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RHINOVIRUS INFECTIONS IN YOUNG CHILDREN: CLINICAL MANIFESTATIONS, SUSCEPTIBILITY, AND HOST RESPONSE

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ABSTRACT

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Rhinovirus infections in young children: Clinical manifestations, susceptibility, and host response

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Rhinoviruses are the most common cause of respiratory infections, but the burden of rhinovirus infections in young children has not been evaluated. A diagnostic marker of virus infections detecting also rhinovirus infections could be useful for avoiding the unnecessary use of antibiotics.

In this prospective birth cohort study, we followed 1570 children for acute respiratory infections from birth to two years of age. We aimed to establish the burden of rhinovirus infections in young children and to study the genetic susceptibility and blood myxovirus resistance protein A (MxA) response to respiratory infections.

Altogether 12 846 episodes of acute respiratory infection were documented with an annual rate of 5.9 per child. Rhinovirus was detected in 59% of acute respiratory infections that were analyzed for viruses. Rhinoviruses were associated with 50% of acute otitis media episodes, 41% of wheezing illnesses, 49% of antibiotic treatments, and 48% of outpatient office visits for acute respiratory infections. The estimated annual rate of rhinovirus infections was 3.5 per child. Children with recurrent respiratory infections were at an increased risk for asthma in early childhood. Genetic polymorphisms of mannose-binding lectin and toll-like receptors were associated with the risk of respiratory infections. Blood MxA protein levels were increased in children with symptomatic virus infections, including rhinovirus infections, as compared to asymptomatic virus-negative children.

Rhinovirus infections impose a major burden of acute respiratory illness and antibiotic use on young children. Genetic polymorphisms may partly explain why some children are more prone to respiratory infections. Blood MxA protein is an informative marker of viral respiratory infections including rhinovirus infections.

Keywords: rhinovirus, children, respiratory tract infections, acute otitis media, single nucleotide polymorphism, mannose-binding lectin, toll-like receptor, MxA protein

TIIVISTELMÄ

LL Laura Toivonen

Rinovirusinfektiot pienillä lapsilla: kliininen kuva, alttius ja immunologinen vaste

Turun yliopisto, Lääketieteellinen tiedekunta, Kliininen laitos, Lastentautioppi, Turun yliopiston tutkijakoulu, kliininen tohtoriohjelma (TKT); Lasten ja nuorten klinikka, Turun yliopistollinen keskussairaala; ja Turun lapsi- ja nuorisotutkimuskeskus, Turun yliopisto, Turku, Suomi

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Rinovirukset ovat yleisimpiä hengitystieinfektioiden aiheuttajia, mutta rinovirusten pienille lapsille aiheuttamaa tautitaakkaa ei ole tarkkaan kuvattu. Virusinfektion diagnostinen merkkiaine, joka tunnistaisi myös rinovirusinfektiot, voisi auttaa välttämään antibioottien tarpeetonta käyttöä.

Tässä prospektiivisessa syntymäkohorttitutkimuksessa seurattiin 1570 lasta äkillisten hengitystieinfektioiden suhteen syntymästä kahden vuoden ikään. Tavoitteenamme oli selvittää rinovirusten aiheuttama tautitaakka pienillä lapsilla ja tutkia geneettistä alttiutta ja veren myksovirusresistenssiproteiini A (MxA) -vastetta hengitystieinfektioille.

Tutkimuksessa todettiin yhteensä 12 846 äkillistä hengitystieinfektiota. Lapset sairastivat keskimäärin 5,9 hengitystieinfektiota vuodessa. Rinovirus todettiin 59 %:ssa hengitystieinfektioista, joista tutkittiin viruksia. Rinovirus liittyi 50 %:iin äkillisistä välikorvatulehduksista, 41 %:iin vinkutaudeista, 49 %:iin antibioottihoidoista ja 48 %:iin lääkärikäynneistä, jotka liittyivät hengitystieinfektioihin. Rinovirusinfektioiden arvioitu määrä oli keskimäärin 3,5 lasta kohden vuodessa. Toistuvia hengitystieinfektioita sairastavilla lapsilla todettiin useammin varhaislapsuuden astma kuin muilla lapsilla. Mannoosia sitovan lektiinin ja tollin kaltaisten reseptorien geneettiset vaihtelut liittyivät hengitystieinfektioiden riskiin. Veren MxA-proteiinin pitoisuus nousi lasten oireisissa hengitystieinfektioissa ja rinovirusinfektioissa oireettomiin virus-negatiivisiin lapsiin verrattuna.

Rinovirusten aiheuttama hengitystieinfektioiden ja niihin liittyvien antibioottihoitojen muodostama tautitaakka pienillä lapsilla on huomattava. Geneettiset vaihtelut voivat osittain selittää, miksi osa lapsista on alttiimpia hengitystieinfektioille. Veren MxA-proteiini on toimiva virusinfektion merkkiaine hengitystieinfektioissa ja rinovirusinfektioissa.

Avainsanat: rinovirus, lapset, hengitystieinfektiot, äkillinen välikorvatulehdus, yhden emäksen polymorfismi, mannoosia sitova lektiini, tollin kaltainen reseptori, MxA-proteiini

TABLE OF CONTENTS

ABBREVIATIONS

AOM	acute otitis media
ARI	acute respiratory infection
AUC	area under the curve
CDHR3	cadherin-related family member 3
CI	confidence interval
CRP	C-reactive protein
DNA	deoxyribonucleic acid
ds	double-stranded
EIA	enzyme immunoassay
hMPV	human metapneumovirus
HRV	human rhinovirus (old name)
ICAM-1	intercellular adhesion molecule 1
IFN	interferon
Ig	immunoglobulin
IL	interleukin
IP-10	IFN-γ-induced protein 10
IQR	interquartile range
LDLR	low-density lipoprotein receptor
MAF	minor allele frequency
MBL	mannose-binding lectin
mRNA	messenger RNA
MxA	myxovirus resistance protein A
OR	odds ratio
PCR	polymerase chain reaction
PIV	parainfluenza virus
RNA	ribonucleic acid
ROC	receiver operating characteristic
RR	rate ratio
RRTI	recurrent respiratory tract infection
rs	reference single nucleotide polymorphism
RSV	respiratory syncytial virus
RT	reverse transcriptase
RV	rhinovirus (current name)
SNP	single nucleotide polymorphism
ss	single-stranded
STEPS	Steps to the Healthy Development and Wellbeing of Children
TLR	toll-like receptor
TNF	tumor necrosis factor
TRAIL	TNF-related apoptosis-inducing ligand
VP	viral protein
WBC	white blood cell

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by the Roman numerals I-IV. Previously unpublished data are also included.

I Toivonen Laura, Schuez-Havupalo Linnea, Karppinen Sinikka, Teros-Jaakkola Tamara, Rulli Maris, Mertsola Jussi, Waris Matti, Peltola Ville. Rhinovirus infections in the first 2 years of life. *Pediatrics. 2016;138(3):e20161309.*

II Toivonen Laura, Karppinen Sinikka, Schuez-Havupalo Linnea, Teros-Jaakkola Tamara, Vuononvirta Juho, Mertsola Jussi, He Qiushui, Waris Matti, Peltola Ville. Burden of recurrent respiratory tract infections in children: a prospective cohort study. *Pediatr Infect Dis J. 2016 Jul 22. [Epub ahead of print]*

III Toivonen Laura, Vuononvirta Juho, Mertsola Jussi, Waris Matti, He Qiushui, Peltola Ville. Polymorphisms of mannose-binding lectin and toll-like receptors 2, 3, 4, 7, and 8 and the risk of respiratory infections and acute otitis media in children. *Submitted.*

IV Toivonen Laura, Schuez-Havupalo Linnea, Rulli Maris, Ilonen Jorma, Pelkonen Jukka, Melen Krister, Julkunen Ilkka, Peltola Ville, Waris Matti. Blood MxA protein as a marker for respiratory virus infections in young children. *J Clin Virol. 2015;62:8-13.*

1 INTRODUCTION

Acute respiratory infections are a major cause of morbidity in infants and young children with a rate of five to six infections per child per year (Monto and Sullivan 1993, Chonmaitree *et al.* 2008, van der Zalm *et al.* 2009a, Byington *et al.* 2015). Rhinoviruses are the most common cause of acute respiratory infections in both children and adults and are detected in one half of acute respiratory infections (Mäkelä *et al.* 1998, van der Zalm *et al.* 2009a, Anders *et al.* 2015). Over 160 types of rhinoviruses have been identified to date (Royston and Tapparel 2016). There is only little cross-protection among serotypes after a rhinovirus infection, and frequent infections by different rhinovirus types occur (Jartti *et al.* 2008b). Rhinoviruses cause an estimated 250 million infections only in the United States per year (Fendrick *et al.* 2003, Ruuskanen and Hyypiä 2005) and a considerable economic burden because of medical visits and missed workdays (Bertino 2002, Bramley *et al.* 2002, Fendrick *et al.* 2003). Direct and indirect costs associated with viral respiratory infections were estimated to be 40 billion dollars per year in the United States, which is similar to that of hypertension and greater than that of asthma or chronic obstructive pulmonary disease (Fendrick *et al.* 2003). Clinical manifestations of rhinovirus infections range from asymptomatic infections and common cold to asthma exacerbations and severe respiratory diseases requiring hospitalization (Miller *et al.* 2007, Peltola *et al.* 2009, Iwane *et al.* 2011, Kieninger *et al.* 2013, Mackay *et al.* 2013), and they are often complicated by acute otitis media in young children (Chonmaitree *et al.* 2008). Rhinovirus infections are more frequent in infants and young children than in older children and adults (Fox *et al.* 1975, Fox *et al.* 1985, Monto *et al.* 1987) and young children transmit rhinoviruses efficiently to other family members (Peltola *et al.* 2008). Despite increasing data on rhinovirus infections, the disease burden of rhinovirus infections in young children in the community is not well evaluated.

While several annual episodes of uncomplicated respiratory infections in young children are typical, particular attention should be paid to children who have unusually frequent or prolonged infections as they and their families carry a substantial burden of respiratory infections and associated outcomes. The definition of recurrent respiratory infections has varied from certain numbers of infection episodes per year to specific diagnoses (Alho *et al.* 1990, Nokso-Koivisto *et al.* 2002, Emonts *et al.* 2007, Jartti *et al.* 2008b), but infections may overlap with each other or be prolonged, especially in those with recurrent infections. Along with environmental risk factors, genetic variations in important factors of the innate immune system may explain the increased susceptibility to respiratory infections (Koch *et al.* 2001, Emonts *et al.* 2007) but the genetic susceptibility to rhinovirus infections is not well characterized.

Currently there is no approved treatment or medical prophylaxis available for rhinovirus infections. Antibiotics are often prescribed for respiratory infections even though the vast majority are caused by viruses (Bertino 2002, Grijalva *et al.* 2009). Antimicrobial resistance has increased dramatically worldwide in recent years due to an excessive use of antibiotics including broad-spectrum antimicrobials. C-reactive protein (CRP), white blood cell (WBC) count, procalcitonin, and other biomarkers can be used in discriminating bacterial from viral infections (Van den Bruel *et al.* 2011) but there is no diagnostic marker specific for virus infections in clinical use. A diagnostic marker for a broad spectrum of virus infections would be useful in order to avoid the unnecessary use of antibiotics in children with acute febrile respiratory virus infections. Blood myxovirus resistance protein A (MxA) is an interferon-induced protein specific for virus infections. Blood MxA protein could be a potential diagnostic marker of a virus infection, but MxA response in rhinovirus infections has not been shown.

In this prospective observational birth cohort study, we aimed to establish the burden of acute respiratory infections caused by rhinovirus during the first two years of life. We followed up the occurrence and clinical outcomes of acute respiratory infections in a cohort of healthy children from birth to two years of age. Home nasal swab sampling by parents was utilized in addition to sampling at the study clinic. We describe the early risk factors, clinical and virologic characteristics, and short-term consequences of recurrent respiratory tract infections in children younger than two years of age. We also assessed the effect of certain genetic variations on the risk of respiratory infections and evaluated blood MxA protein levels in young children with respiratory infections and in a healthy state.

2 REVIEW OF THE LITERATURE

2.1 History of rhinovirus research

Rhinovirus was first isolated as a "DC agent" in 1953 from a nasal washing from a cell biologist with symptoms of upper respiratory tract infection working at the Common Cold Research Unit, England (Andrewes *et al.* 1953). However, this common cold virus was not recognized until 1968 when it was identified as human rhinovirus (HRV) (Conant *et al.* 1968). During the next years, rhinovirus was isolated in 1954 by Pelon and colleagues from recruits with respiratory symptoms at the Great Lakes Naval Training Center in Chicago during an outbreak of common colds (Pelon *et al.* 1957). In 1956, Price and colleagues independently reported about an isolation of an antigenically identical virus from nurses and children with symptoms of common cold (Price 1956). In the early 1960s, new culture methods involving a lowered incubation temperature and a pH neutrality that imitated the conditions of the nose led to an increase in isolation of new rhinovirus strains (Tyrrell and Parsons 1960, Hayflick and Moorhead 1961, Taylor-Robinson and Tyrrell 1962). The name "rhinovirus" (from Greek word *rhino*, "nose") was chosen for the group of biologically related strains because of the specific adaptation of these viruses for growth in nasal epithelium (Tyrrell and Chanock 1963). Large community surveys conducted during the 1960s and 1970s, including the Virus Watch studies in Seattle and New York and the Tecumseh study in Michigan in the United States, revealed the epidemiology and clinical manifestations of rhinovirus infections (Monto and Cavallaro 1972, Fox *et al.* 1975). Implementation of rapid and sensitive reverse transcriptase polymerase chain reaction (RT-PCR) methods in the 1990s revolutionized the diagnostics of rhinovirus infections, and the role of rhinoviruses as respiratory pathogens in infants, in exacerbations of asthma and chronic obstructive pulmonary disease, and in hospitalized and immunocompromised patients was revealed. In 2006, a novel rhinovirus species, group C rhinoviruses, were found by molecular methods from patients with an influenza-like illness that tested negative for influenza (Lamson *et al.* 2006). Molecular dating revealed that these genotypes had been circulating for at least 250 years and were globally distributed (Briese *et al.* 2008). Recent advances in molecular techniques have made rapid PCR based tests for respiratory viruses, including rhinoviruses, available for laboratories and even for bedside testing providing a fast method for diagnostics of rhinovirus infections in the clinical setting.

