (Bochkov et al. 2015, Griggs et al. 2017).

Moreover, the risk allele rs6967330-A is overrepresented in wheezing children less than 4 years of age (Stenberg-Hammar et al. 2018), and this allele is associated with the risk of exacerbations in asthmatic children before the age of six years (Bønnelykke et al. 2014).

2.5 Prevention of recurrent wheezing and asthma

Recognizing the children susceptible for different phenotypes of asthma is important in order to interfere with early development of the disease (Holt & Sly 2012, Nieto et al. 2014, Jackson et al. 2016, Wawrzyniak et al. 2016). Environmental factors during both pregnancy and early childhood play a role in the development of atopic tendency and/or airway physiology of children (Beasley et al. 2015, DeVries et al. 2017). Thus, the essential time for primary prevention is during these time frames.

A varied and healthy diet and the use of vitamin D are recommended for pregnant women for reducing the child's risks for atopic diseases during early childhood (GINA 2016, Christensen et al. 2017, Danielewicz et al. 2017, Wolsk et al. 2017). However, information on the effect of the maternal vitamin D status on the risk of recurrent wheezing and asthma are inconsistent, and thus further studies are needed (Chawes et al. 2016, Jiao & Castro 2015). The maternal use of antibiotics during the last trimester of the pregnancy increases the risk of asthma during early childhood (Mulder et al. 2016, Popovic et al. 2016, Wu et al. 2016). These findings give support to the theory that microbial immune and metabolic programming begins already during pregnancy. As earlier mentioned, maternal smoking during pregnancy increases the risk of wheezing and asthma in the child (Goksör et al. 2007, Prabhu et al. 2010, van der Zalm et al. 2011b, Kalliola et al. 2013, GINA 2016), thus it is important to support the mother's cessation of smoking. Cesarean section and exposure to broad-spectrum antibiotics during the first weeks of life have been noticed to increase the risk of asthma and allergic sensitization in school-age children (Goksör et al. 2013, Alm et al. 2014, Wu et al. 2016, Gerlich et al. 2017, Korhonen et al. 2018), which gives reasons to limit these factors only for justified circumstances.

Recently, the loss of environmental biodiversity has aroused interest worldwide as a risk factor for atopic illness and asthma and also for other non-communicable diseases, such as diabetes and inflammatory bowel diseases (Kondrashova et al. 2005, Lehtinen et al. 2011, Haahtela et al. 2015, Ruokolainen et al. 2015, Jackson et al. 2017, von Mutius 2018). The underlying mechanisms behind how the gut and airway microbiomes and these exposures change the response to allergens and viruses are not well-known, but they are intensively

investigated (Jackson et al. 2017, von Mutius 2018). Breastfeeding can be recommended, since it is associated with a lower risk of asthma symptoms during early childhood and it has many other health benefits (GINA 2016). However, its effect on asthma risk at older ages remains unclear (Bion et al. 2016, den Dekker et al. 2016, Lossius et al. 2018). The role that the gut microbiome plays in allergy and asthma development remains unclear and should be further studied before recommendations about the use of specific probiotics can be given (Luoto et al. 2014, Mennini et al. 2017, Gaufin et al. 2018).

2.5.1 Prevention in high risk children

Early wheezing children have an elevated risk of developing asthma, especially if RV is involved. Thus it can be hypothesized that decreasing the amount of virus infections may decrease the risk of asthma. One probable method for prevention could be a vaccine against RV and/or RSV (Holt & Sly 2012, Rossi & Colin 2015, Stone & Miller 2015, Stobart et al. 2017). However, the development of RV vaccines has been challenging due to the antigenic diversity of circulating viruses. Many candidates are however in clinical trials (Holt & Sly 2012, Stobart et al. 2017).

Sensititized children are at risk of developing asthma, but there are only few available methods for modulating the development of sensitization. Allergen immunotherapy may decrease the risk of new allergen sensitizations in sensitized children aged 5 years or more (Di Bona et al. 2017). Ismail et al. reported that early gut colonization by Bifidobacteria modulates the risk of atopic dermatitis in children a high risk of developing allergic disease (Ismail et al. 2016), which highlights the role of the gut microbiome especially in the group of sensitized children. Omalizumab is a monoclonal antibody that recognizes IgE at the same site as the high-affinity IgE receptor. By forming complexes with free IgE, it blocks the interaction between IgE and mast cells and basophils (Busse et al. 2001). Omalizumab has been noticed to indirectly improve antiviral responses and reduce the frequency of RV-induced colds and asthma exacerbations especially in sensitized patients (Busse et al. 2011, Teach et al. 2015, Esquivel et al. 2017). However, there is no safety data about omalizumab for young children. Thus it is used only as an adjunctive therapy for patients 12 years of age who have sensitization for relevant allergens (e.g. dust mites, cockroaches, cats or dogs) and have severe persistent asthma (NAEPP 2007).

Oral corticosteroid (OCS) treatment has not been found to be effective in children suffering from acute wheezing episode overall (Panickar et al. 2009, Collins & Beigelman 2014). However, OCS may be effective in the prevention of recur-

rent wheezing when directed during acute wheezing episode to children with RV etiology, especially with a high virus load (Jartti et al. 2006, Lehtinen et al. 2007, Lukkarinen et al. 2013, Jartti et al. 2015). It is noteworthy that no other study group has paid attention to the viral etiology when aiming the OCS treatment, and there are no earlier studies about the long-term efficacy of OCS. The efficacy of OCS may be based on the underlying, probably atopy-related inflammation, which is downregulated by OCS (Stellato 2007, de Benedictis & Bush 2012, Holt & Sly 2012). OCS decreases the transcription of many inflammatory genes and their transcription factors and induces the expression of many anti-inflammatory genes (Stellato 2007, de Benedictis & Bush 2012).

2.6 Airway development, lung function and airway reactivity

2.6.1 Airway development and remodeling

A fully developed lung consists of 23 generations of airways from the trachea to the alveoli. Lung development begins by the fourth week of gestation and continues for years after birth. The final generations of the airway start developing prenatally, during the 24th to 25th gestational weeks (Merkus et al. 1996). The final stage in lung differentiation is alveolarization, which begins at term and continues until the age of 2 to 3 years. After differentiation lung growth slows down, but continues until early adulthood (Merkus et al. 1996, Xuan et al. 2000, Gern et al. 2005, Herring et al. 2014). Factors known to affect lung function development prenatally include mechanical anatomical obstacles, maternal hypoxia, maternal use of alcohol or drugs and maternal smoking. Since most of the lung volume expands after birth, many external factors during infancy affect significantly lung growth and lung function development. These factors include respiratory infections, exposure to tobacco smoke and other air pollutants as well as allergic sensitization (Merkus et al. 1996, Merkus 2003).

In asthma, typical airway remodeling changes include reticular basement membrane (RBM) thickening, smooth muscle mass increase in large airways and eosinophilic inflammation (Jeffery 2001, Watelet et al. 2006, Fixman et al. 2007, Papadopoulos et al. 2012). It remains unclear whether a causal relationship between airway inflammation and airway remodeling exists and when the structural changes in asthma first appear (Saglani & Lloyd 2015). In a study with recurrent lower respiratory tract symptoms in Finnish children at the median age of 12 months RBM thickening and eosinophilia were not yet present (Saglani et al. 2005). Re-evaluation of these children showed no correlation of any pathologic

feature in infancy with lung function or airway reactivity at 8 years of age (Malmström et al. 2015). Instead, decreased lung function measured during early infancy was associated with decreased lung function and the use of asthma medication at a school age. Moreover, Saglani et al. reported that the RBM thickness and eosinophilic inflammation were significantly greater in preschool-aged children (mean age 29 months) with recurrent severe wheezing when compared to healthy controls (Saglani et al. 2007). These results may suggest that the mechanisms of the early-onset asthma differ from the process of the later established disease.

2.6.2 Impulse oscillometry

Lung function and airway reactivity testing in young children is challenging. The forced oscillation technique (FOT) for measuring the impedance of the airways was introduced already in 1956 (Dubois et al. 1956). It is a noninvasive method for measuring respiratory mechanics, which uses small-amplitude pressure oscillations superimposed upon normal breathing. Thus it demands only minimal cooperation of the patient (Oostveen et al. 2003). In FOT, the external signals are conducted to the airways through an open mouthpiece and it is a suitable method also for young children (Vogel & Smidt 1994, Marotta et al. 2003, Dencker et al. 2006). Impulse oscillometry (IOS) is a relatively new modification of FOT, which uses a fixed square wave of pressure delivered to the airways at 5 times per second. A continuous spectrum of frequencies is used (Komarow et al. 2011).

The mechanics of FOT and IOS are based on total respiratory impedance (Zrs), which results from the phase and pressure changes of the airflow. The in-phase component of Zrs is called resistance (Rrs), and it describes the mechanichal properties of the respiratory system and reflects the energy loss due to resistive forces to the airflow. Rrs is the key measurement in IOS, since it is clinically interpreted as an indicator of obstruction. Resistance depends on the length of the airways, airway lumen, density of air and turbulence of the airflow. The imaginary out-of-phase component of impedance is expressed by reactance (Xrs), which indicates the elastic properties of the small airways. Both, Rrs and Xrs appear as functions of the frequency of oscillation. The point where the reactance equals zero (Xrs = 0) is characterized as resonance frequency (Fres) (Oostveen et al. 2003). In Finland, there are population-based reference values available for children aged 2-7 years (Malmberg et al. 2002, Malmberg et al. 2008).

During the IOS measurement, the child is sitting and breaths normally through the mouthpiece without coughing or crying. A nose clip is used and the child's cheeks are supported by the technician so that pressure loss to the upper airways

is minimized. An input signal with oscillations at 5-35 Hz is conducted to the airways. A pressure and flow transducer measures inspiratory flow and pressure. Signal filtering is used for separating the resultant signals of pressure and flow. Zrs is the sum of all the forces (Rrs and Xrs) and is calculated from the ratio of pressure and flow at each frequency. Rrs and Xrs are calculated from Zrs as a function of oscillation frequency. Low frequency oscillations ≤ 5 Hz describe the small airways and the high frequency oscillations such as 20 Hz describe the larger airways. When the airway lumen decreases, for example, during bronchoconstriction, Rrs increases. In asthmatics this is seen relatively more with low frequencies. During small airway obstruction, Xrs with low frequencies decreases due to peripheral stiffening. The flow signal should be displayed on the screen during the measurement in order to notice the artifact caused by swallowing, leak, irregular breathing or hyperventilation (Figure 2) (Oostveen et al. 2003, Komarow et al. 2011).

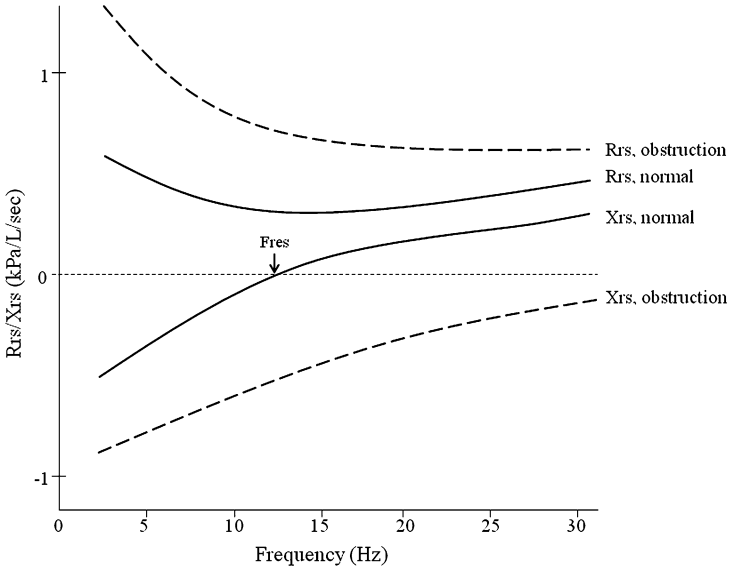

Figure 2 Schematic illustration of the IOS curves as a function of oscillation frequency. Fres, Resonance frequency; Rrs, resistance; Xrs, reactance. Modified from Komarow et al. 2011.