2.2 Viral structure and replication

Rhinoviruses are small non-enveloped viruses that belong to the *Picornaviridae* family and to the genus *Enterovirus*. Rhinoviruses have a positive-sense, single-stranded (ss) RNA genome of approximately 7200 nucleotides long encoding 11 proteins (Royston

and Tapparel 2016). Rhinoviruses infect primarily epithelial cells of the airways by using cell surface receptors and enter the cell via endocytosis. When released to the cytoplasm, viral RNA is translated by the host cell ribosome into a polyprotein, which is cleaved by virally encoded proteases (Jacobs *et al.* 2013). Structural viral protein (VP) 1, VP2, VP3, and VP4 form the viral capsid, and the nonstructural proteins are involved in viral genome replication and assembly. The VP4 anchors the RNA core to the capsid and genetic changes in VP1, VP2, and VP3 account for the antigenic diversity of rhinoviruses. Rhinoviral replication occurs in the cytoplasm and one replication cycle of rhinoviruses is typically completed in 10 to 12 hours. An infected cell may produce even 100 000 infectious particles that are released by lysis of the cell (Ruuskanen and Hyypiä 2005). Of the formed virus particles, only 1 in 200 is a complete infectious virus capable of replicating in the cell culture (Atmar and Englund 2014).

Rhinoviruses are classified into three species, rhinovirus A, B, and recently found group C. Over 160 types of rhinoviruses have been found to date (Figure 1). Species A and B rhinoviruses were originally classified serologically and are based on nucleotide sequence homology in 76 (sero)types in species A and in 25 (sero)types in species B (Savolainen *et al.* 2002). Species C rhinoviruses do not grow on standard cell cultures, and this delayed their discovery. Gern and colleagues were the first to successfully cultivate rhinovirus C *in vitro* in organ culture of nasal epithelial cells (Bochkov *et al.* 2011). Over 50 different type C rhinoviruses have been identified to date based on nucleotide differences in VP1 or VP4/VP2 region (Royston and Tapparel 2016). Rhinoviruses were originally named as human rhinoviruses (HRV), but in 2013, the International Committee on Taxonomy of Viruses (ICTV) renamed these species simply as Rhinovirus A, B, and C (Picornaviridae website. Available online: http://www.picornaviridae.com). After the discovery of species C rhinoviruses, the name “serotype” was changed to “genotype” or simply “type” (Royston and Tapparel 2016).

Rhinoviruses utilize three major types of cellular membrane glycoproteins as receptors to enter the host cell. The majority of the species A and all species B rhinoviruses enter the cells by using intercellular adhesion molecule 1 (ICAM-1) on the cell surface as a receptor, while 12 types of species A rhinoviruses (the minor group) use low-density lipoprotein receptor (LDLR) (Bochkov and Gern 2016). The receptor of species C rhinoviruses was recently identified as cadherin-related family member 3 (CDHR3), which is highly expressed in airway epithelial cells (Bochkov *et al.* 2015, Bochkov and Gern 2016). The biological function of CDHR3 is not known and there is only little information available on mechanisms of interaction between rhinovirus C and CDHR3 (Bochkov *et al.* 2015, Bochkov and Gern 2016).

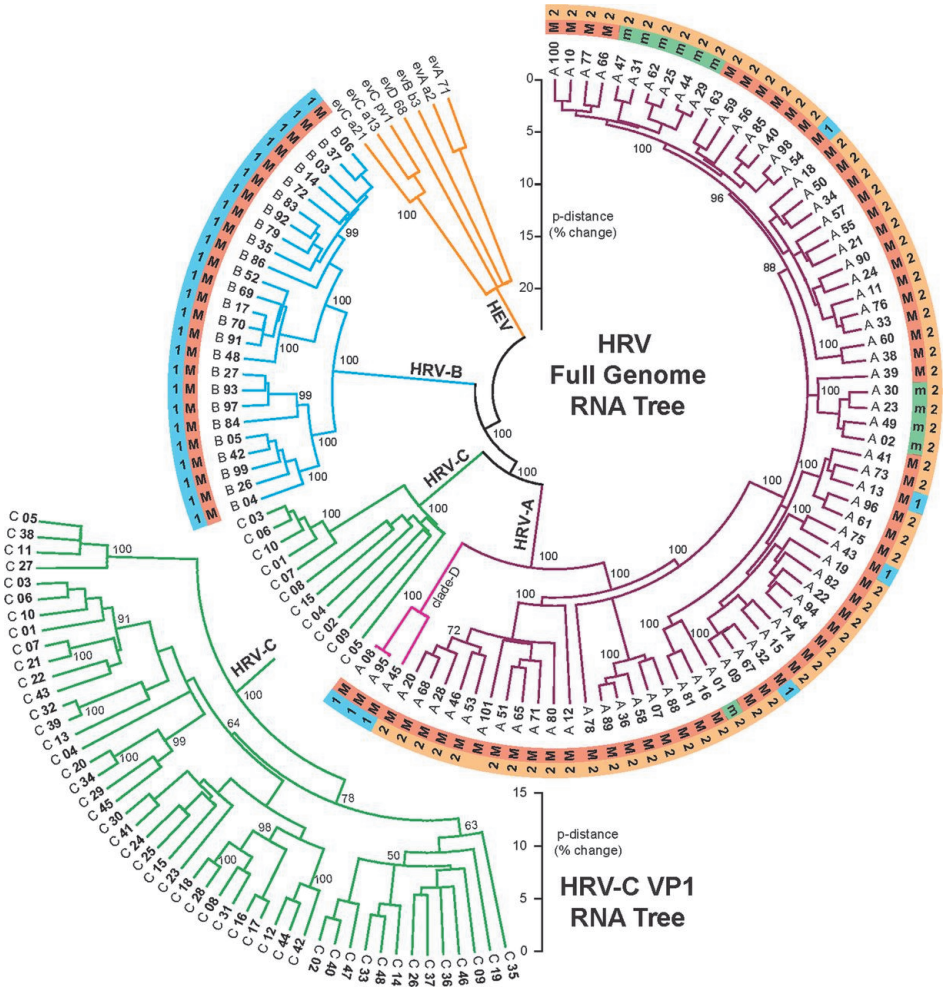

Figure 1. Circle phylogram showing relationships for known genotypes of species A, B, and C rhinoviruses. The inner ring shows members of the major ("M", intercellular adhesion molecule 1 [ICAM-1]) and minor ("m", low-density lipoprotein receptor [LDLR]) receptor groups. The receptor of species C rhinoviruses was recently identified as cadherin-related family member 3 (CDHR3). Bootstrap values are indicated at key nodes. HRV, human rhinovirus; RNA, ribonucleic acid; VP, viral protein. Reprinted from Palmenberg and Gern 2013: Rhinoviruses. In Knipe DM, Howley PM (ed), Fields Virology, 6th edition, and used with the permission of Wolters Kluwer Health.

2.3 Epidemiology

Rhinoviruses are the most common cause of respiratory infections in both adults and children (Monto and Ullman 1974, Monto and Sullivan 1993, Mäkelä *et al.* 1998). With the use of new molecular detection techniques, rhinoviruses have been found in up to 73% of all acute respiratory infections in young children (Table 1). In children under five years of age, rhinovirus infections are at least twice as frequent

as in adults (Fox *et al.* 1985, Monto and Sullivan 1993). The frequency of rhinovirus infections is highest in children under two years of age and correlates inversely with age, with the exception of the age group 20-29 years, which is thought to be due to the high exposure of parents to young children at this age (Fox *et al.* 1985, Monto *et al.* 1987, Linder *et al.* 2013). In a recent cohort study using sensitive PCR methods, the mean rate of rhinovirus infections in Andean children under three years of age was 2.4 per child per year (Budge *et al.* 2014). Rhinovirus infections occur early, as more than 20% of children are seropositive at 6 months of age and have had at least one laboratory-confirmed rhinovirus episode (Blomqvist *et al.* 2002). By the age of 2 years, 79% of the children have had at least one PCR positive rhinovirus episode, and 91% of the children are seropositive for rhinovirus (Blomqvist *et al.* 2002).

The most common complication of a rhinovirus infection in young children is acute otitis media and rhinoviruses have been found in nasopharyngeal aspirates in 41% of acute otitis media episodes (Blomqvist *et al.* 2002). Rhinoviruses are important causes of wheezing illnesses and asthma exacerbations and have been detected in 3-34% of hospitalized bronchiolitis patients (Turunen *et al.* 2014) and in up to 45% of wheezing illnesses in a birth cohort study (Kusel *et al.* 2006). In a recent study, rhinoviruses were found in even 76% of the children with the first wheezing episode (Turunen *et al.* 2014). Rhinoviruses dominate the etiology of wheezing illnesses after 9 to 12 months of age and that of recurrent wheeze (Rakes *et al.* 1999, Jartti *et al.* 2009). Rhinoviruses are an important cause of respiratory infections leading to hospitalization and have been found in up to 30% of children hospitalized for acute respiratory infection with the rate of 4.8 rhinovirus-associated hospitalizations per 1000 children in children under five years (Cheuk *et al.* 2007, Miller *et al.* 2007, Peltola *et al.* 2009).

All species of rhinoviruses are distributed worldwide (Briese *et al.* 2008). Rhinoviruses cause infections throughout the year with a high incidence peak in early fall and a smaller peak in the spring (Monto 2002). Rhinoviruses have been found in up to 90 percent of acute respiratory infections in adults during epidemic seasons in autumn (Arruda *et al.* 1997, Mäkelä *et al.* 1998). The seasonal pattern is identical but opposite in the northern and southern hemispheres (Atmar and Englund 2014). The reasons for this seasonal pattern remain uncertain, as studies intending to assess causality between cold and humid weather have not been able to show causality (Douglas *et al.* 1967). Rhinoviruses have been found to survive better in an environment with a relative humidity of over 50% (Hendley and Gwaltney 1988). The autumn incidence peak coincides with the start of the scholar year, which is thought to enhance transmission. Nevertheless, the spring incidence peak cannot be explained with this hypothesis.

Table 1. Studies assessing the proportion of rhinovirus infections from acute respiratory infections in young children in the community.

Study	Country	Study design	Population	Children, *n*	Age	Proportion of rhinovirus infections in ARIs
Fox *et al.* 1985	USA	Prospective follow-up	Healthy children	*NA*	< 5 yrs	11% (culture only)
de Arruda *et al.* 1991	Brazil	Prospective follow-up	Healthy children	175	< 5 yrs	17% (culture only)
Blomqvist *et al.* 2002	Finland	Prospective cohort	Healthy children	329	≤ 2 yrs	29%
Souza *et al.* 2003	Brazil	Prospective cohort	Healthy children attending daycare center	138	2-24 months	48%
van Benten *et al.* 2003	The Netherlands	Prospective birth-cohort	Healthy children and children with family history of atopy	126	≤ 2 yrs	42%
Kusel *et al.* 2006	Australia	Prospective birth-cohort	Infants at high-risk for atopy	263	≤ 1 yr	49%
Regamey *et al.* 2008	Switzerland	Prospective follow-up	Healthy infants	197	≤ 1 yr	23%
Jartti *et al.* 2008b	USA	Prospective birth-cohort	Children at increased risk for asthma with serial infections	27	≤ 1 yr	43% in mild illnesses, 61% in moderate-to-severe illnesses
van der Zalm *et al.* 2009a	The Netherlands	Prospective birth-cohort	Healthy children	305	≤ 1 yr	73% RV positive, 70% RV alone
van der Zalm *et al.* 2009b	The Netherlands	Prospective, longitudinal	Healthy children	18	< 7 yrs	23%
Ruohola *et al.* 2009	Finland	Prospective	Children with URI without AOM in an out-patient setting	194	0.7-3.9 yrs	71% RV positive, 47% RV alone
Fairchok *et al.* 2010	USA	Prospective cohort	Healthy children attending daycare center	119	≤ 30 months	35%
Mackay *et al.* 2013	Australia	Prospective cohort	Healthy children	234	< 5 yrs	48% (RV or enterovirus)
Miller *et al.* 2013	USA	Prospective cohort	Healthy children	648	≤ 1 yr	26% in all ARIs, 46% in URIs
Linder *et al.* 2013	USA	Prospective birth-cohort	Healthy children	2009	< 5 yrs	36%[a]
Simonen-Tikka *et al.* 2013	Finland	Prospective cohort	Children with increased risk for T1DM	45	≤ 2 yrs	58%
Budge *et al.* 2014	Peru	Prospective follow-up	Healthy children	892	< 3 yrs	44%
Anders *et al.* 2015	Vietnam	Prospective birth-cohort	Healthy children	2459	≤ 1 yr	54-62%[b]
Simpson *et al.* 2016	USA	Prospective	Healthy children with ARIs during influenza seasons	2384	6-59 months	25% RV positive, 16% RV alone

AOM, acute otitis media; ARI, acute respiratory infection; NA, not available; NPS, nasopharyngeal sample; RV, rhinovirus; T1DM, type 1 diabetes mellitus; URI, upper respiratory tract infection.

[a] Virus diagnostics performed for 527 samples during ARIs.

[b] Virus diagnostics performed for 556 samples during ARIs at two different study sites.

With novel molecular detection and typing methods, a high diversity of different rhinovirus genotypes has been found to circulate concomitantly in the community, and multiple rhinovirus types are shown to circulate simultaneously even in families (Peltola *et al.* 2008, Mackay *et al.* 2013). Frequent rhinovirus infections occur and are usually caused by different rhinovirus types (Jartti *et al.* 2008b, van der Zalm *et al.* 2011). Species A and species C rhinoviruses have been most frequently found rhinoviruses followed by species B rhinoviruses (Franco *et al.* 2012, Lee *et al.* 2012, Lauinger *et al.* 2013, Mackay *et al.* 2013, Lu *et al.* 2014). The epidemiology of species C rhinoviruses appears to differ from that of A and B, and they seem to cause a peak of infections in the winter months (Linder *et al.* 2013, Zeng *et al.* 2014). Species A and C rhinoviruses seem to be associated with more severe infections and an increased need for hospitalization (Iwane *et al.* 2011, Lee *et al.* 2012, Linder *et al.* 2013). This suggests biological differences within rhinovirus species that would be independent from their receptor specificity (Bochkov and Gern 2016). *In vitro*, species B rhinoviruses have been shown to have a lower replication rate, cytokine production, and cellular cytotoxicity as compared to rhinovirus A or C (Nakagome *et al.* 2014). Nevertheless, in preschool-aged children in a community setting, no clinical impact attributable to rhinovirus species or genotypes was detected (Mackay *et al.* 2013). Cold winter months are associated with more severe rhinovirus infections independent of the virus prevalence and rhinovirus types (Lee *et al.* 2012).

2.4 Pathogenesis and host factors

2.4.1 Transmission

Rhinoviruses are transmitted by contact and droplets. Infection occurs usually through a respiratory route, but infection by conjunctivae has also been shown (Bynoe *et al.* 1961, Hendley *et al.* 1973). Rhinoviruses are frequently transmitted to other family members from children having a symptomatic rhinovirus infection (Fox *et al.* 1985, Peltola *et al.* 2008), and secondary attack rates are highest among young children (Fox *et al.* 1975, Fox *et al.* 1985, Peltola *et al.* 2008). Rhinoviruses can survive for hours to days in surfaces in an indoor environment in experimental settings, and for two hours on undisturbed skin (Hendley *et al.* 1973, Gwaltney and Hendley 1982, Winther *et al.* 2007, Winther *et al.* 2011).

The only known host for rhinoviruses is humans, although other primates may have asymptomatic rhinovirus infections (Dick and Dick 1968). It has been difficult to develop animal models for rhinovirus infection and most of the detailed data on the pathogenesis of rhinovirus infections have traditionally come from experimental infections in healthy volunteers. In the last 15 years, transgenic mice expressing human ICAM-1 have been successfully infected with rhinovirus, and mouse models can be used for studying rhinovirus infections and rhinovirus-associated exacerbation of allergic airway inflammation (Tuthill *et al.* 2003, Bartlett *et al.* 2008).

2.4.2 Pathophysiology

The incubation period for a rhinovirus infection is usually two to three days (Douglas *et al.* 1966, Hendley *et al.* 1969, Lessler *et al.* 2009). The primary site for a rhinovirus infection is nasal and nasopharyngeal epithelial cells, but rhinoviruses are also able to replicate in the lower respiratory tract (Gern *et al.* 1997, Hayden 2004, Malmstrom *et al.* 2006). The specific tropism of rhinoviruses to the nasal cavity seems to be linked with their optimal growth temperature of 33-35°C, but there is also evidence that certain rhinoviruses are capable of replicating at a higher temperature supporting the role of rhinoviruses in the pathophysiology of lower respiratory tract infections (Papadopoulos *et al.* 1999, Blomqvist *et al.* 2009). Especially some rhinovirus C types can grow in higher temperatures, which may explain the greater propensity of certain rhinovirus C strains to infect lower airways (Ashraf *et al.* 2013, Tapparel *et al.* 2013). For years, it has been hypothesized that exposure to cold air is linked to rhinovirus infections and particularly to upper respiratory tract infections, but even more extensive studies have not been able to show a direct pathophysiological mechanism. Recently, Foxman et al showed in a mouse model that expression of antiviral response genes was higher at 37°C as compared at 33°C in response to rhinovirus infection (Foxman *et al.* 2015). Their results suggest that nasal tropism would not only depend on the characteristics of the virus but also on the host's innate immune response that would be more effective in controlling a rhinovirus infection at higher temperatures, and that the cells would be more vulnerable to a rhinovirus infection at lower temperatures.

Rhinoviral shedding is strongest during the first days of infection (Douglas *et al.* 1966). Rhinovirus shedding lasts usually one to two weeks in immunocompetent individuals and long shedding is rare in infants, but prolonged shedding may occur in immunocompromised hosts (Douglas *et al.* 1966, Peltola *et al.* 2013, Loeffelholz *et al.* 2014).

Rhinoviruses cause relatively little damage to the nasal epithelial cells, and it is rather the local inflammatory reaction to rhinoviruses that results in clinical signs and symptoms of the common cold (Jacobs *et al.* 2013). Low-level rhinovirus viremia may occur during a rhinovirus infection and is found rather frequently during the early course of acute asthma exacerbations caused by rhinovirus (Xatzipsalti *et al.* 2005). Rhinoviral load may be associated with the severity of the symptoms in certain subpopulations or infections caused by certain rhinovirus types (Takeyama *et al.* 2012, Xiao *et al.* 2015), but the data are controversial. In recent studies, rhinovirus loads in nasopharyngeal aspirates were not associated with short-term outcomes in rhinovirus-induced bronchiolitis (Jartti *et al.* 2015a), and the viral loads were similar in nasal washes in rhinovirus positive acute rhinitis and wheezing (Kennedy *et al.* 2014).

2.4.2.1 Innate immune response

The early defense against infectious agents is mediated by surface defense mechanisms and innate immune responses such as production of cytokines, complement activation, and phagocytic responses, which are triggered rapidly in response to exposure to pathogens. Innate immune responses are usually nonspecific, similarly repeating reactions, and the immunological memory is not evolved. Innate immunity is especially important in young children as maternal antibodies derived through the placenta wane during the first year of life, and their adaptive immune responses are still immature.

An undamaged airway epithelium serves as the first line defense and a barrier against respiratory virus infection. Epithelial cells of the airways secrete defensins and other peptides with antimicrobial actions such as lysozyme and cathelicidin (Tosi 2014). Nasal and bronchial ciliary movement and protective reflexes, such as cough and sneezing, transport respiratory pathogens away from the respiratory tract. Recognition and response by the innate immune system occur rapidly after infection of the airway epithelium with rhinovirus. Viruses, unlike bacteria, specifically induce type I (alpha and beta) and III (lambda) interferon (IFN) gene expression in the infected host. Type I and III IFNs have an important role in the innate immune response and have immunomodulatory, antiproliferative, and antiviral functions. IFNs induce many antiviral proteins such as dsRNA-activated protein kinase, 2',5'-oligoadenylate synthetase, RNAse L, and myxovirus resistance protein A (MxA), which all have activity against a wide range of viruses (Samuel 2001).

Pattern-recognition receptors, such as mannose-binding lectin (MBL) and the family of toll-like receptors (TLR), recognize evolutionarily conserved common structures of the pathogens, named as pathogen-associated molecular patterns (PAMP). Recognition of invading pathogens leads to phagocytosis and triggers inflammatory signaling cascades that aim at eradication of the invading pathogen. MBL is a serum lectin that binds to a wide range of micro-organisms, including bacteria and viruses. MBL activates the complement via the lectin pathway and opsonization with MBL leads to phagocytosis of the pathogens (Super *et al.* 1989, Dommett *et al.* 2006). TLRs are transmembrane proteins that recognize specific microbial structures leading to the induction of interferons and pro-inflammatory cytokines, which are important for clearance of pathogens. In addition to initiation of innate immune response, TLRs link innate and adaptive immune systems (Akira *et al.* 2001). The toll gene was identified in the *Drosophila*, the fruit-fly, in 1985 by a German biologist, Christiane Nüsslein-Volhard, and named after her spontaneous comment, *"Das war ja toll!"* meaning "That was great!" (Hansson and Edfeldt 2005). The toll protein of the *Drosophila* was first found to induce the innate immune response in the insect, and similar receptors found in humans derived their name due to the homology to the *Drosophila* toll protein (Medzhitov *et al.* 1997). To date, ten functional TLRs have been recognized (TLR1-

10) in humans (Skevaki *et al.* 2015). The main TLR ligands of respiratory pathogens are presented in Table 2. TLR2 functions as a dimer with TLR1 and TLR6 on the cell surface and recognizes a variety of structures of Gram-positive bacteria and is thus important in immune defense against bacterial infections (Aliprantis *et al.* 1999, Abreu and Arditi 2004). TLR2 has been found to recognize also rhinovirus capsid (Triantafilou *et al.* 2011). TLR4 has a key role in recognizing the bacterial cell wall lipopolysaccharide of Gram-negative bacteria on the cell surface but has a role also in the antiviral response (Arbour *et al.* 2000, Thompson *et al.* 2011). Endosomal TLR3, TLR7, TLR8, and TLR9 recognize viral nucleic acids and induce production of interferons that are critical for antiviral immunity (Thompson *et al.* 2011). TLR3 recognizes double-stranded (ds) RNA (Alexopoulou *et al.* 2001) and TLR7 and TLR8 single-stranded (ss) RNA produced in viral replication cycle (Diebold *et al.* 2004, Heil *et al.* 2004, Lund *et al.* 2004).

Table 2. Role of toll-like receptors (TLRs) in pathogen recognition and pathophysiology of respiratory diseases. Modified from Abreu and Arditi 2004, updated based on Skevaki *et al.* 2015 and Thompson *et al.* 2011.

	Ligand[a]	**Respiratory pathogen or diseases state**
TLR1	Signals as dimer with TLR2 Lipopeptides	*Mycobacterium tuberculosis*
TLR2	Peptidoglycan, lipoteichoic acid, lipoarabidomannan, lipoprotein Rhinovirus capsid	Gram-positive bacteria such as *Streptococcus pneumoniae* *M. tuberculosis*, measles virus Rhinovirus, RSV *Mycoplasma*
TLR3	dsRNA	Viruses
TLR4	Lipopolysaccharide (LPS) RSV F-protein	Gram-negative bacteria RSV *M. tuberculosis*
TLR5	Bacterial flagellin	Unknown
TLR6	Signals as dimer with TLR2 Di-acyl lipopeptides	*Mycoplasma*
TLR7	ssRNA	Viruses
TLR8	ssRNA	Viruses
TLR9	Viral and bacterial DNA as CpG motifs	Bacterial and viral infections, role in respiratory disease unknown
TLR10	Unknown	Unknown

CpG, cytosine-phosphate-guanosine; dsRNA, double-stranded RNA; RSV, respiratory syncytial virus; ssRNA, single-stranded RNA.
[a] Examples of ligands recognized by TLRs.

The initial immune response to a rhinovirus infection is mediated by innate and cell-mediated immunity. Recognition of rhinoviruses results in rapid production of IFNs, which establishes an antiviral state in the infected and surrounding epithelial cells (Royston and Tapparel 2016). An innate immune response induced by a rhinovirus infection leads to the production of cytokines and chemokines that further recruit and activate inflammatory cells (Message and Johnston 2004). During a rhinovirus infection, increased numbers of neutrophils and lymphocytes are present in nasal

secretions, and T cells are detected in bronchial epithelium and submucosa (Jacobs *et al.* 2013). Rhinoviruses upregulate the expression of their major group receptor ICAM-1 in the cell membrane by a nuclear factor (NF)-κβ-dependent mechanism (Papi and Johnston 1999, Winther *et al.* 2002).

Rhinoviruses are recognized by two pattern recognition receptor families: TLRs and retinoic acid-inducible gene-I-like receptors (RLR), a RNA helicase family that includes retinoic acid-inducible gene-I (RIG-I), melanoma differentiation associated gene-5 (MDA-5), and LGP-2 (laboratory of genetics and physiology 2) (Royston and Tapparel 2016). Signal transduction pathways and the activation of the innate immunity in response to rhinovirus infection are shown in Figure 2. Rhinoviral dsRNA is recognized by TLR3 and ssRNA by TLR7 and TLR8 in the endosome (Hewson *et al.* 2005, Slater *et al.* 2010, Triantafilou *et al.* 2011) and rhinoviral capsid is recognized by TLR2 on the cell surface (Triantafilou *et al.* 2011). The signaling is also mediated through RIG-I and MDA-5, which recognize newly synthesized rhinoviral ssRNA and dsRNA in the cytoplasm (Slater *et al.* 2010, Triantafilou *et al.* 2011). Signaling via MDA-5 seems to be more critical in picornavirus infections (Kato *et al.* 2006). Induction of these pathways leads to increased production of interferons and proinflammatory cytokines such as interleukin (IL)-6 and IL-8 (Figure 2). Nasal IL-8 levels have been shown to correlate with the severity of clinical symptoms during a rhinovirus infection (Turner *et al.* 1998). Severity of clinical symptoms has been found to correlate with increased nasal levels of also other inflammatory mediators such as IL-1β, IL-6, regulated upon activation, normal T cell expressed, and secreted (RANTES), and kinins (Atmar and Englund 2014).

2.4.2.2 Adaptive immune response

Adaptive immune responses are activated along with the innate immune responses but develop more slowly in a naïve subject. Adaptive immunity is characterized by a more specific response and long memory mediated by antibodies and T cell responses and is activated quickly in reinfection with the same or similar agent. Both experimental and natural rhinovirus infections induce production of serum type-specific neutralizing immunoglobulin (Ig) G antibodies and nasal secretory IgA antibodies (Jacobs *et al.* 2013). Rhinovirus infections also induce a specific T cell response. Specific antibodies are detectable one to two weeks after an inoculation of an experimental rhinovirus infection and may remain elevated for years (Barclay *et al.* 1989, Jacobs *et al.* 2013). A high level of serotype-specific antibodies confers protection from infection and are associated with milder symptoms after an experimental infection with the same serotype (Alper *et al.* 1998). There is only little cross-protection among serotypes after a rhinovirus infection and due to several types of rhinoviruses circulating in the community, frequent rhinovirus infections by different rhinovirus types occur.