According to earlier studies, IOS can be considered adjunct and, in some cases, even substituent to spirometry (Guilbert et al. 2011, Komarow et al. 2012, Shi et al. 2012, dos Santos et al. 2017). It may give additional information about the functional evaluation of small airways and thus can be considered as a more sensitive method for measuring abnormal pulmonary processes and airway obstruc-

tion (Larsen et al. 2005, Evans et al. 2006). IOS has also facilitated the diagnosis of airway reactivity in young children (Jee et al. 2010, Kalliola et al. 2014). For testing airway reactivity, IOS can be used together with exercise testing, which uses physical activity for inducing probable increased airway reactivity. The subjects are urged to run, for example, and IOS measurement is repeated 1, 5 and 10 minutes after the exercise (Malmberg et al. 2008). Also, a bronchodilatation test can be used together with IOS (Delacourt et al. 2000, Malmberg et al. 2002). The IOS measurement is repeated 15 minutes after the administration of the bronchodilator (Malmberg et al. 2008).

Height-adjusted z-scores can be used for expressing the normal limits of IOS variables (Malmberg et al. 2002). Reference limits (from the 5^{th} to 95^{th} percentile) for baseline measurements are usually set at levels +/-1.65, i.e. in pathological conditions, the Rrs is above +1.65 z-score, and the Xrs is below -1.65 z-score. In airway reactivity no clear consensus on pathologic results exists. The current ATS/ERS guidelines and the Finnish Current Care Guidelines suggest a minimum of 40% increase in resistance in response to exercise or 40% decrease to bronchodilation to be suggestive for asthma (Beydon et al. 2007, Malmberg et al. 2008, Asthma: Current Care Guidelines 2012).

2.6.3 Airway responsiveness

Increased airway responsiveness (AR) (or bronchial reactivity) is defined as a reactive narrowing of the airways leading to airflow limitation (Cockcroft & Davis 2006). It is an excessive reaction for natural or pharmacological stimuli, such as exercise or methacholine. During the reaction smooth muscle contracts inappropriately, which leads to bronchoconstriction. Mechanisms leading to this process are poorly understood, but most probably the smooth muscle mass increase and constriction, airway inflammation and remodeling as well as neurogenic airway tonus controlling are involved (Berend et al. 2008, Papadopoulos et al. 2012).

AR is launched by a direct or indirect stimulus. Direct stimuli, such as methacholine or histamine, work through smooth muscle cells. Indirect stimuli, such as exercise or cold air, work through intermediator cells, which cause the stimulation of the smooth muscle cells. These cells release inflammatory transmitters and thus cause neural activation (Berend et al. 2008).

Reversible obstruction is typical for asthma and usually linked to increased AR. Reversibility and reactivity are however mainly based on different physiologic

phenomena, which do not correlate, even in all asthmatic patients (Suh et al. 2011). Thus, in diagnostics, it is useful to measure both reversibility and AR.

2.6.4 The effect of early life factors on lung function and airway responsiveness

Children with early life wheezing have decreased lung function during later childhood (Martinez et al. 1995). Also, rhinovirus etiology of the wheezing relates to abnormalities in lung function and/or AR at a school age (Kotaniemi-Syrjänen et al. 2008, Guilbert et al. 2011). On the other hand, decreased lung function measured shortly after birth has been found to be associated with an increased risk of wheezing and asthma (Young et al. 2000, Murray et al. 2002, van der Zalm et al. 2011b, Bisgaard et al. 2012), so the causality and the relative importance of hereditary versus infectious factors in the development of asthma remain unclear (Gern et al. 2005). Furthermore, there are no previous data concentrating on the characteristics of the first wheezing episode when concerning the later lung function.

Early-life sensitization has been recognized as an important risk-factor for impairing lung function. Illi et al. reported that sensitization to perennial allergens developed before the age of three years was associated with the increased AR by school age (OR 8.4) (Illi et al. 2006). Further, two other studies have found an association between infant atopy or number of early sensitizations and reduction of lung function between the ages of 1 to 18 years (Belgrave et al. 2014, Turner et al. 2014). In a prospective, population-based birth cohort study, Belgrave et al. reported a significantly poorer lung function in children with multiple early, but not other atopy phenotypes when compared to children without atopy at the age of 3 years. In line with this study, Turner et al. reported a mean reduction of 12.6% in lung function between 1 month and 18 years in children with infant onset atopy (Turner et al. 2014). In this cohort, maternal asthma (mean reduction 9.8%) and maternal smoking (mean reduction 8.1%) were also associated with the reduction in lung function. Moreover, Hyvärinen et al. demonstrated that early aeroallergen sensitization and atopic eczema associate with increased AR at the age of 11 years (OR 12.6) (Hyvärinen et al. 2007). However, the link between atopy and asthma is complex, which is partly shown by the existence of non-atopic asthmatic and non-asthmatic atopic children.

3 AIMS OF THE STUDY

The aims of this study were:

- To study the virus etiology of the first severe wheezing episode and the associations among the virus etiology, atopic characteristics and illness severity (*Study I*).
- To study serum 25-hydroxyvitamin D (25OHD) levels of the children with the first severe wheezing episode, and how the 25OHD level is associated with patient characteristics and virus etiology of the first severe wheezing episode (*Study II*).
- To evaluate the long-term effect of short-course prednisolone treatment on the first rhinovirus induced severe wheezing episode in the follow-up until the age of 5 years (*Study III*).
- To determine the lung function four years after the first severe wheezing episode and how it is associated with the patient characteristics at infancy (*Study IV*).

4 MATERIALS AND METHODS

4.1 Patient enrollment, intervention and protocol

All four studies were parts of a prospective, randomized, double blind, placebo controlled study called Vinku2. Patient recruitment was carried out in the Department of Paediatrics, Turku University Hospital (Turku, Finland) from June 2007 to March 2010. Children aged 3-23 months suffering from their first acute wheezing episode attending the outpatient clinic of the Department of Paediatrics or the Paediatric Infectious Diseases Ward in Turku University Hospital were recruited. Other inclusion criteria were delivery at 36 gestational weeks or later and an informed consent from the guardian. The information about the first wheezing episode was based on the parental report and was confirmed from the medical record. The exclusion criteria included another chronic illness besides atopy, a history of previous systemic or inhaled corticosteroid treatment, varicella contact in a patient without a previous varicella illness, need for intensive care and the guardian's poor understanding of Finnish. The trial was double-blinded until the 12-month follow-up.

At the beginning of the study, the study physician or on-duty physician clinically examined the study subjects and verified the wheezing. Symptoms, medications and the use of supplementary oxygen were recorded daily at the ward. Nasopharyngeal aspirates (NPA) were taken and venous blood samples were drawn. Children with RV etiology and on-going signs of lower respiratory tract symptoms (e.g. cough, noisy breathing or wheezing) were randomized to receive oral prednisolone or placebo when the PCR results were available. Compared to the earlier Vinku study, in our study prednisolone was not initiated until the RV was diagnosed. The first dose of prednisolone (Prednisolon® 5mg tablets, Leiras Takeda, Helsinki, Finland) was 2 mg/kg, followed by 2 mg/kg/day, in 2 divided doses for 3 days. The maximum dose was 60mg/day. The subjects were prospectively followed at scheduled visits 2 weeks, 2 months, 12 months and 4 years after the first wheezing episode. All patient charts were reviewed for the full 4-year follow-up period for asthma symptoms, medications and laboratory tests. The study protocol was registered at ClinicalTrials.gov in August 2008 (ClinicalTrials.gov number NCT00731575)

4.2 Baseline data collection and analyses

4.2.1 Clinical data

Clinical examination was done by the study physician at the study entry. The guardian was interviewed by using a standardized questionnaire concerning other host- and environment-related risk factors for recurrent wheezing and asthma (Appendix 1). The study physician examined and recorded symptoms, medications and the use of supplementary oxygen at the ward. The respiratory symptom score was calculated daily. Patients were discharged from the hospital when the difficulty of breathing had abated (Jartti et al. 2006). After discharge, the guardian recorded symptoms (e.g. rhinitis, cough, breathing difficulty, noisy breathing, and nocturnal wakening because of breathing difficulties) and medication daily in a diary for two weeks. The symptom severity was assessed on a 4-graded scale (Appendix 2).

4.2.2 Laboratory analyses

Routine diagnostic procedures of the Central Laboratory of Turku University Hospital were used for the analyses of blood eosinophil counts, C-reactive protein levels, leukocyte levels and serum levels of allergen-specific IgE from the blood samples.

The NPAs for viral detection were drawn using a standard procedure (Jartti et al. 2004, Allander et al. 2007). The sample was taken through a nostril with a disposable catheter connected to a mucus extractor. A nasopharyngeal swab (nylon flocked dry swab, 520CS01; Copan, Brescia, Italy) was dipped in the NPA, placed in dry tube and transported to the laboratory during the same day. At study entry, NPAs were analyzed within 3 days for RV, EV and RSV. Samples were stored at -70°C before further virus analyses.

An in-house RT-PCR was used for simultaneous detection of rhinovirus A, B and C, enteroviruses and RSV A and B from NPA. A multiplex PCR (Seeplex RV12 ACE Detection; Seegene, Seoul, Korea) test was done for detecting adenovirus, coronavirus (229E, NL63, OC43 and HKU1), influenza A and B viruses, metapneumovirus, parainfluenza virus types 1-3, RV and RSV. PCR products were analyzed by a Screentape machine (Lab901 ScreenTape®System). HBoV was analyzed using PCR and serology. PCR was carried out at the Department of Virology at the University of Turku (Allander et al. 2007). HBoV serology was analyzed from paired serum samples collected 2-3 weeks apart at the Haartman

Institute, Helsinki, Finland (Söderlund-Venermo et al. 2009). An enzyme immunoassay was used for detecting IgG and IgM antibodies against HBoV. Diagnosis of acute HBoV infection was based on seroconversion or ≥4-fold increase in virus-specific IgG antibody levels in paired serum samples and a positive IgM result.

RV load was analyzed from RNA of RV-positive samples by a quantitative RT-PCR, using known concentrations of RV-B14 plasmid. The plasmid with a known quantity was received from Glyn Stanway at the University of Colchester (Essex, United Kingdom). Serum 25OHD levels were measured by means of liquid chromatography tandem mass spectrometry at Massachusetts General Hospital (Boston, MA).

4.3 Follow-up visits and long-term data collection

4.3.1 Clinical data

The follow-up visits were arranged at 2 weeks, 2 months, 12 months and 4 years after the first wheezing episode. The guardian was also instructed to bring the child to the hospital each time the child had a breathing difficulty during the first 12-month follow-up period. For the first two weeks, the guardian was asked to assess the symptom severity on a 4-score-graded scale. The guardian fulfilled a symptom and medication diary for the first two months. Thereafter, until the 12-month follow-up, they were asked to fill in the dates of breathing difficulties, respiratory medications and visits to health care providers (Appendix 2 and 3). A standardized questionnaire was used for the parental interview at the 4-year follow-up visit (Appendix 4). The study physician clinically examined the children at every follow-up visit. Medical records were reviewed until the end of the follow-up period for symptoms suggestive of atopy and asthma. The use of asthma therapies was registered.