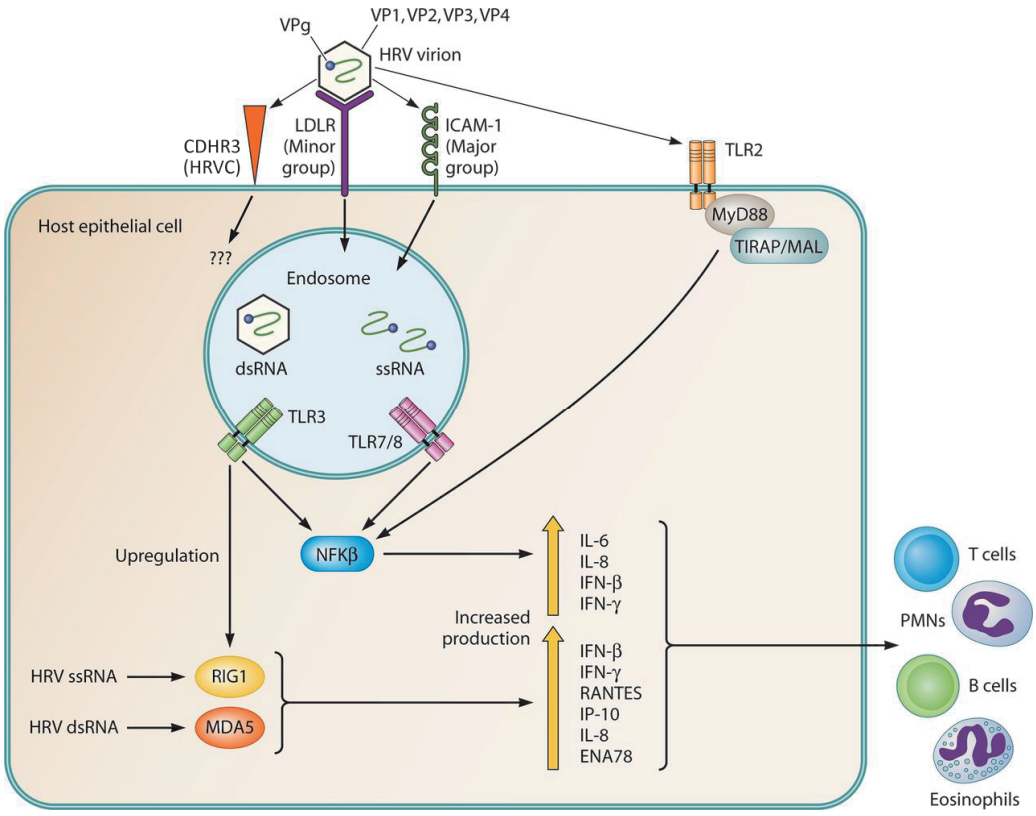

Figure 2. Signal transduction pathways and activation of the innate immune response in a rhinovirus infection. The major group rhinoviruses enter the cells by using intercellular adhesion molecule 1 (ICAM-1) on the cell surface as a receptor, while the minor group uses low-density lipoprotein receptor (LDLR). The receptor of species C rhinoviruses was recently identified as cadherin-related family member 3 (CDHR3) with yet unknown biological function. Rhinoviral dsRNA is recognized by toll-like receptor (TLR) 3 and ssRNA is recognized by TLR7 and TLR8 in the endosome leading to induction of antiviral and proinflammatory cytokines via nuclear factor (NF)-κβ pathway. Retinoic acid-inducible gene 1 (RIG-1) and melanoma differentiation-associated gene 5 (MDA-5) recognize newly synthesized viral dsRNA and ssRNA in the cytoplasm and are upregulated by activation of TLR3. TLR2 recognizes rhinovirus capsid on the cell surface which triggers a proinflammatory cytokine response via a MyD88-dependent pathway. HRV, human rhinovirus; VP, viral protein; TIRAP/MAL, Toll–interleukin-1 receptor domain containing adapter protein/MyD88 adapter-like; IL, interleukin; IFN, interferon; PMNs, polymorphonuclear leukocytes; RANTES, regulated, normal T cell expressed, and secreted; IP-10, IFN-γ-induced protein 10; ENA78, epithelial cell-derived neutrophil-activating peptide 78. Modified from Jacobs *et al*. 2013 and used with the permission of American Society for Microbiology.

2.4.3 Risk factors

Environmental factors and characteristics of the host, such as age, immunological, genetic, and anatomical characteristics, may affect the susceptibility to respiratory infections. Age is inversely associated with the risk of respiratory infections, and the

highest incidence is found in infants during the first years of life (Monto and Ullman 1974). Infants and young children are especially vulnerable to respiratory infections and acute otitis media from approximately 6 to 24 months of age as maternal antibodies derived through the placenta wane during the first year of life and adaptive immune responses are still immature (Tregoning and Schwarze 2010). Host response to infectious agents in children at this age is mediated mainly by innate immunity, while adaptive immune responses develop by increasing exposure to pathogens. Immunological defects, such as lowered interferon production, and genetic variations such as single-nucleotide polymorphisms (SNPs) in genes related to innate immunity may be especially important in young children and have been found to increase the risk for respiratory infections (Pitkaranta *et al.* 1999, Koch *et al.* 2001, Emonts *et al.* 2007, Revai *et al.* 2009). Preterm infants have an increased risk for respiratory infections and for developing serious infections and complications as their immune system is more immature than that of full-term infants, they may lack maternal antibodies that pass the placenta mainly from the 32nd gestational week and onward, and due to respiratory support and chronic conditions associated to prematurity. Chronic conditions and medications, especially those affecting respiratory tract or the immune system such as bronchopulmonary dysplasia, cystic fibrosis, and immunosuppressive treatments, predispose children to respiratory infections. Male sex has been found to increase the risk for respiratory infections (Badger *et al.* 1953, Simoes 2003, Anders *et al.* 2015).

Children are frequently exposed to respiratory pathogens because of close contact to other children and transmit rhinoviruses efficiently to other family members, especially to their siblings (Fox *et al.* 1985, Peltola *et al.* 2008). Siblings are an important risk factor for respiratory and rhinovirus infections (Badger *et al.* 1953, Fox *et al.* 1985, Peltola *et al.* 2008, Anders *et al.* 2015, Chonmaitree *et al.* 2016). Close contacts to other children in group daycare increase the risk for respiratory infections (Ståhlberg 1980, Wald *et al.* 1988, Alho *et al.* 1990, Louhiala *et al.* 1995, Ball *et al.* 2002, Copenhaver *et al.* 2004, von Linstow *et al.* 2008, Côté *et al.* 2010) and infections can be prevented effectively by implementing an infection prevention program in daycare centers (Uhari and Mottonen 1999). An increased risk of respiratory infections has been associated with lower socioeconomic status (Biering-Sorensen *et al.* 2012, Anders *et al.* 2015), but also a higher educational level of the family has been reported to associate with an increased infection rate (Monto and Ullman 1974). Longer exclusive breastfeeding seems to associate with a decreased risk for upper respiratory tract infections, pneumonia, and acute otitis media (Alho *et al.* 1990, Chantry *et al.* 2006, Duijts *et al.* 2010, Chonmaitree *et al.* 2016). This protective effect is conveyed via immunoglobulin A, oligosaccharides, antimicrobial lactoferrin, and immunomodulatory and other agents of the breastmilk (Newburg 2005). Passive smoke exposure seems to increase the risk for respiratory infections, although data are partly controversial (Simoes, 2003, Chonmaitree *et al.* 2016). Maternal smoking during pregnancy was an independent risk factor for wheezing and respiratory infections in a

study comprising 22 390 Norwegian children (Haberg *et al.* 2007). Independently of maternal smoking during pregnancy, paternal smoking after birth was also associated with these outcomes. In adults, psychological stress and poor sleeping have been shown to increase susceptibility to common cold (Cohen *et al.* 1991, Pedersen *et al.* 2010). In contrast, a diverse social network has been shown to protect from respiratory infections (Cohen *et al.* 1997).

Young children are at a high risk for developing acute otitis media as a complication of a respiratory virus infection. The anatomy of the developing nasopharynx, such as the short Eustachian tube, predisposes young children to acute otitis media (Bluestone 2008). Risk factors for acute otitis media include a family history of acute otitis media, outside-the-home daycare, passive smoke exposure, the lack of breastfeeding, use of a pacifier, genetic and immunological variations, frequent viral upper respiratory tract infections, and nasopharyngeal colonization with pathogenic bacteria (Uhari *et al.* 1996, Chonmaitree *et al.* 2016). In addition to acute otitis media, previous studies have linked early nasopharyngeal bacterial colonization to recurrent wheezing and asthma (Faden *et al.* 1997, Bisgaard *et al.* 2007). Allergic sensitization increases the risk of rhinovirus-induced wheezing illnesses and asthma exacerbations (Jartti *et al.* 2010, Rowe and Gill 2015).

2.4.3.1 Single nucleotide polymorphisms

As exposure to pathogens is frequent in young children and because their defense system relies mainly on innate immunity, aberrant innate immune responses may result in an increased susceptibility to infections. Single nucleotide polymorphisms (SNPs) are common genetic variants found at a frequency of over 1% within a population and may alter the amino acid sequence, affect promoter characteristics, or be completely silent. To the host, SNPs may have a detrimental or a protective role, or both, or they may have no clinical effect. SNPs in genes involved in innate immunity may be especially important during the first years of life and may result in altered susceptibility to infectious or inflammatory diseases (Koch *et al.* 2001, Wiertsema *et al.* 2006, Mittal *et al.* 2014). Risk of respiratory infections has been associated with genetic variants of innate immune factors such as MBL, IL-6, and tumor necrosis factor (TNF) α (Koch *et al.* 2001, Revai *et al.* 2009). Studies in twins suggest a heritability for acute otitis media (Casselbrant *et al.* 1999), and many possible otitis media candidate genes have been studied (Mittal *et al.* 2014). For example, polymorphisms in IL-6, TLR4, and TNFα have been found to associate with otitis media or recurrent otitis media (Emonts *et al.* 2007, Alper *et al.* 2009, Revai *et al.* 2009). Alper and colleagues found that the high production IL-10 phenotype and the low production IL-6 phenotype were associated with an increased risk of otitis media during a rhinovirus infection (Alper *et al.* 2009). A single nucleotide polymorphism (rs6967330, C529Y) in the rhinovirus C receptor CDHR3 is linked to the increased expression of CDHR3 protein at the surface of airway epithelial cells and is associated

with an increased risk of wheezing illnesses and hospitalizations for childhood asthma and may promote rhinovirus C infections (Bonnelykke *et al.* 2014, Bochkov *et al.* 2015, Bochkov and Gern 2016). There is little information about genetic susceptibility to rhinovirus infections in the clinical setting.

Mannose-binding lectin

Heterozygous polymorphisms of mannose-binding lectin (MBL) result in decreased serum levels of MBL and a partial functional defect of the lectin pathway, as combined heterozygous and homozygous polymorphisms cause very low serum MBL levels and non-functional lectin pathway (Cedzynski *et al.* 2004, Wiertsema *et al.* 2006). Also polymorphisms in the promoter region influence serum MBL levels (Madsen *et al.* 1995, Cedzynski *et al.* 2004, Wiertsema *et al.* 2006). An MBL level of less than 500 ng/mL is considered MBL deficient, although definitions vary (Eisen *et al.* 2008). Polymorphisms of the MBL structural gene, *MBL2*, in exon 1 in codons 52, 54, and 57 are designated as D, B, and C variants, respectively, and the wild-type allele is designated as A (Table 3). Polymorphisms in MBL and the resulting low serum levels of MBL have been found to associate with an increased susceptibility to severe infections such as invasive pneumococcal disease, meningococcal disease, and sepsis and pneumonia in neonates (Summerfield *et al.* 1997, Hibberd *et al.* 1999, Frakking *et al.* 2007, Munoz-Almagro *et al.* 2014). MBL deficiency may also increase susceptibility to respiratory infections and acute otitis media in children (Koch *et al.* 2001, Cedzynski *et al.* 2004, Wiertsema *et al.* 2006, Bossuyt *et al.* 2007, Chen *et al.* 2009, Eisen 2010). The main results of clinical studies of MBL polymorphisms and respiratory infections are presented in Table 4. A meta-analysis including five eligible studies found no association between MBL codon 54 polymorphisms and susceptibility to recurrent respiratory infections in children, but other structural or promoter polymorphisms were not included (Atan *et al.* 2016).

Toll-like receptors

SNPs within the toll-like receptor (TLR) genes may result in decreased recognition of and response to TLR ligands and thus in altered susceptibility to infections (Arbour *et al.* 2000, Mittal *et al.* 2014, Skevaki *et al.* 2015). Examples of SNPs in TLRs with a potential role in the pathogenesis of respiratory infections are shown in Table 3. The main results of clinical studies assessing TLR polymorphisms and respiratory infections are presented in Table 4. TLR polymorphisms have been linked to the susceptibility of children to recurrent febrile bacterial infections (TLR2) (Kutukculer *et al.* 2007), recurrent acute otitis media (TLR4) (Emonts *et al.* 2007), severe respiratory syncytial virus (RSV) infection (TLR4) (Puthothu *et al.* 2006), and wheezing illnesses (TLR3) (Nuolivirta *et al.* 2012b), but the results have been varying. A meta-analysis found no association between TLR4 polymorphisms and risk of severe RSV infection (Zhou *et al.* 2016). Polymorphisms in TLR7 and TLR8 have been linked to asthma (Moller-Larsen *et al.* 2008), but their association with respiratory infections has not been studied in clinical settings.

Table 3. Single nucleotide polymorphisms in mannose-binding lectin (MBL) and toll-like receptors (TLR) 2, 3, 4, 7, and 8 with a possible role in the pathophysiology of respiratory disease.

Gene	Reference SNP	Chromosome	Functional consequence	Nucleotide change	Amino acid change[a]	Potential effect	Global minor allele frequency[b]	Minor allele frequency in European population[c]
MBL2	rs5030737	10:52771482	missense	C -> T	52 Arg -> Cys	Decreased serum levels of	2.7%	6.0%
MBL2	rs1800450	10:52771475	missense	G -> A	54 Gly -> Asp	MBL and a functional defect	12.2%	14.1%
MBL2	rs1800451	10:52771466	missense	G -> A	57 Gly -> Glu	of the lectin pathway[d]	8.1%	1.2%
TLR2	rs5743708	4:153705165	missense	G -> A	753 Arg -> Gln	Compromised signaling[e]	0.7%	2.4%
TLR3	rs3775291	4:186082920	missense	C -> T	412 Leu -> Phe	Compromised signaling[f]	23.2%	32.4%
TLR4	rs4986790	9:117713024	missense	A -> G	299 Asp -> Gly	Impaired response[g]	6.0%	5.7%
TLR7	rs179008	X:12885540	missense	A -> T	11 Gln -> Leu	May alter the processing of TLR7 at the endoplasmic reticulum[h]	11.8%	17.6%
TLR8	rs2407992	X:12920993	intron variant, synonymous codon	G -> C	651 Leu -> Leu	Alternative splicing of TLR8[h]	27.7%	28.9%

Missense mutation, a point mutation in which a single nucleotide change results in a codon that codes for a different amino acid; rs, reference single nucleotide polymorphism; SNP, single nucleotide polymorphism.

Reference SNPs and minor allele frequencies based on the 1000Genome data (Abecasis *et al.* 2012) from the Single Nucleotide Polymorphism database (dbSNP) by the National Center for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov/snp/).

[a] Number refers to the position of the amino acid in the protein. For MBL, SNP in codon 52 is referred as allele D, in codon 54 as allele B, and in codon 57 as allele C, and wild-type as allele A.

[b] Reported by SNP database in a default global population (1000Genome phase 3 genotype data from 2500 worldwide individuals, released in 2013). For example, for TLR2 rs5743708, minor allele is 'A' and has a frequency of 0.7% in the sample population.

[c] Based on the sample of 1006 chromosomes from a European population reported in the SNP database.

[d] Cedzynski *et al.* 2004, Wiertsema *et al.* 2006

[e] Xiong *et al.* 2012

[f] Ranjith-Kumar *et al.* 2007

[g] Ohto *et al.* 2012

[h] Moller-Larsen *et al.* 2008

Table 4. Clinical studies on associations between single nucleotide polymorphisms (SNPs) in mannose-binding lectin (MBL) and toll-like receptors (TLRs) 2, 3, 4, 7, and 8 and acute respiratory infections and otitis media in children.

Gene	Studied SNPs	Study	Study design	*n*, cases (*n*, controls)	Outcome	Effect of variant type
MBL2[a]	Structural, *promoter*[b]	Koch *et al.* 2001	Prospective cohort	252	Acute respiratory infections	Increased risk
	rs1800450, rs1800451	Ozbas-Gerceker *et al.* 2003	Case-control	69 (100)	Recurrent respiratory infections	Decreased risk
	Structural, *promoter*[b]	Cedzynski *et al.* 2004	Case-control	335 (78)	Recurrent respiratory infections	Increased risk
	Structural, *promoter*	Bossuyt *et al.* 2007	Case-control	55 (43)	Recurrent respiratory infections	Increased risk
	Structural, *promoter*	Muller *et al.* 2007	Prospective cohort	749	Respiratory infections	*NS*
	Structural, *promoter*	Ruskamp *et al.* 2008	Prospective cohort	987	Respiratory infections	*NS*
	Structural, *promoter*[b]	Chen *et al.* 2009	Case-control	70 (120)	Recurrent respiratory infections	Increased risk
	Structural, *promoter*[b]	Tao *et al.* 2012	Case-control	57 (105)	Recurrent respiratory infections	*NS*
	rs1800450	Atan *et al.* 2016	Meta-analysis		Recurrent respiratory infections	*NS*
	rs1800450	Nokso-Koivisto *et al.* 2014	Prospective and retrospective cohort	653	Upper respiratory tract infection, OM during URI, OM proneness	*NS*
	Structural[b]	Garred *et al.* 1993	Prospective	76	Recurrent otitis media	*NS*
	Structural	Homoe *et al.* 1999	Retrospective	82	AOM, Recurrent AOM	*NS*
	Structural, *promoter*[b]	Wiertsema *et al.* 2006	Prospective cohort	244	Otitis media	Increased risk at 12-24 mo
	Structural, *promoter*	Nuytinck *et al.* 2006	Case-control	17 (172)	Recurrent/persistent otitis media	Increased risk
	Several SNPs	Sale *et al.* 2011	Prospective	618	Chronic OME or recurrent OM	*NS*
	rs1800450	Esposito *et al.* 2015	Case-control	200 (200)	Recurrent AOM	*NS*
	Structural, *promoter*[b]	Kristensen *et al.* 2004	Case-control	55 (113)	LRTI caused by RSV	*NS*
	Structural	Nuolivirta *et al.* 2012a	Prospective	129	Clinical characteristics of bronchiolitis	*NS*
	Structural	Koponen *et al.* 2012	Prospective follow-up	141	Asthma after bronchiolitis	Increased risk at 5-7 yrs

Gene	Studied SNPs	Study	Study design	*n*, cases (*n*, controls)	Outcome	Effect of variant type
	Structural	Esposito *et al.* 2014	Prospective follow-up	119 (119)	Wheezing	Increased risk
TLR2	rs5743708	Emonts *et al.* 2007	Case-control	348 (463)	Recurrent AOM	*NS*
	Several SNPs	Sale *et al.* 2011	Prospective	618	Chronic OME or recurrent OM	*NS*
	rs5743708	Carroll *et al.* 2012	Case-control	70 (70)	Otitis proneness	*NS*
	rs5743708	Kutukculer *et al.* 2007	Case-control	52 (91)	Recurrent febrile bacterial infections	Increased risk
	rs5743708	Esposito *et al.* 2012b	Prospective	272 (164)	Influenza	*NS*
	rs5743708	Nuolivirta *et al.* 2013	Prospective follow-up, controls	129 (318)	Bronchiolitis severity Postbronchiolitis wheezing	*NS*
TLR3	rs3775291	Nuolivirta *et al.* 2012b	Prospective follow-up	129	Bronchiolitis Postbronchiolitis wheezing	Increased risk Decreased risk
	rs5743313, rs5743315	Esposito *et al.* 2012b	Prospective	272 (164)	A/H1N1/2009 influenza associated pneumonia	Increased risk (rs5743313)
TLR4	rs4986790, *rs4986791*	Badolato *et al.* 2004	Prospective	20	Recurrent respiratory infections, pneumonia	Increased risk
	rs4986790	Nuolivirta *et al.* 2009	Prospective follow-up	129	Respiratory infections, ear infections, wheezing	*NS*
	rs4986790, *rs3732378*	Nokso-Koivisto *et al.* 2014	Prospective and retrospective cohort	653	Upper respiratory tract infection, OM during URI, OM proneness	*NS*
	rs4986790, *rs4986791*	Emonts *et al.* 2007	Case-control	348 (463)	Recurrent AOM	Decreased risk (rs4986790)
	Several SNPs	Sale *et al.* 2011	Prospective	618	Chronic OME or recurrent OM	*NS*
	rs4986790, *rs4986791*	Carroll *et al.* 2012	Case-control	70 (70)	Otitis proneness	*NS*
	rs4986790, *rs4986791, rs2737191*	Esposito *et al.* 2015	Case-control	200 (200)	Recurrent AOM	*NS*
	Several SNPs	Hafren *et al.* 2015	Five prospective cohorts	929 (882) and 1708 families	Otitis media	Increased risk in Finnish subpopulation
	rs4986790, *rs4986791*	Tal *et al.* 2004	Case-control	181 (90)	Severe RSV bronchiolitis	Increased risk
	rs4986790, *rs4986791*	Puthothu *et al.* 2006	Case-control	131 (270)	Severe RSV infection	Increased risk
	rs4986790, *rs4986791*	Mandelberg *et al.* 2006	Prospective	52	Severe RSV infection	Increased risk

Gene	Studied SNPs	Study	Study design	*n*, cases (*n*, controls)	Outcome	Effect of variant type
	rs4986790	Paulus *et al.* 2007	Case-control	236 (219)	Severe RSV disease	*NS*
	rs4986790, *rs4986791*	Awomoyi *et al.* 2007	Case-control	105 (97)	Symptomatic RSV disease	Increased risk
	rs4986790	Helminen *et al.* 2008	Case-control	97 (400)	RSV bronchiolitis	*NS*
	rs4986790	Löfgren *et al.* 2010	Case-control	312 (356)	Severe RSV bronchiolitis	*NS*
	rs4986790, *rs4986791*	Kresfelder *et al.* 2011	Case-control	296 (113)	RSV disease	*NS*
	rs4986790, *rs4986791*	Goutaki *et al.* 2014	Case-control	50 (99)	Bronchiolitis requiring hospitalization, RSV bronchiolitis	*NS*
	Several SNPs	Zhu *et al.* 2015	Case-control	196 (311)	Hospitalized RSV bronchiolitis	Increased risk (rs41426344)
	rs4986790	Zhou *et al.* 2016	Meta-analysis	1009 (1348)	Severe RSV infection	*NS*
	rs4986791	Zhou *et al.* 2016	Meta-analysis	473 (481)	Severe RSV infection	*NS*
	rs4986790, *rs4986791*	Esposito *et al.* 2012b	Prospective	272 (164)	Influenza	*NS*
TLR7	rs179008	*No clinical studies of respiratory infections*				
TLR8	rs2407992	*No clinical studies of respiratory infections*				

AOM, acute otitis media; LRTI, lower respiratory tract infection; OM, otitis media; NS, not significant; rOM, recurrent otitis media; RRTI, recurrent respiratory tract infections; RSV, respiratory syncytial virus; URI, upper respiratory tract infection. SNPs not studied in the STEPS cohort are shown in *italics*.

[a] For the *MBL2* gene, structural polymorphisms in codons 52, 54, and 57 (rs5030737, rs1800450, rs1800451, respectively) and promoter variants.

[b] MBL serum levels measured.

2.5 Clinical presentation

2.5.1 Common cold

Rhinoviruses are the most common cause of acute respiratory infections in both children and adults (Monto and Ullman 1974, Monto and Sullivan 1993, Mäkelä *et al.* 1998). In prospective studies using sensitive PCR methods, rhinoviruses have been found in up to 73% of acute respiratory infections in young children (Table 1). The mean rate of detected rhinovirus infections was 2.4 per year in children under three years of age in a prospective study in Andean children (Budge *et al.* 2014). Rhinovirus infections are usually mild upper respiratory tract infections and self-limiting. The duration of respiratory symptoms is usually 7 to 10 days and may be longer. In a prospective study, the mean duration of respiratory symptoms in children was even 15 days (Peltola *et al.* 2013). The symptoms present usually as rhinorrhea, nasal congestion, sneezing, sore throat, cough, and malaise, and fever may be present especially in children.

2.5.2 Rhinosinusitis

Paranasal sinuses are frequently affected in the course of a rhinovirus infection and resolve usually without treatment. Sinusitis occurs during the early days of common cold (Puhakka *et al.* 1998). Rhinoviral RNA may be detected in maxillary aspirates or sinus biopsies in 40-50% of the patients with acute sinusitis (Pitkaranta *et al.* 1997, Pitkaranta *et al.* 2001). Sinus abnormalities are frequently detected during the common cold with computed tomography or magnetic resonance imaging and often resolve spontaneously (Turner *et al.* 1992, Gwaltney *et al.* 1994, Kristo *et al.* 2003). In a study by Kristo and colleagues, 60% of the children aged 4-7 years had major abnormalities such as mucosal swelling in their maxillary and ethmoidal sinuses during uncomplicated acute respiratory infection, 35% in the sphenoidal sinuses, and 18% in the frontal sinuses. Maxillary and ethmoidal sinuses are most frequently infected in young healthy adults with an experimental rhinovirus infection (Turner *et al.* 1992). Gwaltney and colleagues detected abnormalities in one or both maxillary sinuses by computed tomography in even 87% of healthy adults with the common cold and the changes resolved or improved without antibiotic treatment in 79% of the patients in two weeks. (Gwaltney *et al.* 1994).

2.5.3 Acute otitis media

The most common complication of a rhinovirus infection is acute otitis media. Acute otitis media occurs usually as a complication of a viral upper respiratory tract infection and mainly within the first week after the onset of respiratory symptoms. Acute otitis media complicates one-third of acute respiratory infections in young children and is the most frequent reason for antimicrobial prescriptions in resource-rich countries (Nokso-

Koivisto *et al.* 2015, Chonmaitree *et al.* 2016). In a prospective study, the mean rate of acute otitis media was 1.72 episodes per child-year (Chonmaitree *et al.* 2008). The most common bacterial causes of acute otitis media are Gram-positive *Streptococcus pneumoniae* and Gram-negative non-typeable *Haemophilus influenzae* and *Moraxella catarrhalis* which may colonize the nasopharynx from an early age and particularly during respiratory infections. Nasopharyngeal colonization with pathogenic bacteria increases the risk for acute otitis media (Ruohola *et al.* 2013, Chonmaitree *et al.* 2016). Viral infection plays an important role in the pathogenesis of acute otitis media by causing inflammation of the nasopharynx and Eustachian tube, increasing bacterial colonization and adherence to the epithelial cells, and leading to the dysfunction of Eustachian tube (Nokso-Koivisto *et al.* 2015). The resulting negative middle ear pressure facilitates the entrance of both bacteria and respiratory viruses from the nasopharynx into the middle ear. Viruses alone may cause acute otitis media, although usually they increase the risk for bacterial infection and may worsen the clinical outcome of bacterial acute otitis media. RSV is frequently, even in half of the infections, complicated by acute otitis media (Heikkinen *et al.* 1999, Chonmaitree *et al.* 2008, Nokso-Koivisto *et al.* 2015). Rhinovirus infections can be complicated with acute otitis media in up to 30% of the infections (Chonmaitree *et al.* 2008). In a Finnish Otitis Media Cohort study, even 41% of all acute otitis media episodes were associated with a rhinovirus infection (Blomqvist *et al.* 2002). Both viruses and bacteria can be detected in even 66% of middle ear exudates, and picornaviruses are the most frequently found viruses (Ruohola *et al.* 2006). Also novel species C rhinoviruses have been found in middle ear effusions during acute otitis media (Savolainen-Kopra *et al.* 2009).