4.3.2 Laboratory analyses

Nasopharyngeal swab samples were taken at each follow-up visit and also at visits for acute episodes. The swab samples were collected using a sterile cotton swab which was placed into dry and sterile vials and transported at room temperature to the laboratory and stored at -70°C. Serum samples were taken at 2-week, 12-month and 4-year follow-up visits and stored at -70°C. Laboratory analyses

included blood eosinophil counts and allergen-specific IgE levels (codfish, cow's milk, egg, peanut, soybean, wheat, cat, dog, horse, birch, mugwort, timothy, *Cladosporium herbarum* and *Dermatophagoides pteronyssinus*; fluoro-enzyme immunoassay, CAP FEIA, Phadiatop Combi®, Phadia, Uppsala, Sweden).

4.3.3 Impulse oscillometry

At the 4-year follow-up visit, which was carried out from November 2011 to October 2012 at the Research Centre of Applied and Preventive Cardiovascular Medicine, University of Turku, the lung function was tested by IOS (Jaeger GmbH, Würzburg, Germany) (Vogel & Smidt 1994, Malmberg er al. 2002, NAEPP 2007). The caretakers were instructed to discontinue the child's regular asthma control medication for 4 weeks and to withhold salbutamol for 12 hours before the IOS testing. The device was calibrated daily, the system was checked against reference impedance, air temperature and humidity were measured. The IOS results were adjusted by the height of the child. The measurements were accepted when the child remained still at an appropriate posture for at least 20 seconds, and when the breathing pattern was regular and quiet. The study pediatrician judged the IOS curves for the whole measurement time. The measurements having an artifact were rejected. Three acceptable measurements were obtained.

After the baseline measurement, an exercise test was conducted. The children ran for 6-8 minutes and the heart rate was held at 85-90% of their estimated maximum heart rate [205- (1/2) x age], which was assessed with a heart rate monitor (Polar Sport Tester, Polar Elektro Ltd, Kempele, Finland). An exercise test was performed outside when air temperature was ≥5°C, otherwise the test was performed inside (Malmberg et al. 2008). An IOS measurement was repeated 1, 5 and 10 minutes after running and 15 minutes after the bronchodilation with inhalation of 400 micrograms of salbutamol (Ventoline®) administered through a spacer (Babyhaler®, both from Glaxo Smith Kline, Brentford, UK).

Baseline, post-exercise and post-bronchodilator respiratory system Zrs, Rrs and Xrs were acquired. The frequency dependency of resistance (dRrs/df) was determined by using linear regression through data points Rrs5 and Rrs10. Reference limits for z-scores were set at levels +1.65 for Rrs and -1.65 for Xrs. When testing AR and reversibility, Rrs was categorized abnormal if the exercise-induced increase in mean crude values was ≥ 35% or if bronchodilator-induced decrease in mean crude values was ≥ 35%.

4.4 Definitions

The respiratory symptom score was the sum of scores for the degree of dyspnea (0 = none, 1 = mild, 2 = moderate, 3 = severe), type of breathing (0 = normal, 1 = use of stomach muscles, 2 = use of intercostal muscles, 3 = nasal flaring), severity of auscultation findings (0 = none, 1 = expiratory, 2 = inspiratory and expiratory, 3 = audible without a stethoscope) and assessment of expiratory : inspiratory time ratio (0 = 1:2; 1 = 1:1, 2 = 2:1, 3 = 3:1).

Sensitization was defined as positive for IgE antibodies against common allergens (cut-off level 0.35 kU/L) (Jartti et al. 2015). Aeroallergen sensitization was defined as IgE antiobodies to cat, dog, horse, birch, mugwort, timothy, *Cladosporium herbarum* and/or *Dermatophagoides pteronyssinus*. B-eos was expressed as cells $x10^9$/L, and the cut-off limit for the elevated B-eos value was 0.4 cells x 10^9/L (Jartti et al. 2010b). Eczema was a physician-made diagnosis with typical symptoms of pruritus, typical distribution and chronicity of disease (NAEPP 2007). Eczema was defined as atopic if IgE antibodies to any of the allergens were present.

4.5 Outcomes

4.5.1 Patient characteristics and illness severity (I)

In *Study I*, the associations between patient characteristics and virus etiology as well as the severity of infection and virus etiology were studied. Virus etiology included RV, RSV, HBoV and coinfection. Patient characteristics included age, sex, atopic sensitization, total IgE level, blood eosinophil count, eczema, atopic eczema, parental rhinitis, asthma and smoking. Illness severity included patient status (i.e. inpatient vs. outpatient), severity score (score ≥6 vs. <6), duration of hospitalization (≥24h vs. <24h) and the total duration of wheezing (≥3 days vs. <3 days) and cough (≥14 days vs. <14 days). Patients receiving prednisolone (n = 38) were excluded from the illness severity analyses, since prednisolone is associated with the short-term outcomes of acute wheezing.

4.5.2 Vitamin D concentration (II)

In *Study II,* the associations between vitamin D level and virus etiology and atopic characteristic during the first wheezing episode were studied. Vitamin D level

was expressed as serum 25OHD concentrations (nmol/l). The use of vitamin D supplements was asked from the guardian by standardized questionnaires. Patient characteristics and virus etiology consisted of the same variables as in the *Study I.*

4.5.3 Time to initiation of regular asthma control medication (III)

In *Study III,* the asthma control medication was initiated as soon as the children fulfilled the criteria based on NAEPP guidelines for the initiation of asthma therapy in children less than 5 years of age (NAEPP 2007). The criteria consisted of ≥4 wheezing episodes (≥1 diagnosed by a physician) within a year that lasted >1 day and affected sleep, in addition to 1 major risk factor (i.e. physician diagnosed atopic eczema, aeroallergen sensitization or parental history of asthma) or 2 minor risk factors (wheezing apart from colds, blood eosinophil count $\geq 0.40 \times 10^9$/L or food sensitization) and/or prolonged symptoms lasting >4 weeks and requiring symptomatic treatment >2 days per week and/or two exacerbations requiring systemic corticosteroids within 6 months (NAEPP 2007). In some children, asthma control medication was started after the third acute wheezing episode according to the Finnish guidelines (Asthma: Current Care Guidelines 2012).

4.5.4 Lung function and airway reactivity

In *Study IV,* lung function was measured by IOS four years after the first acute wheezing episode. Baseline, post-exercise and post-bronchodilator values of Rrs and Xrs at the frequencies of 5-20 Hz were measured, and the associations between the values, virus etiology and atopic characteristics of the first wheezing episode were studied. The reference limits for Rrs and Xrs at baseline were defined as a z-score +1.65 or -1.65, respectively. After the exercise ≥35% increase of Rrs5 was defined as abnormal. A positive bronchodilation response was considered if Rrs decreased ≥35% from the baseline.

4.6 Statistical methods

Statistical power calculations were done for the 12-month follow-up (Jartti et al. 2015) but not for the 4-year follow-up. Analyses were performed using JMP software (Version 8.0.2, SAS Institute, Gary, NC, USA) for *Studies I* and *II* and SPSS software (Versions 23 and 24, SPSS Inc, Chicago, III, USA) for *Studies III* and *IV.* Basic statistics were analyzed using t-test, one-way ANOVA or Mann-

Whitney U-test for continuous data and Pearson's Chi square, Fisher's exact and Kruskal-Wallis tests for dichotomous data. Two-sided p-values less than 0.05 were considered significant.

In *Study I,* univariate and multivariate logistic regression analyses were used for analyzing the associations between the virus etiology, patient characteristics and illness severity. The results were expressed as OR and 95% CI. Multivariable analysis included age and sex. Univariate and multivariate linear regression analyses were used for analyzing the associations between 25OHD concentration and patient characteristics in *Study II*. RV load was log-transformed for creating a normal distribution before inclusion in the analysis. The results were expressed as mean difference and 95% CI for unadjusted analyses and regression coefficient beta and 95% CI for adjusted analyses.

In *Study III,* the Cox model was used for testing the effect of prednisolone on the time to the initiation of asthma control medication. The model included the main effects of dichotomized RV genome load and intervention group and the interaction effect of RV genome load by intervention group. The Cox model included no covariates, since no significant differences in baseline patient characteristics were found. RV load was dichotomized due to the skewed distribution. The cut-off level for the RV genome load was identified by testing different copy number levels and considering the significance of the p-value for RV load vs. group interaction and the threshold used in our previous report (Jartti et al. 2015).

In *Study IV,* logistic and linear regression analyses were used for studying the associations between patient characteristics and lung function variables. Results were expressed as OR and 95% CI for logistic regression and as regression coefficient beta and 95% CI for linear regression analyses. Multivariable models included age, OCS treatment and RV at study entry and depending on the dependent variable also allergic sensitization, seasonal sensitization, atopic eczema, hospitalization at study entry and/or inhaled corticosteroid (ICS) within 4 weeks. Natural logarithmic change was applied for Rrs5.

4.7 Ethics

Written informed consent was obtained from the guardian of the participating children. The study was approved by the Ethics Committee of the Hospital District of Southwest Finland, Turku, Finland.

5 RESULTS

5.1 Study populations and patient characteristics

5.1.1 Study I and II

In *Studies I* and *II*, 125 consecutive children were eligible for the study. Twelve children were declined, and two were excluded due to misdiagnoses. Further, four children were excluded from the *Study II* due to the lack of vitamin D analyses. Finally, 111 and 107 children were included in the *Studies I* and *II*, respectively (Figure 2).

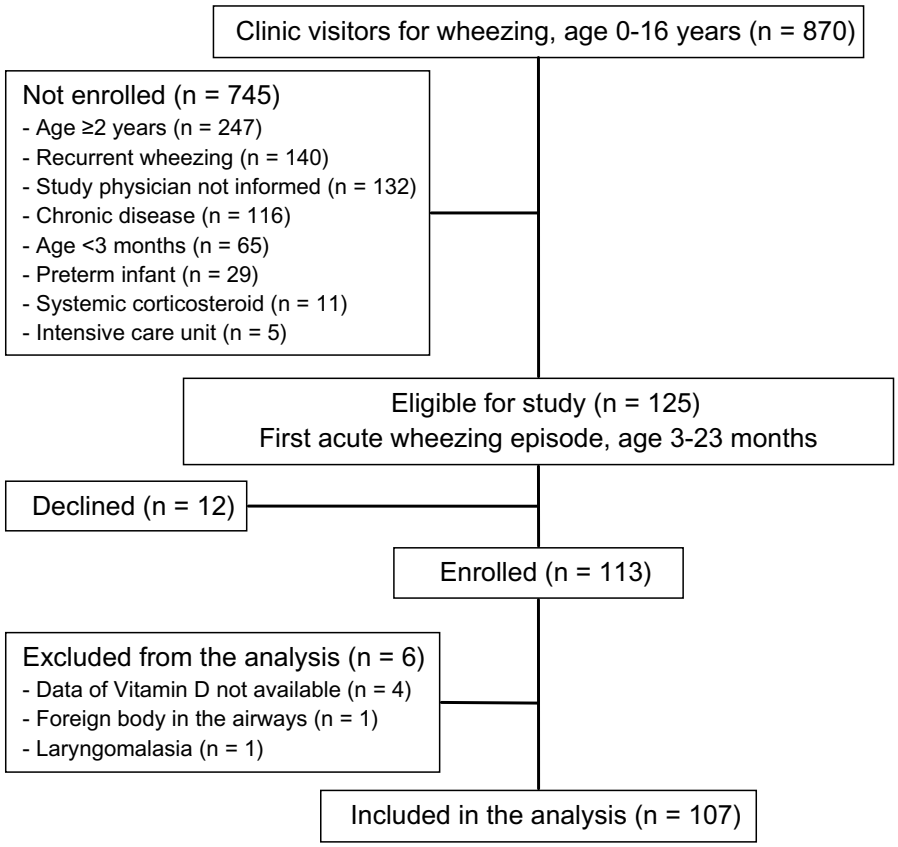

Figure 3 Simplified study flow chart for Studies I-II.

In *Study I,* the mean age of the 111 children was 12 months (SD 6.0), and 67% were boys. Seventy-nine percent needed hospitalization. Any sensitization was diagnosed in 23% of the patients, 29% had eczema, atopic eczema was present in 16%, blood eosinophilia ≥0.4 $x10^9$/l in 41% and parental asthma in 20% of the children. The median duration of the symptoms before the recruitment was two days.

At least one virus was detected from all 111 (100%) children. The most common etiology was RV (76%), followed by RSV (28%), HBoV (18%) and other viruses (<10% each). In single-virus infections (n = 69), RV was the most common agent (72%) followed by RSV (16%). Coinfection was found in 38% of the patients. Two viruses were found in 71% of the coinfections. The most common 2-virus infection included RV and HBoV (33%). Three viruses were found in 24% and four viruses in 5% of the coinfections (Table 2).

In *Study II*, the mean age of the 107 included children was 12 months (SD 6.0), and 67% were boys. Any atopic characteristic (i.e. sensitization, blood eosinophilia or atopic eczema) was diagnosed in 55% of the children. RV was detected in 77%, RSV in 29% and HBoV in 18% of the children. The mean serum 25OHD concentration was 86 nmol/l (SD 21, range 35-150). Serum 25OHD concentration <50 nmol/l, thought to be the lower normal level, was detected in 5 (5%) children and <75 nmol/l in 34 (33%) children. Twenty-eight (26%) children had serum 25OHD concentration 100 nmol/l or higher (Table 2).

Table 2 Patient characteristics for studies I-IV

	Study I n = 111	**Study II n = 107**	**Study III n = 59**	**Study IV n = 76**
Age (months)	12 (6.0)	12 (6.0)	13 (6.0)	12 (6.0)
Male sex, no.	74 (67%)	72 (67%)	46 (78%)	50 (66%)
Serum 25OHD, nmol/l		86 (21)	85 (24)	88 (20)
Atopic eczema, no.	17/108 (23%)	17 (16%)	11/74 (19%)	13/74 (18%)
B-eos, $x10^9$/l	0.34 (0.09-0.57)	0.35 (0.09-0.59)	0.46 (0.21, 0.72)	0.40 (0.16-0.58)
B-eos >0.4 $x10^9$/l, no.	45/107 (41%)	44 (41%)	30/56 (51%)	32/74 (43%)
Any sensitization, no.	25/108 (22%)	25 (23%)	16/57 (28%)	22/74 (28%)
Food sensitization, no.	24/108 (22%)	24 (22%)	15/57 (25%)	21/74 (28%)
Aeroallergen sensitization, no.	12/108 (11%)	12 (11%)	8/57 (14%)	11/74 (15%)
In-patients, no.	88 (79%)	86 (80%)	47 (80%)	62 (82%)
Virus etiology, no.				
RV, no.	84 (76%)	82 (77%)	59 (100%)	57 (75%)
RV load, copies/ml		median 3200 (IQR 84, 16000)	median 4300 (IQR 79, 16000)	median 4000 (IQR 49-17000)
RSV, no.	31 (29%)	31 (29%)	8 (14%)	20 (26%)
HBoV, no.	20 (18%)	19 (18%)	6 (10%)	10 (13%)
Coinfection, no.	42 (38%)	41 (38%)	20 (34%)	26 (34%)
Parental smoking, no.	45 (41%)	45 (42%)	23 (39%)	30 (40%)
Maternal smoking, no.	21 (19%)	21 (20%)	9 (15%)	15 (20%)
Regular ICS medication at 4-year follow-up, no.				37 (75%)

25OHD, serum 25-hydroxyvitamin D; B-eos, blood eosinophil count; HBoV, human bocavirus; ICS, inhaled corticosteroid; RV, rhinovirus. Values shown as mean (standard deviation) or number (%) unless otherwise noted.

5.1.2 Study III

At study entry, all 79 RV-positive children were randomized to receive a short course of prednisolone or placebo. For *Study III,* 10 children were excluded due to an insufficient follow-up time, nine due to insufficient data about RV load and one due to initiation of ICS for another reason. Hence, 59 children were included in the analysis (Figure 3).

At study entry, the mean age of the 59 patients was 13 months (SD 6.0), 31% were sensitized, 23% had eczema and 34% had coinfection. A high (>7000 copies/ml) RV genome load was detected in 39% of the children. The prednisolone and placebo groups did not differ in terms of the baseline characteristics. Asthma control medication was initiated in 68% of the children, in 69% of the prednisolone group and in 67% of the placebo group (Table 2).

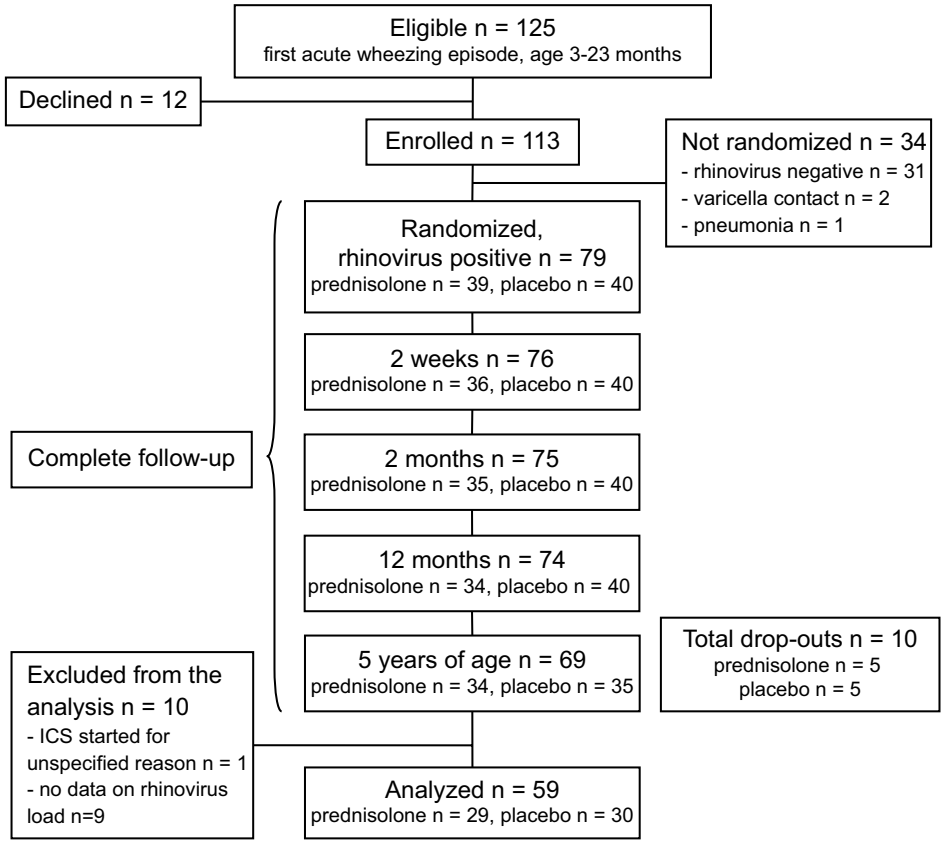

Figure 4 Study flow chart for Study III. ICS, inhaled corticosteroid (From *Study III*).

5.1.3 Study IV

For *Study IV*, 77 (62%) children attended the follow-up visit 4 years after the first wheezing episode. IOS was conducted for 76 children. An exercise test was not conducted in 3 children due to refusal or for difficult asthma symptoms. All 76 children with bronchodilation and/or exercise test were included.

At study entry, the mean age of the 76 children was 12 months (SD 6.0), 82% were hospitalized and 66% were boys. RV was the most common detected virus (75%) followed by RSV (26%). During the first wheezing episode, coinfection was detected in 34% and allergic sensitization from 30% of the children. Median delay in starting the study drug was 45 hours (IQR 41-71). At the follow-up visit, the mean age was 60 months (SD 7.9), and 49% of the children needed ICS treatment during the preceding 12 months. ICS was discontinued at least for 4 weeks prior to the exercise testing in 25 children. The dropouts did not differ from included patients (Table 2).

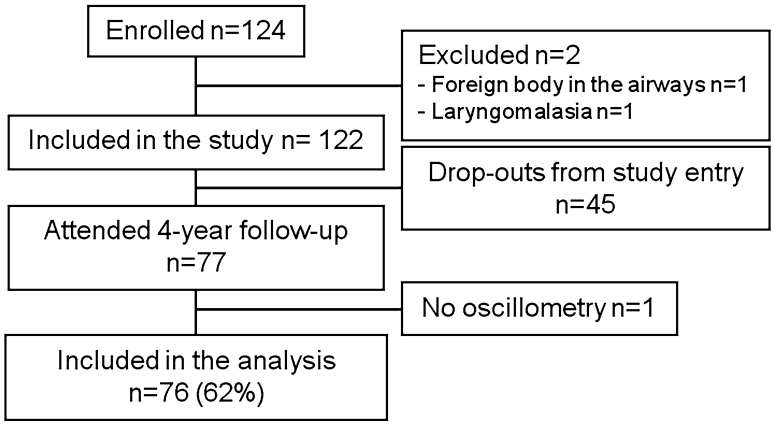

Figure 5 Study flow chart for Study IV.

5.2 Atopic characteristics, illness severity and virus etiology (I)

During the first wheezing episode, RV etiology was positively associated with age, blood eosinophil count ≥0.4 x 10^9/l, eczema, atopic eczema, prolonged cough, parental allergic rhinitis and parental smoking (all $p < 0.05$). In age-adjusted analyses, the association remained significant with all other but eczema and atopic eczema. RSV etiology was positively associated with hospitalization and negatively associated with age, male sex, eczema, blood eosinophilia and parental smoking (all $p < 0.05$). In age- and sex-adjusted analyses, all these associations remained significant. Virus coinfection was associated with maternal allergic rhinitis and prolonged wheezing, both in unadjusted and in age-adjusted analyses (all $p < 0.04$) (Table 3).

5.3 Vitamin D, atopic characteristics and virus etiology (II)

Serum 25OHD concentration was inversely associated with age, female sex, HBoV etiology and blood eosinophil count > 0.4 x 10^9/l (all $p < 0.05$) but not with other atopic characteristics or virus etiology. When adjusted with age, 25OHD concentration was inversely associated with female sex but not with any atopic characteristics or virus etiology.

Table 3 Associations between patient characteristics and virus etiology for *Study I*.