2.5.4 Wheezing illnesses

Rhinoviruses are the second most common causative agents of bronchiolitis leading to hospitalization after RSV (Meissner 2016). Rhinovirus dominates in wheezing children hospitalized after 12 months of age, while RSV is more frequent during the first year of life (Jartti *et al.* 2009). Rhinovirus infections are associated with one-third to one-half of wheezing illnesses and asthma exacerbations (Kusel *et al.* 2006, Jackson *et al.* 2008, Jartti *et al.* 2009, Piotrowska *et al.* 2009, Busse *et al.* 2010) and are an important cause of day-to-day respiratory symptoms in children with asthma (Tovey *et al.* 2015). In children with the first wheezing episode, rhinovirus was detected in 76% of the infections (Turunen *et al.* 2014).

Early rhinovirus-associated wheezing is associated with recurrent wheezing and the development of asthma in children (Kotaniemi-Syrjanen *et al.* 2003, Lemanske *et al.* 2005, Jackson *et al.* 2008, Jartti and Gern 2011, Midulla *et al.* 2012, van der Gugten *et al.* 2013, Jackson *et al.* 2016). Allergic sensitization increases the risk of rhinovirus-induced wheezing and asthma exacerbations (Jartti *et al.* 2010, Rowe and Gill 2015).

In a cohort of children with a parental history of atopy, wheezing during a RSV infection was associated with a threefold increased risk for asthma at six years of age, whereas wheezing during a rhinovirus infection was associated with almost tenfold increased risk (Jackson *et al.* 2008). Especially, a wheezing illness later in childhood is associated with an increased risk for asthma, as nearly 90% of children who had a rhinovirus-associated wheezing illness in their third year of life, had asthma at six years of age (Jackson *et al.* 2008). In a high-risk birth cohort, rhinovirus-associated wheezing illnesses occurring during early childhood were associated with decreased lung function in children up to eight years of age (Guilbert *et al.* 2011).

Rhinovirus infections and allergy in infancy synergistically increase the risk for developing asthma (Rowe and Gill 2015). The direct damage to developing airways and modulation of immune responses due to virus infections has been thought to explain this interaction (Bochkov and Gern 2016). Rhinovirus infections have also been proposed to have only a secondary role as indicators of abnormal physiology of the airways or aberrant antiviral immune responses. A combination of the previous two hypotheses proposes that early respiratory virus infections and a genetic predisposition to allergies and atopy are combined with complex interactions in promoting asthma. In a prospective birth cohort study, Jackson and colleagues demonstrated that allergic sensitization precedes rhinovirus wheezing but in contrast, viral wheezing does not lead to an increased risk of subsequent allergic sensitization (Jackson *et al.* 2012).

Several studies have shown impaired innate and adaptive immune responses such as deficient IFN production and impaired Th1 responses in subjects with asthma (Wark *et al.* 2005, Contoli *et al.* 2006, Message *et al.* 2008, Bartlett *et al.* 2012, Durrani *et al.* 2012). Th2 cytokines associated with asthma and allergies have been found to impair innate immune responses to rhinovirus in respiratory epithelial cells (Contoli *et al.* 2015). A weak interferon response to virus infection may result in increased viral replication, enhanced type 2 inflammation in the airways, more severe wheezing illness, and exacerbation of pre-existing asthma (Jackson *et al.* 2016). Evidence for the multifactorial effect of genetic variations and rhinovirus infections to the risk of childhood-onset asthma has accumulated in recent years. The association between rhinovirus wheezing illness during early life and an increased risk of childhood-onset asthma is associated with genetic variation at the chromosome 17q21 locus (Caliskan *et al.* 2013).

2.5.5 Pneumonia, severe infections, and hospitalizations

Experimental and clinical data show that rhinoviruses are able to replicate in and infect the lower respiratory tract (Gern *et al.* 1997, Papadopoulos *et al.* 2000, Hayden 2004, Mosser *et al.* 2005, Malmstrom *et al.* 2006). Several clinical studies have shown that rhinovirus is a common pathogen in children hospitalized with community-acquired

pneumonia. Rhinoviruses are present in 18-40% of community-acquired pneumonia, although a causative role is difficult to establish as viral-bacterial co-infections are common (Juven *et al.* 2000, Tsolia *et al.* 2004, Cilla *et al.* 2008, Nascimento-Carvalho *et al.* 2008, Ruuskanen *et al.* 2011, Esposito *et al.* 2012a, Honkinen *et al.* 2012, Jain *et al.* 2015). In a large population-based study covering 2222 children with radiographically proven community-acquired pneumonia, viruses were detected in 66% and rhinovirus in 27% of the children (Jain *et al.* 2015). Of note, rhinovirus was found frequently also in asymptomatic children (17% vs. 22% in asymptomatic children and children with pneumonia after adjustment for age and site). In a prospective study, rhinoviruses were most frequently found in children under one year of age with community-acquired pneumonia (Esposito *et al.* 2012a). Although accumulating data suggest that rhinoviruses contribute to the development of community-acquired pneumonia, their role in the pathogenesis of pneumonia still remains unclear. Interestingly, a temporal association has been shown between increased rhinovirus circulation in the community and invasive pneumococcal disease in children younger than five years suggesting that rhinoviruses may contribute to the risk of invasive pneumococcal disease (Peltola *et al.* 2011).

Rhinovirus infections are a frequent cause of hospitalization in young children. Rhinoviruses have been found in 14-35% of children hospitalized for an acute respiratory infection (Cheuk *et al.* 2007, Miller *et al.* 2007, Peltola *et al.* 2009, Lu *et al.* 2014, Zeng *et al.* 2014). Rhinovirus infections are the second most common respiratory virus infections leading to hospitalization after RSV (Kusel *et al.* 2006, Calvo *et al.* 2007), and in one study, rhinoviruses were the most frequently found viruses in children hospitalized for respiratory symptoms or fever (Miller *et al.* 2007). In that prospective study, a mean of 4.8 rhinovirus-associated hospitalizations per 1000 children per year were documented (Miller *et al.* 2007). The most common reason leading to hospitalization during a rhinovirus infection is an acute wheezing illness (Calvo *et al.* 2007, Peltola *et al.* 2009). Hospitalizations due to rhinoviruses are most frequent in children under six months of age and with a history of atopy, wheezing, or other underlying conditions (Miller *et al.* 2007, Peltola *et al.* 2009). Rhinoviruses are common also in the intensive care unit (Peltola *et al.* 2009). Co-infections are frequently detected in rhinovirus-positive hospitalized patients (Cheuk *et al.* 2007, Lu *et al.* 2014). Rhinovirus A and C are linked with more severe infections and hospitalizations than rhinovirus B (Miller *et al.* 2009, Peltola *et al.* 2009, Iwane *et al.* 2011, Esposito *et al.* 2012a, Franco *et al.* 2012, Lee *et al.* 2012, Lu *et al.* 2014). In some studies, species C rhinoviruses seem to be more often associated with lower respiratory tract infections than rhinovirus A (Lauinger *et al.* 2013, Linder *et al.* 2013), but rhinovirus A was the most prevalent rhinovirus in a study on children with community-acquired pneumonia (Esposito *et al.* 2012a). In some studies, no differences between disease severity and rhinovirus species have been found in hospitalized children (Zeng *et al.* 2014).

Rhinovirus outbreaks with a fatal outcome have been documented (Hai le *et al.* 2012) and rare extra-pulmonary rhinovirus infections such as pericarditis and a disseminated fatal rhinovirus infection have been reported in children (Tapparel *et al.* 2009, Lupo *et al.* 2015). Rhinoviruses may cause or predispose to lower respiratory tract infections especially in immunocompromised hosts, and infections may be more severe and even fatal and persistent infections have been described (Ghosh *et al.* 1999, Ison *et al.* 2003, Kaiser *et al.* 2006, Kainulainen *et al.* 2010, Jacobs *et al.* 2015).

2.5.6 Recurrent respiratory tract infections

Healthy, young children have a mean rate of five to six acute respiratory infections per child per year (Badger *et al.* 1953, Monto and Ullman 1974, Chonmaitree *et al.* 2008, von Linstow *et al.* 2008). While several annual episodes of uncomplicated respiratory infections are typical in young children, some children have unusually frequent or prolonged infections. Only limited data are available about recurrent respiratory infections, and the definition of recurrent respiratory infections has varied from certain numbers of infection episodes per year to specific diagnoses (Alho *et al.* 1990, Nokso-Koivisto *et al.* 2002, Emonts *et al.* 2007, Jartti *et al.* 2008b). Rhinoviruses have been shown to be the most common cause of recurrent respiratory tract infections in children (Nokso-Koivisto *et al.* 2002, Jartti *et al.* 2008b).

2.5.7 Asymptomatic infections

With the use of sensitive and specific molecular detection methods, rhinoviruses have been detected in approximately 15% of asymptomatic subjects, and coinfection rates have been high (Jartti *et al.* 2008a). The rate of asymptomatic rhinovirus infections has been diverse in different studies being the highest in infants, from 11% up to 47% in children under one year of age (Kusel *et al.* 2006, Jansen *et al.* 2011). Similar to symptomatic rhinovirus infections, rhinovirus A and C are found more frequently than rhinovirus B in asymptomatic children (Hasegawa *et al.* 2015). Frequent detection of rhinoviruses in asymptomatic subjects has raised concern about the clinical relevance of the rhinovirus-positive PCR finding. The detection of a rhinovirus, especially with molecular tests, may reflect previous infection, ongoing asymptomatic or mild infection, or an incubation period preceding the onset of symptoms. Viral genetic analyses with repeated sampling suggest that high prevalence rates are due to a high infection rate with different rhinovirus subtypes rather than prolonged shedding or carriage of the same rhinovirus type in immunocompetent individuals (Jartti *et al.* 2008b, van der Zalm *et al.* 2011, Peltola *et al.* 2013).

2.6 Laboratory diagnosis

2.6.1 Sample collection

Specimens for rhinovirus analyses should be collected as soon as possible after the onset of symptoms as rhinovirus copy numbers are highest in the respiratory tract during the first two days of infection, although rhinovirus may be detected at least from one day before to six days after the onset of symptoms (Jacobs *et al.* 2013). Nasopharyngeal swabs or aspirates, nasal swabs, and nasal wash specimens are feasible for the detection of rhinoviruses in upper respiratory tract infections (Heikkinen *et al.* 2002, Jacobs *et al.* 2013, Waris *et al.* 2013). Nasal samples can be taken at home and sent by standard mail to the laboratory in research settings (Lambert *et al.* 2007, Peltola *et al.* 2008, Waris *et al.* 2013). Specimens stay stable at room temperature for at least four days and survive mailing without loss in rhinovirus detectability (Waris *et al.* 2013). Flocked nasal swabs have an equal diagnostic sensitivity to rhinovirus as compared to nasopharyngeal aspirate with no significant quantitative difference, and they are more comfortable to the patient. Flocked nasal swabs have better quantitative sensitivity for rhinovirus as compared to cotton swabs. (Waris *et al.* 2013) Rhinoviruses may be detected also from samples from the lower respiratory tract such as sputum and bronchoalveolar lavage.

2.6.2 Virus detection methods

The viral culture was the first method for detecting rhinoviruses. The viral culture in a suitable cell line is still important in studies on viral characteristics, pathogenesis of rhinovirus infections, and in vaccine development. Rhinoviruses require specific culture media, temperature and prefer roller tube culture. Conventional cultures take up to 14 days, and the detection of rhinovirus is based on the cytopathic effect and subsequent specific identification tests. Rapid culture methods have been described including virus antigen detection using immunofluorescence at 48 hours post-infection (Jacobs *et al.* 2013). Species C rhinoviruses do not grow in standard cell cultures but can be cultivated in an organ culture of nasal epithelial cells (Bochkov *et al.* 2011).

RT-PCR is the most sensitive method for diagnosing rhinovirus and detects also species C rhinoviruses. Identification of rhinoviruses is currently based mainly on RT-PCR, because viral culture is laborious, has a lower sensitivity, and due to the important role of species C rhinoviruses. In RT-PCR, the extracted RNA is transcribed to complementary deoxyribonucleic acid (cDNA) by a reverse transcriptase enzyme, and cDNA is exponentially amplified by the polymerase enzyme in cyclically changing temperatures. Many primers used in RT-PCR target a highly conserved non-coding region shared by rhinoviruses and enteroviruses and further methods are required for the differentiation of the two species or rhinovirus-specific methods may be used (Jacobs *et al.* 2013, Österback *et al.* 2013, Atmar and Englund 2014). With quantitative

real-time RT-PCR, rhinoviral RNA can be quantitated by measuring fluorescence signals emitted from the hybridization of rhinovirus-specific fluorescent probes once every cycle, and the data are retrieved in real time (Jartti *et al.* 2013). Rhinoviruses can be detected by respiratory multiplex-PCR panels where more than one PCR analysis is performed at the same time. Due to the development of automated techniques, the results may be available within the same working day, which has increased the use of RT-PCR in the clinical setting as rapid results are crucial for clinical decisions. Genotyping of rhinoviruses is usually performed by RT-PCR amplification and sequence analysis of either the VP1 or VP4/VP2 gene coding regions (Jacobs *et al.* 2013).

Point-of-care tests for respiratory viruses based mainly on antigen detection have become more common and are useful in diagnostics of influenza-, parainfluenza-, and RS-virus infections (Ivaska *et al.* 2013). Due to the high diversity of rhinovirus types and lack of common antigen among rhinoviruses, there are no antigen detection tests available for rhinoviruses. Recently, rapid and automated PCR tests suitable for bedside testing have been developed for the detection of respiratory viruses, including rhinovirus, and are already commercially available.