	RV n = 84	**RSV n = 31**	**HBoV n = 20**	**Coinfection n = 42**
Age, months	***1.1 (1.0, 1.2)***	***0.90 (0.83, 0.97)***	***1.1 (1.0, 1.2)***	0.98 (0.92, 1.0)
Male sex	2.3 (0.95, 5.7)	***0.33 (0.14, 0.79)***	0.54 (0.20, 1.5)	0.94 (0.45, 2.3)
Atopic character-	***5.4 (2.1, 12)***	***0.26 (0.10, 0.61)***	1.2 (0.45, 3.3)	0.72 (0.33, 1.6)
istics	***4.0 (1.5, 12)***	***0.37 (0.14, 0.97)***	0.70 (0.23, 2.1)	0.73 (0.32, 1.7)
Any sensitization	2.8 (0.87, 13)	0.55 (0.17, 1.5)	1.2 (0.36, 3.7)	0.90 (0.34, 2.2)
	1.6 (0.42, 7.5)	0.99 (0.27, 3.3)	0.68 (0.18, 2.2)	0.98 (0.35, 2.6)
Food sensitiza-	2.6 (0.81, 12)	0.59 (0.17, 1.6)	1.8 (0.19, 1.7)	1.2 (0.47, 3.1)
tion	1.6 (0.43, 7.6)	0.98 (0.27, 3.2)	1.2 (0.35, 3.7)	1.4 (0.50, 3.6)
Aeroallergen	p = 0.066*	***p = 0.018****	0.39 (0.021, 2.2)	0.29 (0.043, 1.2)
sensitization			***0.14 (0.0069, 0.93)***	0.29 (0.040, 1.3)
Total IgE, >45	1.5 (0.18, 2.0)	0.29 (1.1, 16)	2.7 (0.88, 8.0)	0.83 (0.30, 2.1)
kU/l	0.86 (0.24, 3.5)	0.46 (0.090, 1.8)	1.7 (0.51, 5.5)	0.98 (0.34, 2.7)
B-eos, >0.4x10^9/l	***15 (4.0, 94)***	***0.17 (0.080, 0.78)***	1.3 (0.47, 3.5)	0.53 (0.23, 1.2)
	11 (2.9, 72)	***0.27 (0.068, 0.63)***	0.76 (0.25, 2.3)	0.51 (0.21, 1.2)
Dermatitis	***4.2 (1.3, 19)***	***0.19 (0.042, 0.59)***	0.79 (0.24, 2.3)	0.44 (0.17, 1.1)
	3.4 (1.0, 15)	***0.23 (0.050, 0.73)***	0.52 (0.14, 1.6)	0.45 (0.17, 1.1)
Atopic dermatitis	***6.1 (1.1, 110)***	0.29 (0.04, 1.1)	1.0 (0.21, 3.5)	0.45 (0.12, 1.4)
	3.2 (0.54, 62)	0.56 (0.077, 2.6)	0.48 (0.090, 1.9)	0.45 (0.11, 1.5)
Parental allergic	***2.8 (1.1, 6.9)***	0.53 (0.23, 1.2)	2.4 (0.83, 7.7)	1.6 (0.74, 3.7)
rhinitis	***2.5 (1.0, 6.5)***	0.69 (0.28, 1.7)	2.0 (0.68, 6.7)	1.7 (0.77, 3.9)
Parental asthma	2.3 (0.71, 11)	0.51 (0.14, 1.5)	0.67 (0.15, 2.3)	1.2 (0.44, 3.0)
	2.4 (0.66, 12)	0.51 (0.13, 1.7)	0.65 (0.14, 2.3)	1.2 (0.44, 3.1
Parental smoking	***3.0 (1.2, 9.0)***	0.50 (0.20, 1.2)	0.57 (0.19, 1.6)	0.61 (0.27, 1.3)
	3.4 (1.2, 10)	***0.38 (0.14, 1.0)***	0.55 (0.17, 1.5)	0.61 (0.27, 1.3)

B-eos, Blood eosinophil count; HBoV, human bocavirus; IgE, immunoglobulin E; RSV, respiratory syncytial virus; RV, rhinovirus. Data expressed as odds ratio (95% confidence interval), results from both univariable (first line) and multivariable (second line) analyses are expressed. Multivariable analyses were adjusted to age (RV, HBoV, coinfection) or to age and sex (RSV). Bold and italic indicates a significant result, $p<0.05$. B-eos and IgE were log-transformed. *Odds ratios for RV or RSV etiology and aeroallergen sensitization were not calculable since, there were no aeroallergen-sensitized patients in RV-negative or RSV-positive group.

5.4 The long-term efficacy of prednisolone (III)

When compared to placebo, prednisolone did not affect the time to initiation of asthma control medication overall (p = 0.99), however, the RV genome load at study entry modified the effect of prednisolone (RV load x study drug interaction p = 0.04, Figure 5). In children with a RV genome load of >7000 copies/ml, the risk for initiation of the medication was lower in the prednisolone group compared to the placebo group (hazard ratio 0.38, 95% CI 0.14-1.01, p = 0.054). In the placebo group, asthma control medication was initiated to all the 9 children with a high RV genome load during the subsequent 14 months after the first wheezing episode.

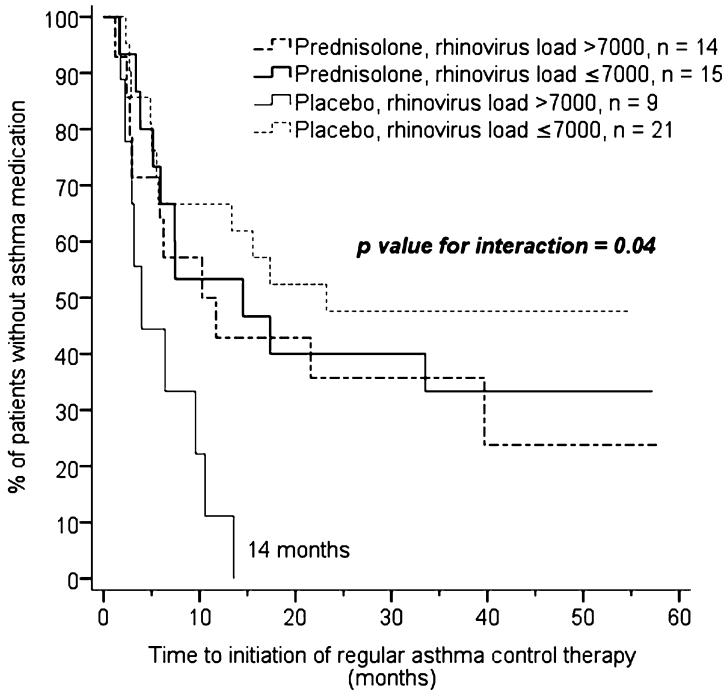

Figure 6 The time to initiation of asthma control medication in children randomized to receive prednisolone or placebo for the first RV-induced wheezing episode. Data are represented according to the RV genome load. Children with a RV genome load of >7000 copies/ml had longer time to initiation of asthma control medication in prednisolone group when compared with the placebo group. In the placebo group, asthma medication was initiated to all children with a high RV genome load (n = 9) during the 14 months after the first wheezing episode (Modified from *Study III)*.

5.5 Lung function and airway responsiveness (IV)

Of 76 children, one child had a pathological Rrs 5Hz value and one had pathological Xrs 5Hz value in the IOS baseline measurement. Children with atopic eczema (β -0.74; 95% CI -1.4 to -0.11; p = 0.022) or seasonal sensitization (β -1.1; 95% CI -2.1 to -0.14; p = 0.025) at study entry had lower Xrs 5Hz at baseline measurement than children without these characteristics. However, these results did not remain statistically significant in multivariable analyses with age, OCS for the first wheezing episode, RV etiology and ICS use within 4 weeks, in which only age remained statistically significant (β -0.052; 95% CI -0.095 to -0.009; p = 0.019).

Increased AR in the exercise test was diagnosed in 8 (10%) children. Increased AR was positively associated with atopic eczema (OR 14; 95% CI 2.7 to 72; p = 0.002) and allergic sensitization (OR 10; 95% CI 1.8 to 55; p = 0.008). The association with allergic sensitization remained in multivariable analysis, which included age at study entry, OCS for the first wheezing episode, allergic sensitization and RV etiology at study entry (OR 8.8; 95% CI 1.2 to 64; p = 0.032). The exercise-induced change in lnRrs 5Hz was positively associated with allergic sensitization (β 0.74; 95% CI 0.28 to 1.2; p = 0.002), atopic eczema at study entry (β 0.90; 95% CI 0.35 to 1.4; p = 0.002), hospitalization at study entry (β 0.73; 95% CI 0.18 to 1.3; p = 0.010) and ICS use within 4 weeks (β 0.70; 95% CI 0.080 to 1.3; p = 0.028). Allergic sensitization remained statistically significant in multivariable analyses, which included age at study entry, hospitalization, OCS at study entry, ICS use within 4 weeks, allergic sensitization at study entry and RV etiology (β 0.54; 95% CI 0.027 to 1.1; p = 0.027). Abnormal positive response for brochodilation with salbutamol was diagnosed in one (1%) child. The changes in post-bronchodilator values were not affected by any of the patient characteristics. The OCS treatment during the first wheezing episode did not affect the lung function or airway reactivity (Table 4).

Table 4 Lung function *vs.* patient characteristics, *Study IV*.

	Post-exercise Rrs 5Hz change ≥35% (n = 8)			
	Univariable		**Multivariable**	
Age at study entry, months	1.1 (0.97, 1.3)	p = 0.15	0.98 (0.84, 1.1)	p = 0.83
Age at 4-year follow-up, months	1.7 (0.55, 5.5)	p = 0.35		
Male sex	3.7 (0.43, 32)	p = 0.23		
OCS at study entry	2.9 (0.65, 13)	p = 0.16	1.7 (0.34, 8.9)	p = 0.51
ICS within 4 weeks	4.4 (0.87, 22)	p = 0.074		
Atopic eczema at study entry	***14 (2.7, 72)***	***p =0 .002***		
Sensitization at study entry	***10 (1.8, 55)***	***p = 0.008***	***8.8 (1.2, 64)***	***p = 0.032***
Food sensitization at study entry	***11 (2.0, 60)***	***p = 0.006***		
Aeroallergen sensitization at study entry	***9.8 (1.9, 50)***	***p = 0.006***		
Seasonal sensitization at study entry	***11 (1.2, 88)***	***p = 0.031***		
Perennial sensitization at study entry	***5.9 (1.1, 31)***	***p = 0.036***		
Parental smoking at study entry	0.92 (0.20, 4.2)	p = 0.92		
Maternal smoking at study entry	0.53 (0.060, 4.7)	p = 0.57		
Rhinovirus at study entry	n/a	p = 1.0	n/a	p = 1.0
Rhinovirus genome load >7000 copies/mL	0.17 (0.019, 1.5)	p = 0.11		
RSV	n/a	p = 1.0		
Co-infection	0.58 (0.11, 3.1)	p = 0.53		
25OHD, nmol/l	1.0 (0.96, 1.03)	p = 0.88		

B-eos, blood eosinophil count; 25OHD, 25-hydroxyvitamin D; ICS, inhaled corticosteroid; n/a, not applicable; OCS, oral corticosteroid; Rrs, resistance; RSV, respiratory syncytial virus. For baseline values, 2-sample t test and linear regression analysis were used, and results are expressed as regression coefficient beta or mean difference and 95% confidence intervals. For post-exercise values, logistic regression analysis was used, and results are reported as odds ratios and 95% confidence intervals. Multivariable model included age, OCS treatment, sensitization and rhinovirus etiology at study entry. Significant associations are shown in bold and italic.

6 DISCUSSION

6.1 Virus etiology of the first wheezing episode

In this study, RV was found to be the most common virus etiology in the airways of the first time wheezing children. The detection rate was 76%, which is higher than in earlier studies (Jartti et al. 2009, Marguet et al. 2009). This finding emphasizes the role that RV plays as an important causing agent of acute wheezing episode. On the other hand, the associations among RV etiology, wheezing, atopic characteristics and increasing age are well documented (Jartti et al. 2009, Lukkarinen et al. 2013, Midulla et al. 2010). Compared to previous studies on bronchiolitis (Bosis et al. 2008, Midulla et al. 2010, Mansbach et al. 2012), the present study included children with relatively old age (mean 12 months), had wheezing as an inclusion criterion and also consisted of a high incidence of atopic characteristics. This may explain the high detection rate of RV in our study population. The most probable explanation for the high detection rate is nevertheless the use of sensitive real-time PCR instead of the less sensitive conventional PCR (Jansen et al. 2011).