Serological diagnosis of rhinovirus infection is based on demonstrating a rise in specific antibody titers in paired serum samples. Antibodies may be measured in both serum and nasal secretions by neutralization, plaque reduction, complement fixation, and enzyme-linked immunosorbent assays (ELISA) (Jacobs *et al.* 2013). The use of serology is restricted mainly in epidemiological studies as the usefulness in diagnostics of acute rhinovirus infections is limited by the vast number of rhinovirus serotypes and that antibodies are only detectable after one to three weeks from the onset of symptoms.

With the use of novel methods for analyzing blood gene expression profiles, rhinovirus infections can be diagnosed based on characteristic RNA transcriptional signature (Zaas *et al.* 2009), and symptomatic rhinovirus infections can be differentiated from asymptomatic rhinovirus infections (Heinonen *et al.* 2015).

2.6.3 General markers of a virus infection

C-reactive protein (CRP), white blood cell (WBC) count, procalcitonin, IL-6, or other biomarkers can be used in discriminating bacterial from viral infections (Gendrel *et al.* 1999, Gilbert 2010, Van den Bruel *et al.* 2011). Point-of care tests are feasible and relatively accurate for measurement of CRP level and WBC count in febrile children in an emergency department (Ivaska *et al.* 2015b). However, there may be discrepancies between different markers and their usefulness depends on the clinical setting (Gendrel *et al.* 1999, Peltola *et al.* 2007, Gilbert 2010, Van den Bruel *et al.* 2011, Ivaska *et al.* 2015a), and there is no diagnostic marker specific for virus infections in clinical use. A

general marker of virus infections would be useful in differentiating viral from bacterial infections especially in febrile children both in outpatient and inpatient settings. Potential diagnostic markers of virus infections include blood proteins specific for virus infections such as myxovirus resistance protein A (MxA), TNF-related apoptosis-inducing ligand (TRAIL), and IFN-gamma-induced protein 10 (IP-10) (Forster *et al.* 1996, Wark *et al.* 2007, Oved *et al.* 2015).

2.6.3.1 Blood MxA protein

Viruses, unlike bacteria, specifically induce type I (alpha and beta) and III (lambda) interferon (IFN) gene expression in the infected host. IFN-inducible antiviral proteins that reliably predict the presence of IFNs in the body could be used as markers of a virus infection. Myxovirus resistance protein A (MxA) is an intracellular, cytoplasmic GTPase expressed in high levels in peripheral blood mononuclear cells (PBMCs), is induced specifically by type I and III IFNs, and has activity against a wide range of viruses (von Wussow *et al.* 1990, Simon *et al.* 1991, Ronni *et al.* 1998, Kotenko *et al.* 2003, Holzinger *et al.* 2007, Haller and Kochs 2011). Mx protein was first discovered in mice that were genetically resistant to influenza virus and was named according to the property of conducting resistance against myxoviruses (Lindenmann 1962, Haller and Lindenmann 1974, Horisberger *et al.* 1983, Staeheli *et al.* 1986). In humans, two Mx proteins, myxovirus resistance protein 1 or A (MxA) and myxovirus resistance protein 2 or B (MxB), have been described, but only MxA has intrinsic antiviral activity (Haller and Kochs 2011). MxA has been suggested as a marker of virus infection because it is induced exclusively by type I and III IFNs, basal levels of MxA in healthy immunotolerant individuals are low, and its half-life is rather long (2.3 days *in vitro*) (Ronni *et al.* 1993, Maria *et al.* 2014). In febrile children, blood MxA protein can discriminate between viral and bacterial infection (Forster *et al.* 1996, Halminen *et al.* 1997, Nakabayashi *et al.* 2006, Engelmann *et al.* 2015). MxA response has been shown in respiratory infections caused by influenza-, adeno- and RS-viruses (Halminen *et al.* 1997, Chieux *et al.* 1999), but response to rhinovirus infections has not been shown.

2.7 Treatment

Currently there is no approved treatment available for rhinovirus infections. Antibiotics are often unnecessarily prescribed for viral respiratory infections with no evidence of bacterial complications (Bertino 2002, Grijalva *et al.* 2009). Rhinovirus infections are mostly self-limited, and the treatment is mainly symptomatic with over-the-counter medications and supportive care at the wards.

In two phase 3 multicenter studies, pleconaril was associated with a one day reduction in duration of symptoms of common cold and with a more rapid loss of cultivable picornaviruses (Hayden *et al.* 2003), but the license was declined due to concerns

about resistance and safety, including interactions with hormonal contraception (Senior 2002). Other antivirals such as vapendavir and pirodavir have been studied, but the trials have been limited because of drug toxicity and a lack of effect in the clinical setting (Jacobs *et al.* 2013). Although intranasal IFN-α2 had some effect in preventing rhinovirus infections, a placebo-controlled trial of IFN-α2 for treatment of common cold showed no benefit to placebo (Hayden *et al.* 1988). In the contrary, the subjects receiving a higher IFN dose experienced longer symptoms and more nasal congestion and sore throat likely due to the toxicity of the IFN. Due to limited clinical efficacy and side effects, IFN-α2 has not been adopted in clinical use. However, preliminary observations in three patients with hypogammaglobulinemia suggest that short-term subcutaneous pegylated IFN-α combined with oral ribavirin treatment would be beneficial in recurrent or chronic rhinovirus infections in immunocompromised individuals (Ruuskanen *et al.* 2014).

Controversy remains about the evidence of effects of herbal medicine, *Echinacea* (family *Asteraceae*), on the common cold. A Cochrane and another meta-analysis concluded that there is no evidence that *Echinacea* prevent or treat the common cold even though some studies show benefit (Karsch-Volk *et al.* 2014, Karsch-Volk *et al.* 2015). Zinc has activity against rhinovirus and may reduce the duration and severity of symptoms of common cold when administered within 24 hours from the beginning of symptoms (Singh and Das 2013). A Cochrane meta-analysis concluded that in adults, antihistamines have a limited, one to two days, beneficial effect on severity of overall symptoms of common cold, but no clinically significant effect on nasal obstruction, rhinorrhea or sneezing, and there is no evidence of their effectiveness in children (De Sutter *et al.* 2015). The use of intranasal corticosteroids for common cold symptom relief is not supported by the current evidence (Hayward *et al.* 2015). Systemic corticosteroids may be beneficial in rhinovirus-induced wheezing especially in children with high viral loads (Lukkarinen *et al.* 2013, Jartti *et al.* 2015b). In a double-blind, placebo-controlled trial, use of aspirin and acetaminophen was associated with suppression of serum neutralizing antibody response and increased nasal symptoms and signs in healthy adults with an experimental rhinovirus infection (Graham *et al.* 1990).

Antibiotics are not effective in rhinovirus infections. However, the use of azithromycin early during a respiratory tract infection compared with placebo reduced the risk of progression to severe lower respiratory illness in children with a history of lower respiratory tract infection (Bacharier *et al.* 2015). The mechanism for this could be either the anti-inflammatory or antimicrobial effects of macrolide antibiotics (Jackson *et al.* 2016). Antibiotic treatment is beneficial in bacterial complications of rhinovirus infections, such as acute otitis media (Hoberman *et al.* 2011, Tahtinen *et al.* 2011).

2.8 Prevention

2.8.1 Non-pharmaceutical prophylaxis

As no effective medical prophylaxis is available currently, the most effective preventive methods are strategies for avoiding rhinovirus infections including social distancing, use of respiratory masks, and hand hygiene (Jacobs *et al.* 2013). Social distancing, such as avoiding public gatherings and the use of respiratory masks, have been primarily evaluated in prevention of influenza A and influenza-like illness, and depending on the infectivity of the pathogen, may reduce attack rates significantly. The use of masks is an established effective intervention to reduce respiratory virus infections, especially among health-care workers (Jefferson *et al.* 2011). A Cochrane meta-analysis concluded that the spread of respiratory viruses may be prevented by hygienic measures, such as handwashing, especially around younger children (Jefferson *et al.* 2011). Daycare in smaller child groups and at home decreases risk for respiratory infections at young age (Louhiala *et al.* 1995).

Rhinoviruses are pH sensitive and are inactivated below pH 5-6 (Royston and Tapparel 2016). Rhinoviruses are resistant to lipid solvents due to the lack of a lipid envelope (Atmar and Englund 2014). Alcohol disinfectants are not effective against rhinoviruses, but organic acids added into hand cleansers have virucidal effects against rhinovirus (Turner *et al.* 2004, Turner *et al.* 2012). Rubbing of the hands with an ethanol-based hand disinfectant was found to be ineffective against rhinovirus, but washing with soap and water was found to remove viruses efficiently (Savolainen-Kopra *et al.* 2012).

2.8.2 Pharmaceutical prophylaxis

To date, there has not been any rhinovirus vaccine evaluated in clinical trials (Jacobs *et al.* 2013, Glanville and Johnston 2015). Vaccine development against rhinoviruses has been difficult due to the existence of more than 100 rhinovirus serotypes with highly variable antigenic sites. The first vaccine studies showed some promise of serotype-specific protection against cold symptoms, but the diversity of rhinovirus types has posed a challenge to vaccine development as there is only modest cross-neutralization among serotypes, and little progress has been made in over 50 years (Papi and Contoli 2011, Glanville and Johnston 2015). Other challenges to the development of an effective vaccine are the limited epidemiological data of most commonly circulating rhinovirus strains, and an incomplete understanding of virulence of different rhinoviruses and of antigenic differences between species A, B, and recently found species C (Jacobs *et al.* 2013). Limited animal models of rhinovirus infection have made vaccine development difficult, but progress in immunization studies has been made with mouse models for rhinovirus infections (Glanville and Johnston 2015). Recent advances in vaccine development have been obtained by generating cross-

reactive antibodies and T cell responses against conserved regions of rhinovirus using peptide immunogens (Glanville *et al.* 2013, Glanville and Johnston 2015).

In 1980s, intranasal IFN-α2 was found to reduce respiratory illness, but local adverse effects such as nasal bleeding and irritation limited its use (Farr *et al.* 1984, Hayden *et al.* 1986, Monto *et al.* 1986). Better-tolerated IFN-β serine was not efficient in preventing the common cold as compared to placebo (Sperber *et al.* 1989). *Echinacea* plant preparations have been found to be ineffective in preventing a rhinovirus infection or respiratory infections in several double-blinded, placebo-controlled studies (Turner *et al.* 2005, Karsch-Volk *et al.* 2014). A Cochrane meta-analysis confirmed these negative findings, but another meta-analysis suggested that *Echinacea* products may be associated with a small reduction in the incidence of colds (Karsch-Volk *et al.* 2014, Karsch-Volk *et al.* 2015). Intranasal zinc gluconate has been found ineffective for the prevention of experimental rhinovirus colds (Turner 2001), and Cochrane meta-analyses found no evidence of its efficacy in preventing a common cold (Singh and Das 2013) or acute otitis media (Gulani and Sachdev 2014). The effect of vitamin C (ascorbic acid) in preventing and treating the common cold has been a subject of controversy for 70 years. A Cochrane meta-analysis found no difference in the incidence of colds between subjects receiving vitamin C or placebo, although a modest decrease in duration and severity of symptoms was found most pronounced in individuals exposed to brief periods of severe physical exercise (Hemila and Chalker 2013).

In a randomized, double-blind, placebo-controlled trial, prebiotics (galacto-oligosaccharide and polydextrose mixture) and probiotics (*Lactobacillus rhamnosus* GG) administered between days three and 60 of life were associated with a significantly lower incidence of respiratory tract infections and rhinovirus infections in preterm infants (Luoto *et al.* 2014). In another randomized, double-blind, placebo-controlled trial on formula-fed infants, probiotics (*Lactobacillus rhamnosus* GG and *Bifidobacterium lactis* Bb-12) administered daily from two to 12 months of age were associated with a decreased risk of acute otitis media before seven months of age and with a decreased risk for recurrent respiratory infections during the first year of life (Rautava *et al.* 2009). A systematic review concluded that probiotics have a modest effect both in diminishing the incidence of upper respiratory tract infections and the severity of the infection symptoms in immunocompetent children (Ozen *et al.* 2015). A Cochrane meta-analysis involving children and adults indicated that probiotics may be more beneficial than placebo for preventing acute upper respiratory tract infections (Hao *et al.* 2015).

3 AIMS OF THE STUDY

The main objective of this thesis was to assess clinical manifestations and associated morbidity of rhinovirus infections in children during the first two years of life in an observational prospective birth cohort study. We also aimed to study the genetic susceptibility to rhinovirus infections and evaluated the use of the blood MxA protein as a marker for respiratory infections in children younger than two years of age.

The specific aims of this study were:

1. To characterize the burden and the clinical effects of acute respiratory infections caused by rhinovirus and to evaluate the prevalence of asymptomatic rhinovirus infections in children under two years of age. (I)

2. To identify young children with recurrent respiratory tract infections in order to characterize the burden of respiratory infections in these children as well as clinical manifestations, viral etiology, risk factors, and short-term consequences of recurrent respiratory infections. (II)

3. To assess the effects of MBL and TLR gene polymorphisms on the susceptibility to respiratory infections, rhinovirus infections, and acute otitis media in children during the first two years of life. (III)

4. To study the MxA levels during respiratory virus infections, especially in rhinovirus infections, as compared to those in healthy young children. (IV)

4 MATERIALS AND METHODS

4.1 Study design

This study was conducted within the prospective observational birth cohort study called Steps to the Healthy Development and Well-being of Children (the STEPS Study) (Lagstrom *et al.* 2013). Families of 1827 children, including 30 pairs of twins, were recruited during the first trimester of pregnancy or during the first days after the birth from a cohort of all children born in the Hospital District of Southwest Finland between January 2008 and April 2010 to their Finnish or Swedish speaking mothers (eligible cohort, n = 9811 mothers; n = 9936 children). No other selection criteria than language were applied.