The detection rate of RSV was only 29% in the children, which is lower than in other studies concentrating on children aged less than 24 months (Rakes et al. 1999, Bosis et al. 2008, Backman et al. 2018). In our study, again, the age of the study population (mean age 12 months) was relatively old when compared to the earlier studies. Since the peak incidence of the RSV etiology takes place between the ages of 3-6 months (Jartti & Gern 2017), the high mean age of our study partly explains the low detection rate. Using wheezing as an inclusion criterion decreased the detection rate of RSV. The next frequent virus etiology was HBoV, which is in line with the earlier studies using serodiagnosis (Jartti et al. 2009, Midulla et al. 2010). The detection rate of coinfections (38%), as well, is in line with earlier studies (Jartti et al. 2009, Nascimento et al. 2010, Calvo et al. 2015, Garcia-Garcia et al. 2017a).

6.2 The associations between virus etiology and patient characteristics

In this study, we found that RV etiology was associated with atopic characteristics such as eczema and blood eosinophilia, which are usually considered as the two first manifestations of the atopic march. This finding is supported by earlier studies (Jartti et al. 2010b, Nascimento et al. 2010), which suggests that the asso-

ciation between RV infection and recurrent wheezing may be clearer in children with atopic characteristics (Lukkarinen et al. 2013, Midulla et al. 2014). Interestingly, in this study, RSV etiology did not seem to be associated with atopic characteristics, which emphasizes the role that RV etiology plays in atopic patients. The lack of the association may again be explained by the relatively high mean age of our study children, since RSV usually dominates earlier (Mansbach et al. 2012, Meissner 2016, Jartti & Gern 2017).

Atopic sensitization leads to an increase of the Th2 cell response and release of cytokines, such as IL-13 (Ingram & Kraft 2012, Lee et al. 2016). These changes cause an upregulation of ICAM-1 expression, which is used by some RV-A species for entering the cells. An increased number of ICAM-1 receptors on the cell surface of the bronchial epithelium leads to increased RV proliferation and replication (Blaas & Fuchs 2016). Increased IL-13 release also impairs the immune response to RV through inhibition of TLR3 expression (Contoli et al. 2015). Additionally, both RV infections and atopic sensitization have been connected to poor interferon response, such as deficient IFN-λ induction, which partly explains the association between atopic characteristics and RV etiology (Wark et al. 2005, Contoli et al. 2006, Johnston 2007, Baraldo et al. 2012, Moskwa et al. 2018). On the other hand, atopic characteristics together with a poor interferon response exacerbate clinical RV infection and thus highlight the children, who are at risk for developing asthma.

Prolonged cough was found to be associated with RV etiology. Children susceptible to RV infection are likely to have chronic, partly asthma-related inflammation in the airways. This inflammation causes clinical symptoms, mostly cough which is exacerbated by RV infection. Poor interferon responses are known to be related to more severe RV infection (Contoli et al. 2006, Sykes et al. 2012), which could also explain the association between RV and prolonged cough. In the present study, children with RSV infection were more often hospitalized during the first wheezing episode when compared to other children, which is in line with earlier studies (Marguet et al. 2009, Mansbach et al. 2012).

In our study, coinfection was associated with prolonged (≥3 days) wheezing, which is in line with some previous studies (Mansbach et al. 2012, Marcone et al. 2014), but not all (Marguet et al. 2009, Calvo et al. 2015, Petrarca et al. 2018). This association in the present study was found with coinfections overall but not with specific viruses such as RSV or RV, which have earlier been connected with illness severity when detected as a part of a coinfection (Mansbach et al. 2012, Calvo et al. 2015). Recently, in a systematic review and meta-analysis, no difference in illness severity between coinfections and single virus infections was found (Asner et al. 2014). Instead an increased risk of mortality in preschool-

aged children with coinfections was detected. However, further studies are needed to evaluate the clinical significance of coinfections.

6.3 The role of vitamin D

Age was the most important predicting agent for serum vitamin D level in our study population, with the vitamin D level being higher in younger children, which is in line with the earlier studies (Stenberg-Hammar et al. 2014, Heimbeck et al. 2013). Almost all parents reported regular use of vitamin D supplements, but the reported correlation with age may suggest that the use becomes more irregular as the child grows older.

We did not find any associations between the virus etiology or atopic characteristics and vitamin D status. Some earlier studies have reported a link between vitamin D levels and atopic characteristics (Mullins & Camargo 2012, Heimbeck et al. 2013, Wang et al. 2014). Furthermore, Dogru et al. recently reported that in wheezing children, a lower 25OHD level was associated with longer illness duration and a greater number of wheezing episodes (Dogru & Seren 2017). Compared to these studies, our negative finding may be explained by the higher serum vitamin D level in our study (86 nmol/l vs. 29-54 nmol/l) (Heimbeck et al. 2013, Wang et al. 2014), and a rather low detection rate of vitamin D deficiency. Further, in the study of Stenberg-Hammar et al., the serum vitamin D level of the study population was relatively good (mean 82 nmol/l in wheezing children). They reported an association between vitamin D levels and wheezing, but not with atopic characteristics. However, in that study the virus etiology was not evaluated (Stenberg-Hammar et al. 2014). The lack of the findings about the association between vitamin D level and atopic characteristics may suggest that the serum vitamin D level defined as sufficient for healthy children may be adequate also for children at high risk of developing asthma in terms of the pathogenesis of allergic diseases.

There is great variability in the studies concerning the associations between vitamin D and respiratory infections or atopic status. Many studies have measured only the use of the supplement or dietary intake (Urashima et al. 2010) instead of the actual serum 25OHD, which can be considered as a strength in our study. Also the study populations have diversity especially in age and study region latitude. These differences make it difficult to make conclusions about the sufficient vitamin D status and dietary intake. Thus, further studies about the role that vitamin D plays in wheezing and asthmatic patients are needed.

6.4 Efficacy of prednisolone at 4-year follow-up

This study is the first randomized, double-blinded, placebo-controlled trial studying the short- and long-term effect of short-course oral prednisolone for the first RV-induced wheezing episode. In the earlier analysis, prednisolone had a significant effect on a subgroup of children with a high RV load in a 12-month follow-up (Jartti et al. 2015). An earlier *post hoc* analysis (Lehtinen et al. 2007, Lukkarinen et al. 2013) supports our hypothesis by showing a long-term effect of prednisolone in RV-positive children with first acute wheezing episode in terms of reducing recurrent wheezing. Compared to the earlier Vinku study, in our study, prednisolone was not initiated until the RV was diagnosed with PCR which caused a delay (median of 45 hours) in initiating the study drug. This delay may be an explanation for the lack of overall effectiveness. Early administration may be critical, since viral load peaks during the first days of the infection (Kennedy et al. 2014). Also a different method used for the RV diagnostics may be an explanation for the differences. Conventional PCR used in Vinku, was in Vinku2 replaced by the quantitative RT-PCR, which can detect RVs at a lower level (Jartti et al. 2013). Thus, in the Vinku2 study, there were most probably children with a lower viral genome load diagnosed as RV than in the Vinku study, which highlights the role that a viral load plays.

Asthma-prone children have an underlying inflammation in the airways, which predisposes to RV infection. OCS reduces this underlying inflammation, which may be an explanation for the association between RV genome load and efficacy of OCS (Stellato 2007, de Benedictis & Bush 2012, Holt & Sly 2012). Moreover, a high RV genome load is found to be associated with more severe inflammation (Jartti et al. 2010b, Baraldo et al 2012, Sykes et al 2012, Xiao et al. 2015, Bruning et al. 2015). Underlying inflammation is associated with deficient interferon responses against virus infection. This leads to ineffective viral clearance, increased virus replication, promoted type 2 T-cell responses and, thus, more severe inflammation (Baraldo 2012, Contoli et al. 2015). Furthermore, RV infection may intensify inflammation by increasing the expression of eotaxin and interleukins 4 and 13 as well as by stimulating the immigration of eosinophils, macrophages and neutrophils (Stone & Miller 2015). OCS strengthens the epithelial barrier and thus protects it from viral infections. OCS also represses the transcription of the inflammatory genes and transcription factors as well as expresses anti-inflammatory genes (Stellato 2007, de Benedictis & Bush 2012). Further, OCS inhibits RV-induced ICAM-1 upregulation (Papi et al. 2000).

Our study underlines the role that RV plays in early acute wheezing episodes, since the effect of prednisolone on acute wheezing episode was reported previously, but these studies did not include viral detection (Jartti et al. 2002b, Plint et

al. 2009, Alansari et al. 2013). All the earlier studies have not confirmed the effect of prednisolone on early wheezing, but again, viral etiology was not determined (Oommen et al. 2013, Panickar et al 2009). The clinical challenge is to find the children who are at a high risk for developing asthma, and out of them, those who could benefit from early interventions. Our study suggests that the virus genome load may be one possible marker. However, the RV genome load measurement is not as widely used in clinical practice. These findings also suggest that OCS may have potential as an early intervention, but further studies are needed (Lukkarinen et al. 2015).

In Finland, a national Allergy Program was initiated in 2008 in order to change the common attitude towards allergy, recognize and focus on severe allergies and treat the underlying inflammation early (Haahtela et al. 2017). In the Vinku2 study, we used an early intervention with OCS for children at high risk of developing asthma. The efficacy of OCS is based on its potential in decreasing the underlying, probably atopy-related inflammation (Stellato 2007, de Benedictis & Bush 2012, Holt & Sly 2012). By treating the inflammation early with OCS, in Vinku2 with a follow-up until the age of 5 years, we were able to reduce the incidence of asthma in a subgroup of children with high rhinovirus genome load (71% in the prednisolone group vs. 100 % in placebo group) (Study III). This finding is in line with the Vinku study, where OCS treated RV-positive children had 40% less asthma (Lukkarinen et al. 2013). Both of these findings fit nicely with the aims of the Finnish Allergy Program.

6.5 Lung function and airway reactivity

Our finding about the association between atopic sensitization diagnosed during the first acute wheezing episode and increased AR 4 years later, at the age of five years, strongly suggests that atopic sensitization is a risk factor for asthma and calls attention to diagnosing the sensitization as early as possible. Earlier studies have shown that early atopic sensitization is associated with decreased lung function during later childhood (Illi et al. 2006, Belgrave et al. 2014, Turner et al. 2014). To our knowledge, Vinku2 is the first study concentrating on the development of lung function after the first virus-induced wheezing episode. Thus these results extend the earlier findings by showing the association between atopic sensitization at the time of the first wheezing episode and increased airway reactivity four years later.

The mechanisms between the atopic sensitization and AR are most probably based on the balance of type 1 and type 2 inflammation. Increased IL-4 and IL-13 production is linked to both AR and atopic sensitization and represent a Th2-

mediated mechanism (Ingram & Kraft 2012, Lee et al. 2016). The Th1-mediated mechanism may be based on reduced IFN-λ production both in atopic patients and patients with reduced lung function (Contoli et al. 2006, Baraldo et al. 2012).

In our study, the detection rates of decreased lung function and increased AR were relatively low when compared to earlier studies (Marotta et. al. 2003, Belgrave et al. 2014, Morales et al. 2015). According to Finnish guidelines (Asthma: Current Care Guideline, 2012), the ICS treatment for asthma is started earlier, when compared to international guidelines (NAEPP 2007). This may lead to better management of the symptoms and thus reduce the abnormal findings in lung function testing. In our study, 12 children were not able to discontinue the ICS treatment before the IOS testing, which also partly explains the good lung function results and increased AR in only 10% of the children.

6.6 Methods

6.6.1 Detection of viruses

For virus detection, we used real-time PCR which has been noticed to be more sensitive for virus detection when compared to conventional PCR (Jansen et al. 2011). Some previous studies have also used in situ hybridization from bronchial epithelium biopsies for RV diagnostics from lower airways (Papadopoulos et al. 2000, Malmström et al. 2006). However, there is still a lack of a sensitive, quick and non-invasive bed-side test for RV diagnostics. In this study, RV genome load was measured using quantitative RT-PCR. Since the high RV genome load has been found to be associated with more severe inflammation (Jartti et al. 2010b, Baraldo et al 2012, Sykes et al 2012, Xiao et al. 2015, Bruning et al. 2015), determining the viral load may help in the interpretation of the clinical significance of the PCR positivity. Other methods for determining the levels of rhinovirus following infection in airway epithelium include immunocytochemistry or Western blotting for capsid proteins (Mosser et al. 2002, Lopez-Souza et al. 2004, Lachowicz-Scroggins et al. 2010). These methods are useful for determining relative levels of virus, but they do not measure absolute levels of viral RNA. The number of infectious particles is only a small fraction of the total. Moreover, estimating of viral load by real-time PCR has several technical challenges, such as the low interassay reproducibility and variability of deficiency depending on the genotype being amplified (Schibler et al. 2012). Thus, using the quantitative PCR, new information about the disease pathogenesis, progression and clinical management may be found (Sachs et al. 2011, Sikazwe et al. 2016). However,

the sample collection and the method should be standardized. At the moment, there are no commercial quantitative PCR assays.

We used nasopharyngeal aspirate for collecting the samples for virus detection. Concerning the detection rates of the pathogens, Spyridaki et al. reported the detection rate from aspirate to be lower than from wash and higher than from brushes, but these differences were not statistically significant (Spyridaki et al. 2009). Concerning the discomfort, washes and aspirates were comparable, while swabs caused the least discomfort (Spyridaki et al. 2009). Earlier studies have also reported that swab has the same diagnostic sensitivity for virus detection as aspirate (Lambert et al. 2008, Spyridaki et al. 2009, Waris et al. 2013), which makes swab preferable when compared to aspirate.

6.6.2 Detection of vitamin D

Different markers are used for detecting vitamin D status. The 25-hydoxyvitamin D level is agreed to be the best available indicator of the net incoming contributions from cutaneous synthesis and total intake (Davis et al. 2007, Brannon et al. 2008, Ross et al. 2011). Thus, the serum 25OHD levels may function as a biomarker of exposure, but its role as a biomarker of effect is not clearly understood. The half-life of 25OHD is weeks, which makes it a relatively good marker of long-term vitamin D status (Ross et al. 2011). Ergocalciferol (Vitamin D_2) and cholecalciferol (Vitamin D_3) can also be measured separately. They both are prohormones, and it has been assumed that they are 25-hydroxylated at similar rates (Strushkevich et al. 2008), which makes 25OHD more useful method. On the other hand, d, calcitriol, i.e. 1,25-dihydroxyvitamin D, is the active hormonal form of vitamin D, hydroxylated from 25OHD. It is not a useful measurement, since the half-life is short, the levels are regulated by other factors, the formation is not directly regulated by vitamin D intake and the levels may be normal even during severe vitamin D deficiency due to the up-regulation of the 1α-hydroxylase enzyme (Ross et al. 2011).

6.6.3 Measurements of lung function and airway reactivity

For lung function and AR testing we used IOS together with exercise and bronchodilation testing. Due to the requirement of suitability to young children (e.g. less cooperation), IOS appears to be a reliable method starting from the age of 2 to 3 years (Vogel & Smidt 1994, Marotta et al. 2003, Dencker et al. 2006). However, it has some limitations. Even though the performance is easier than in spi-

rometry, it still requires some cooperation, which may be a problem especially in young children. In our study, the children cooperated well, IOS was sufficiently conducted in 99% if the children, which is partly explained by the relatively high age of the children (i.e. mean of 60 months). However, the good success rate in our study consolidates the role that IOS plays as a primary lung function method for preschool aged children. When compared to spirometry, IOS is less studied, and the interpretation of the results is less familiar for many practitioners (Beydon et al. 2007, Pellegrino et al. 2005, Rosenfeld et al. 2013). IOS is suitable for diagnosing obstructive diseases, but its reliability in other conditions, such as restrictive states, is uncertain, and more research is needed (Oostveen et al. 2003, Beydon et al. 2007). Other probable tests for measuring lung function in young children include specific airway resistance and functional residual capacity measured by whole-body plethysmography, maximal airflow at functional residual capacity by rapid thoraco-abdominal compression, interrupter resistance, functional residual capacity using gas dilution techniques and impedance pneumography (Beydon et al. 2007, Seppä et al. 2011). The recommendable test in every situation depends on the clinical/research question being asked. Systematic studies comparing a number of tests are needed for standardizing the methods and understanding the role of each test (Beydon et al. 2007).

We used a free running test for evaluating AR. It is an indirect measurement highly specific for asthma and reflecting airway inflammation. It has many advantages when compared to pharmacological challenges: it is natural, it stimulates the normal exercise pattern and it requires no complicated instrumentation. However, the free running test is susceptible to some factors, such as temperature and humidity (Malmberg et al. 2008). These effects were minimized by conducting the test inside when outside temperature was below +5°C. Our good success rate is in line with the earlier studies showing that the exercise test is practical method for testing airway reactivity already in pre-schoolers (Malmberg et al. 2008).

6.6.4 Detection of atopy with IgE

Atopic sensitization was defined as IgE antibodies to any of the common allergens included in the Phadiatop Combi®, which is generally accepted and widely used in scientific literature. We did not use skin prick testing due to its discomfort for the child and more difficult and time-consuming technical realization. The main clinical outcome of the study was the diagnosis of asthma, which was based on the NAEPP criteria (NAEPP 2007). In some children, ICS medication was initiated already after the third wheezing episode, which is based on the

Finnish guidelines (Asthma: Current Care Guidelines 2012). This may have lead to better management of the disease and thus reduce the findings concerning the lung function impairment.

6.7 Strengths and limitations

This is the first randomized placebo-controlled study about the effect of the short-course of oral corticosteroid given for the first RV-induced wheezing episode. No earlier studies have focused exclusively on the first wheezing episode. Other strengths include consecutive, detailed and careful data collection and characterization of the children, prospective study design and comprehensive viral diagnostics. We used a sensitive quantitative RT-PCR for viral diagnostics in an experienced laboratory. Serum 25OHD measurement was used for defining vitamin D status. For *Study III,* the probable asthma diagnoses were investigated from medical records also from children, who did not attend the follow-up visits, if possible, which reduces the selection bias. In this age-group, the use of IOS instead of spirometry can be considered as a strength. The IOS measurement had good quality, and the majority of the patients were able to discontinue the ICS before the testing.

However, this study has some limitations. The sample size was relatively small, which precluded some of the analyses. 80% of the children were hospitalized during the first wheezing episode, which probably makes these results not applicable for out-patient application or children with a mild wheezing illness. Children, aged less than 3 months, were excluded due to the intervention. RV diagnostics with RT-PCR caused a delay of 45 hours in the initiation of the study drug, which may have affected on the lack of findings concerning the overall effect of OCS.

7 SUMMARY AND CONCLUSIONS

First, at least one virus was detected from the airways from all children suffering from the first acute wheezing episode. RV was the most common agent followed by RSV and HBoV. RV etiology was associated with age, blood eosinophil count and eczema, which underlines the synergism between atopic characteristics and RV etiology already during the first wheezing episode. RV etiology was also associated with parental smoking and prolonged cough.

Second, the mean serum 25OHD level of the first-time wheezing children was normal when considering the target level set for the healthy children. Age was the major determinant of the serum 25OHD level, which was not associated with any atopic characteristics or virus etiology of the first acute wheezing episode. This may suggest that the target level set for the healthy children may be adequate also for the children at an elevated risk for asthma.

Third, a subgroup of children with high RV genome load benefitted from the short course of OCS given for the first RV-induced wheezing episode considering the time to initiation of the regular asthma control medication in a follow-up until the age of 5 years. This takes notice on the different phenotypes of the wheezing and the directing of the probable early interventions that could modify the natural course of asthma.

Fourth, atopic sensitization diagnosed during the first severe wheezing episode was associated with increased AR four years later. This finding emphasizes the role that atopic sensitization plays as a risk factor for asthma and suggests that detecting atopic sensitization early may be important in predicting the development of lung function of asthma-prone children.

In conclusion, RV is an important etiologic factor of the first wheezing episode and associates with atopic characteristics. Our long-term follow-up data suggests that the natural course of asthma may be modified by early and carefully targeted anti-inflammatory treatment. Development of lung function may be predictable according to some early characteristics, which may also direct the interventions. More carefully directed prospective clinical trials are needed for determining the correct intervention for asthma-prone children and for selecting the correct target group.

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APPENDICES

Appendix 1. Parental questionnaire*

The key questions
To be filled by study physician at parental interview

Name: _____
Social security number: _____
Names of the parents / guardians: _____
_____
Address: _____
Phone: _____
Email: _____

Does the child fulfill inclusion criteria of the study: age 3-23 months, >h37+0, first episode of breathing difficulty and written informed consent from the parents?
Yes □ No □

Does the child fulfill inclusion criteria of the intervention trial: rhinovirus PCR positive and still signs of lower respiratory infection (breathing difficulty, noisy breathing or cough)?
Yes □ No □

Randomized to receive the study drug: Yes □ No □
If yes, when (day, time) _____

Any exclusion criteria: chronic other than atopy related illness, previous systemic or inhaled corticosteroid treatment, participation to another study (excluding long-term follow-up studies in childhood), varicella contact if previously intact, need for intensive care unit treatment, or poor understanding of Finnish No □

Parents / guardians have received routine hospital wheezy questionnaires (2 forms) and symptom diaries (3 forms): Yes □

Height _____ cm and weight _____ kg
Still breastfeeding Yes □ No □
Duration of breastfeeding ___ months
Duration of exclusive breastfeeding ___ months
Does the child have doctor-diagnosed atopic eczema: Yes □ No □

	Mother	Father
Doctor-diagnosed asthma:	Yes □ No □	Yes □ No □
Allergic rhinitis:	Yes □ No □	Yes □ No □
Smoking:	Yes □ No □	Yes □ No □
Furry pets:	Yes □ No □	

Number of children in the family: ___ children
Daycare: Home □ Small group □ Kindergarden □

Wheezy questionnaire

To be filled by a parent/guardian

1. Does your child have a family doctor?
   No □ Yes □,Dr _____ practicing in _____

2. Type of daycare?
   1) Home □ 2) Family day care □ 3) Day care center □ 4) Other □ , what?_____

3. Type of home?
   1) Apartment building □ 2) House □ 3) Row house □ 4) Farm □
   5) Other □ what? _____

4. Number of children in the family? _____

5. Parental smoking? No □ Yes □ , if yes, smoking:
   1) inside No □ Yes □
   2) in the car No □ Yes □

6. Pets at home? dog No □ Yes □
   cat No □ Yes □
   other animals No □ Yes □ , what? _____

7. Other allergen sources at home?
   feather pillows/blankets No □ Yes □
   fitted carpet No □ Yes □

8. At day care pets/animals? No □ Yes □ , what? _____
   smoking? No □ Yes □

9. At other places, weekly exposure to
   animals? No □ Yes □
   smoking? No □ Yes □

10. Are there allergic symptoms in the family?
    eczema No □ Yes □ , underline: mother / father / sibling
    rhinitis No □ Yes □ , underline: mother / father / sibling
    asthma No □ Yes □ , underline: mother / father / sibling

11. Does the child have allergic symptoms? Please, mark the suspected source on the reverse side.
    eczema No □ Yes □
    rhinitis No □ Yes □
    asthma No □ Yes □

12. Does your child have an “allergy diet”?
    No □ Yes □ Please, specify the diet to the study nurse.

13. Has your child ever undergone skin prick tests?
    No □ Yes □ , when ___/___(month/year), where _____

14. Information about allergies (please circle the suspected sources):
    1) Dietary: chocolate, cocoa, citrus, egg, fish, tomato, strawberry, pea, apple, carrot, nuts, pear, peach, cow´s milk, breast milk substitute, rye, barley, oats, wheat, other_____
    2) Animals: dog, cat, horse, cow, guinea pig, feather, other _____
    3) Pollen: birch, alder, conifer, hay, mugworth, other _____
    4) Other causes: room dust, fungal spore, other_____

15. Information about the child´s respiratory infections:
    During the last 12 months:
    1) "common cold" ___ times
    2) antibiotic prescription ___ times
    3) pneumonias ___ times
    4) bronchitis ___ times
    5) otitis ___ times
    6) parasenthesis ___ times
    7) other, what? _____

16. Adenoidectomy
    No □ Yes □ , when ___/___ (month/year), where _____

17. Maxillary sinus puncture
    No □ Yes □ , when ___/___ (month/year), where _____

18. Information about breathing difficulty symptoms:
    Were there "common cold" symptoms during the current difficulty in breathing?
    No □ Yes □ I can't say □
    If you suspect other causes, please name them: _____

19. The duration of respiratory symptoms before study entry?
    1) rhinitis _____ days
    2) cough _____ days
    3) rhinitis _____ days

20. Have other family members had "common cold" symptoms? No □ Yes □

21. Is this your child's first episode of breathing difficulties? No □ Yes □

22. Does your child have any regular medication? No □ Yes , what? _____

*The key questions are directly translated from Finnish study form. The wheezy questionnaire contains selected questions from 2 page standard wheezy questionnaire and 7 page standard allergy questionnaire used at Turku University Hospital.

Appendix 2. Symptom diary 1, Vinku2 study.

Name **Social security number** _____

Daily symptom and medications until 2-week visit

Date (fill in one column per day)	An example 1.1.07							
Hospitalization for expiratory breathing difficulty, yes or no	Yes							
Cough, 0 (no) - 3 (severe)	2							
Expiratory breathing difficulty, 0 (no) - 3 (severe)	1							
Noisy breathing, 0 (no) - 3 (loud)	0							
Rhinitis, 0 (no) - 3 (severe)	0							
Night wakening for breathing difficulties, 0 (no), 1 (once), 2 (often), 3 (continuously)	1							
Temperature, exact or on scale 0 (no) - 3 (high)	Fever 37.9, or 1							
Any other symptom (report any deviation from normal)	Fell in stairs, tearful							
Other notes (e.g. cause of symptom)	acute otitis media, playing with a cat							
Study drug taken (tally)	111							
Bronchodilator (name, dose, number of doses; tally)	Ventoline 0.1 mg, puffs 1111							
Other medication	Amorion mixt 80 mg/ml; 3.7ml							
Doctor's appointment: where, why, name of the doctor, and treatment	Healthcenter Mäntymäki 1, fever, cough, tearful, Amorion for acute otitis media							

If any questions, do not hesitate to contact study physician by phone.

Appendix 3. Symptom diary, Vinku2 study

Name _____ **Social security number** _____

Report every episode of expiratory breathing difficulty during the 12 month follow-up period (one episode may last more than a day) and check also whether the patient was treated solely at home, as outpatient or as inpatient.

Episode number	Date	Treated solely at home	Doctor's appointment	Hospitalization	Corticosteroid	
					Oral	Inhaled
1						
2						
3						
4						
5						
6						
7						
8						
9						
10						
11						
12						
13						
14						
15						
16						
17						
18						
19						
20						

If any questions, do not hesitate to contact study physician by phone.

Appendix 4. Parental questionnaire for 4 year visit

Health during the last 12 months

1) Has the child had wheezing during the last 12 months?
   a. yes, times:_____
   b. no

   1b) If yes, has the wheezing occured
      a. at night, times:_____
      b. during ”common cold”, times:_____
      c. during exercise, times:____
      d. during animal exposure, times:_____
      e. during flower, hay or tree pollen exposure, times:____
      f. during dust exposure, times:_____
      g. in any other situation, what:_____

2) Has the child had doctor visits for wheezing during the last 12 months?
   a. yes
   b. no
   2b) If yes, where?
   a. outpatient department, times:___
   b. at ward, times: _____

3) Does the children have doctor diagnosed asthma?
   a. yes, when diagnosed?_____ where?_____
   b. no

4) How often the child has been waking up due to wheezing during the last 12 months?
   a. never
   b. less than once a month
   c. less than once a week
   d. at least once a week

5) Has the child’s breathing had a wheezing sound during running or other exercise during the last 12 months?
   a. yes, times:_____
   b. no

6) Has the child needed medication inhaled or orally during the last 12 months?
   a. yes
   b. no
   6b) If yes, has he used any of the following mediaction?
      a. yes: Becotide, Cortivent, Seretide, Flixotide, Pulmimcort, or any other corticosteroid
      b. no
   6c) If yes, how long was the medication used continuously?
      a. less than 2 weeks, times:_____

b. 2-4 weeks, times:_____
c. more than 4 weeks, times:____

7) Has the child needed this medication for difficulty in breathing only during ”common cold”?

a. yes
b. no

8) Has the child needed following bronchodilators for difficulty in breathing?

a. yes, liquid Ventoline, or Salbuvent, inhaled Ventoline; Serevent or Bricanyl
b. no

8b) Has the child needed the bronchodilator only during ”common cold”?

a. yes
b. no

9) How often the child has needed bronchodilator?

a. less than once a month
b. once or twice a month
c. once a week or more often

10) How many respiratory infections the child has had during the last 12 months?

a. none
b. times:_____

10b) If yes, what infections?

a. ”common cold”, _____times
b. pneumonia, _____times
c. laryngitis, _____times
d. tonsillitis, _____times
e. other, what?_____

11) How many otitis the child has had during the past 12 months?

a. none
b. times:_____

12) Has the child had cough that has continued more than 4 weeks during the past twelve months?

a. yes
b. no

13) Has the child had dry cough other than during a respiratory infection?

a. yes
b. no

14) Has the child had dry cough during night other than during a respiratory infection?

a. yes
b. no

14b) If yes, how long has it continued?

a. more than 4 weeks, times:____

b. less than 4 weeks, times:_____

15) Has the child had itching eczema during last 12 months?

a. yes

b. no

15b) If yes, has it occured in any of the following places: crook of the arms, hollow of the knee, front of the ankles, buttocks, neck, around the ears or eyes

a. yes

b. no

16) Has the eczema disappeared during the last 12 months?

a. yes

b. no

16b) If yes, how long has it lasted?

a. less than 2 weeks

b. 2-4 weeks

c. more than 4 weeks

16c) Has the eczema repeated during the last 12 months?

a. yes, times:_____

b. no

17) Do you have any pets?

a. yes, what?_____

b. no

17b) If yes, are they outside of the house?

a. yes

b. no

18) Is the child in weekly contact with animals?

a. yes, what?_____

b. no

19) Does anyone smoke at your home?

a. yes (father, mother, nanny)

b. no

19b) If yes, does the smoking happen

a. mostly inside

b. mostly outside

c. always outside

20) Where is the child's day nursery?

a. home

b. family day care

c. day-care center

d. other, what?_____

21) Total time of day nursery
   a. home _____months
   b. family day care _____months
   c. day care center_____months

22) Number of children in the family:_____

23) Has the child has allergic rhinitis?
   a. yes
   b. no
   If yes, when started?(mm/yy):_____/_____
   What is the most probable cause:_____

24) At any time of the year, has the child been in weekly contact with pets?
   a. yes
   b. no
   If yes, when started? (mm/yy)_____

25) Has the child got corticosteroid treatment orally, intramuscularry or intravanously?
   a. yes, how many?_____
   b. no

26) Does the child have any other diseases or medications?
   a. yes
   b. no
   If yes, what_____
   When started_____
   Where diagnosed_____
   Duration_____

Earlier than the last 12 months, has the child had

Doctor diagnosed atopic eczema Yes____ No_____, if yes, when started (mm/yy)____/____
Regular asthma medication Yes____No_____, if yes, when started (mm/yy)____/____
Doctor diagnosed asthma Yes____No_____, if yes, when started (mm/yy)____/____
Allergic rhinitis Yes____No_____, if yes, when started (mm/yy)____/____

Any other disease, what and when started

_____

Allergy and asthma questions to mother

Has mother ever had allergic rhinitis (respiratory symptoms due to allergens such as pollen, animals or dust) Yes_____No_____

If yes: What caused?_____

Pollen or animals Yes_____No_____

Symptoms as a child, but not after 16 years of age Yes_____No_____

Still symptoms, but no doctor dianosis Yes_____No_____

Still symptoms and need for follow-ups Yes_____No_____

Has mother ever had doctor diagnosed asthma Yes_____No_____

If yes:

Symptoms as a child, but not after 16 years of age Yes_____No_____

Still symptoms, but no doctor diagnosis Yes_____No_____

Still symptoms and need for medication Yes_____No_____

What causes symptoms (e.g. allergens, exercise, cold air, flu, medication etc.)

_____

Where diagnosed?_____

Has mother ever had doctor diagnosed food allergy Yes_____No_____

If yes,

What diagnosed by skin prick test or blood samples?_____

What diagnosed by doctor supervised predisposition?_____

Other food allergies?_____

Symptoms as a child but not after 16 years of age Yes_____No_____

What still causes symptoms?_____

Where diagnosed?_____

Has mother ever had doctor diagnosed atopic eczema Yes_____No_____

If yes

Symptoms as a child, but not after 16 years of age Yes_____No_____

Still symptoms and need for skin cream or mild corticosteroid cream? Yes_____ No_____

Still symptoms and need for medium or strong corticosteroid cream / tacrolimus or pimecrolimus cream or light therapy? Yes_____No_____

Where diagnosed?_____

Allergy and asthma questions to father

Has father ever had allergic rhinitis (respiratory symptoms due to allergens such as pollen, animals or dust) Yes_____No_____

If yes: What caused?_____

Pollen or animals Yes_____No_____

Symptoms as a child, but not after 16 years of age Yes_____No_____

Still symptoms, but no doctor dianosis Yes_____No_____

Still symptoms and need for follow-ups Yes_____No_____

Has father ever had doctor diagnosed asthma Yes_____No_____

If yes:

Symptoms as a child, but not after 16 years of age Yes_____No_____

Still symptoms, but no doctor diagnosis Yes_____No_____

Still symptoms and need for medication Yes_____No_____

What causes symptoms (e.g. allergens, exercise, cold air, flu, medication etc.)

_____

Where diagnosed?_____

Has father ever had doctor diagnosed food allergy Yes_____No_____

If yes,

What diagnosed by skin prick test or blood samples?_____

What diagnosed by doctor supervised predisposition?_____

Other food allergies?_____

Symptoms as a child but not after 16 years of age Yes_____No_____

What still causes symptoms?_____

Where diagnosed?_____

Has father ever had doctor diagnosed atopic eczema Yes_____No_____

If yes

Symptoms as a child, but not after 16 years of age Yes_____No_____

Still symptoms and need for skin cream or mild corticosteroid cream? Yes_____ No_____

Still symptoms and need for medium or strong corticosteroid cream / tacrolimus or pimecrolimus cream or light therapy?

Yes_____No_____

Where diagnosed?_____

*The parental questionnaire questions are directly translated from Finnish study form

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