Enrolment and follow-up of the study children are shown in Figure 3. Children were followed from birth to two years of age for respiratory infections by daily diaries. Parents recorded all respiratory and other symptoms, physician visits with associated diagnoses and treatment, and illness-related absenteeism of the child from day-care and the parent from work to the diary. The daily diary was available for the parents both in paper and electronic format. Children were invited to scheduled visits to the study clinic at the age of 13 and 24 months. Background information was gathered by structured question forms during pregnancy or soon after birth and at the ages of 13, 18, and 24 months. Data on atopic and allergic conditions were collected with qualified forms (The International Study of Asthma and Allergies in Childhood, ISAAC) at the age of 24 months. Vaccination histories of the children were collected from the electronic registries of regional well baby clinics.

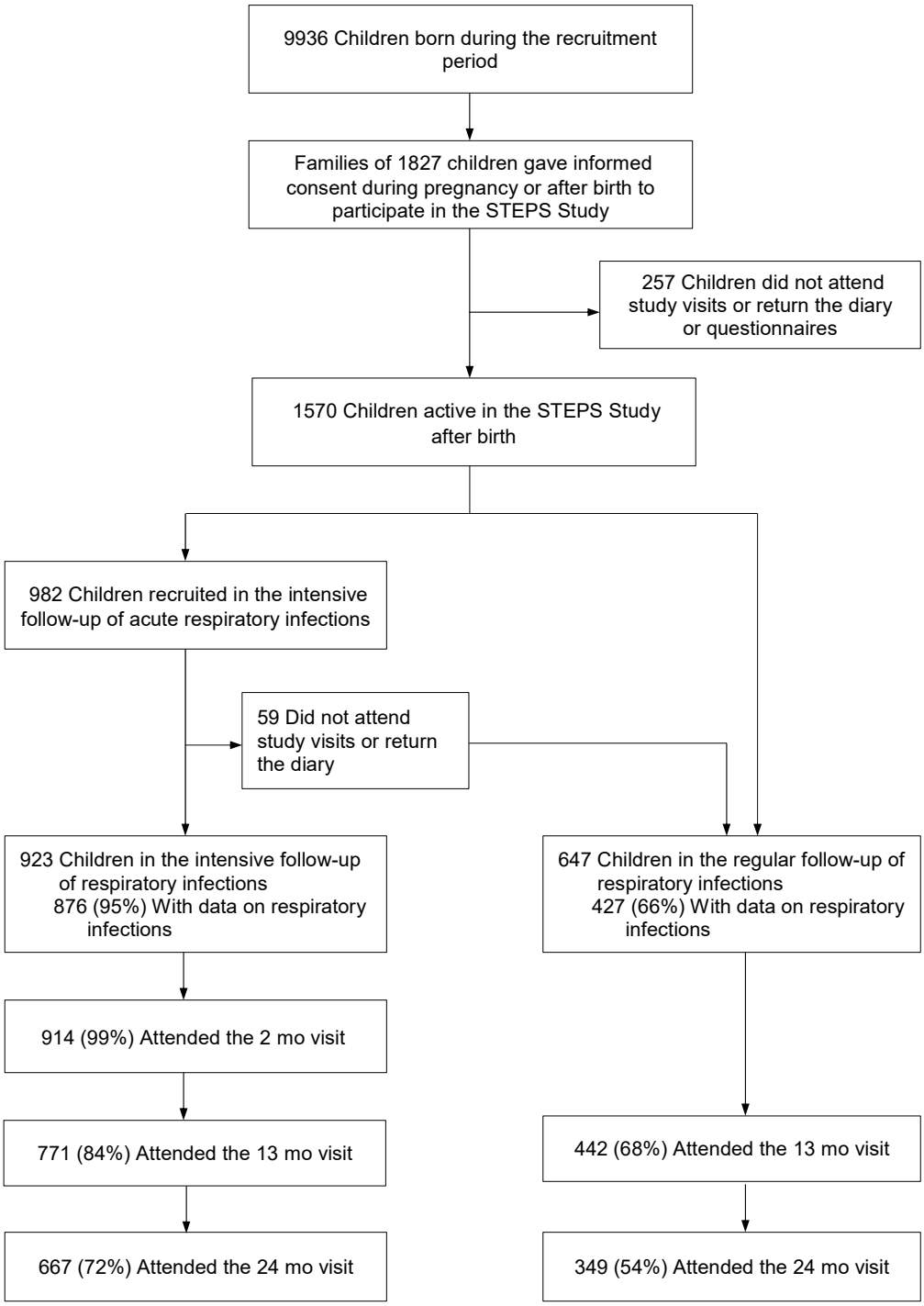

Figure 3. Enrolment and follow-up of the study children. Children were recruited during the first trimester of pregnancy or during the first days after the birth from a cohort of all children born in the Hospital District of Southwest Finland between January 2008 and April 2010 to their Finnish or Swedish speaking mothers. Modified from the Studies I and II.

A subgroup of 982 children were recruited after birth without selection criteria to an intensive follow-up on respiratory infections from birth to two years of age. In addition to scheduled visits at the ages of 13 and 24 months, the children participating in the intensive follow-up were invited to a scheduled visit at the age of two months, and blood samples and a nasopharyngeal sample were obtained. From the children in the intensive follow-up, nasal swab samples were obtained from both nostrils at the depth of 2-3 cm using flocked nylon swabs (Copan, Brescia, Italy) by study personnel at each visit, and any current respiratory symptoms were documented. Parents were trained to collect the nasal swab samples at the first visit to the study clinic by the study personnel. Families were encouraged to visit the study clinic if the child had an acute respiratory infection, and if they felt that an evaluation by a physician was needed. At the study clinic, nasal swab samples were collected, and the child was examined by a study physician. Clinical findings were documented in a structured form. Otitis media was diagnosed by using pneumatic otoscopy and tympanometry. If the family visited a physician elsewhere or they felt that an evaluation by a physician was not needed, the parents took the specimens at home at the onset of illness and sent them to the laboratory by standard mail as described earlier (Peltola *et al.* 2008, Waris *et al.* 2013). Data on emergency department visits and hospitalizations of the child including results of routine virus diagnostics were collected from the Electronic Registry of Hospital District of Southwest Finland, which comprises information from both hospitals providing inpatient pediatric care in the area (Turku University Hospital and Salo District Hospital).

Blood samples for MxA, CRP, and WBC count determinations were collected from children 1 to 24 months of age presenting to the study clinic with symptoms of an acute respiratory tract infection and fever or ill appearance from February 2009 through April 2011. Blood samples for MxA were collected also from children at the age of 2 and 13 months at scheduled visits to the study clinic and from 44 asymptomatic parents of the children. Any samples collected at different time points from the same child were analyzed as separate cases.

4.2 Laboratory methods

4.2.1 Respiratory virus detection (I, II, III, IV)

The nasal swab specimens were stored at -80°C before analysis. Swabs were suspended in phosphate buffered saline and nucleic acids were extracted by a NucliSense easyMag (BioMerieux, Boxtel, Netherlands) or MagnaPure 96 (Roche, Penzberg, Germany) automated extractor. Extracted RNA was reverse transcribed with specific primers for rhino-/enterovirus and RSV, and the cDNA was amplified using real-time, quantitative PCR for rhinovirus, enteroviruses, and RSV as described earlier (Österback *et al.* 2013) with the modification that primers and probe for RSV F gene

were included in the same assay. In rhinovirus detection, primers and probes from the highly conserved 5' non coding region of the genomic RNA were used for simultaneous amplification and differentiation of rhino- and enteroviruses. The method allows detection of all known human rhino- and enterovirus types with superior sensitivity (McLeish *et al.* 2012, Österback *et al.* 2013). Laboratory developed antigen detection tests were performed for influenza A and B viruses, parainfluenza (PIV) type 1, 2, and 3 viruses, RSV, adenovirus, and human metapneumovirus (hMPV) for samples collected in January 2009 or later (89% of all samples). All specimens collected during the influenza seasons were subjected to RT-PCR for influenza A and B viruses (Jokela *et al.* 2015). The first and last day of each influenza season were defined on the basis of the influenza antigen test results and data from the infectious disease surveillance registry of the National Institute for Health and Welfare, Finland. Routine respiratory viral diagnostics was performed by PCR or rapid antigen detection methods for 26 patients as part of their care at the Turku University Hospital.

In the Study IV, Seeplex RV12 multiplex PCR assay (Seegene, Seoul, Korea) was performed according to the manufacturer's instructions for the detection of rhinovirus, RSV-A, RSV-B, adenovirus, influenza A and B viruses, PIV 1-3, hMPV and coronaviruses 229E/NL63 and OC43/HKU1. A separate PCR test was used for detection of human bocavirus (Koskenvuo *et al.* 2008). For rhinovirus and RSV, a positive result in either one of the tests was considered as a positive test result.

4.2.2 Bacterial culture (II)

A semi-quantitative bacterial culturing method was performed for nasopharyngeal samples collected at the age of two months from 312 children visiting on Monday or Tuesday. A sample of bacterial suspension was plated with a 10 µl loop and spread over one-quarter of the plate, and then the sample was streaked onto the remaining three quadrants by using the same 10 µl loop. Four different culture plates were used: a blood agar plate containing 5% sheep blood, a heated blood agar (chocolate agar) plate, a *H. influenzae* selective plate (a heated blood agar plate containing 300 mg/l bacitracin), and a *S. pneumoniae* selective plate (sheep blood agar plate containing 5 mg/l colistin and 2.5 mg/l oxolinic acid). Plates were incubated in 5% CO_2 at 35°C for 48 hours and inspected daily. *S. pneumoniae* isolates were identified by using the optochin disk susceptibility test (Oxoid, Basingstoke, England), *H. influenzae* isolates by the X, V and X+V factor test (Oxoid), and *M. catarrhalis* isolates by the oxidase and Tributyrin test (Rosco Diagnostica, Taastrup, Denmark).

4.2.3 Genetic analyses (III)

Candidate genes and SNPs were selected on the basis for their role in innate host defense and acute inflammatory response and for prior evidence of their involvement in respiratory infections in the literature.

DNA was extracted from 200 µl of whole blood by QIAGEN QIAamp DNA Blood Mini Kit 250 (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The genotyping of MBL structural gene (*MBL2*) in codons 52 (allele D, reference single nucleotide polymorphism (rs) 5030737), 54 (allele B, rs1800450) and 57 (allele C, rs1800451), TLR2 Arg753Gln (rs5743708), TLR3 Leu412Phe (rs3775291), TLR4 Asp299Gly (rs4986790), and TLR8 Leu651Leu (rs2407992) was performed by pyrosequencing (PSQ^{TM}96MA Pyrosequencer, Biotage, Uppsala, Sweden), using a PSQ^{TM}96 PyroGold Q96 reagent kit according to the manufacturer's protocol as previously described (Vuononvirta *et al.* 2011, Nuolivirta *et al.* 2012b). Presence of the PCR products was verified on a stained agarose gel. For genotyping of TLR7 Gln11Leu (rs179008), the PCR products were first purified using the QIA quick PCR Purification Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. PCR products with low deoxyribonucleic acid (DNA) content were eluted to 30 µl of elution buffer. The purified PCR products (5 µl) were pipetted to 96-well plates together with *TLR7* rs179008 forward primer (1.6 µl). Sequencing was done at the Institute for Molecular Medicine, Helsinki, Finland. All the primers were purchased from Sigma-Aldrich, Finland.

4.2.4 Blood MxA protein, CRP, and WBC measurements (IV)

MxA protein levels were determined by an enzyme immunoassay (EIA) from a capillary whole blood sample taken by a fingertip prick (children visiting for respiratory tract infection) or from a peripheral venous whole blood sample taken by antecubital venipuncture (scheduled visits). Blood samples were diluted 1:20 in hypotonic buffer and stored at −70°C until EIA was performed using a set of capture and detection antibodies as described in the supplementary data of the Study IV and in earlier studies (Vallittu *et al.* 2008, Maria *et al.* 2014, Ruuskanen *et al.* 2014). Briefly, lysed and thawed sample and biotinylated detector antibody were added into microtiter well coated with capture antibody. After overnight incubation and washing steps, bound biotin was detected by streptavidin-peroxidase catalyzed color reaction with tetramethylbenzidine. Recombinant human MxA protein produced with a baculovirus expression system was used as the standard (Ronni *et al.* 1993). MxA concentrations (µg/l) were calculated from a standard curve corresponding blood MxA concentrations of 50–1600 µg/l. Higher concentrations were measured after additional dilution of the samples. The test has been validated to measure MxA concentration as an indication of bioavailability of interferon in beta-interferon treated multiple sclerosis patients (Vallittu *et al.* 2008).

CRP and WBC count were measured from capillary whole blood samples. A rapid CRP test was performed with Orion QuikRead 101 (Orion Diagnostica, Espoo, Finland) and rapid WBC count was done using HemoCue WBC (HemoCue, Ängelholm, Sweden) point-of-care analyzer according to the manufacturer's instructions.

4.3 Definitions

Studies I-III

An episode of acute respiratory infection was defined as the presence of rhinitis or cough, with or without fever or wheezing, documented in the diary by parents, or as a diagnosis of an acute respiratory infection by a physician. Episodes were defined separate if there was at least one day without respiratory symptoms in between. If the diary data were missing, outpatient visits and hospitalizations for an acute respiratory infection one day before through 14 days after the nasal swab collection were linked with the virologic result into an episode of an acute respiratory infection. If several nasal samples were collected during continuous respiratory symptoms, the date of a nasal swab taken more than 14 days from the first one was considered as the first day of a new episode. *Co-infections* were defined as detection of two or more viruses from one or several specimens during the same episode within 14 days from each other.

Diagnoses of *acute otitis media*, *wheezing illness* (bronchiolitis, obstructive bronchitis, or acute asthma), *laryngitis*, *pharyngitis*, and *pneumonia* were performed by the study physician, or recorded into the diary or medical records by a physician at other outpatient office or hospital. If there were repeated diagnoses during continuous respiratory symptoms, parallel diagnoses within 14 days were calculated as one diagnosis.

When calculating the *annual days with symptoms of respiratory infection associated with rhinovirus infections*, the length of an acute respiratory infection was limited to a maximum of 60 days. When analyzing *outcomes of rhinovirus infections*, data were included from the symptomatic period with a maximum duration of 14 days before and 14 days after the collection of the nasal sample. Co-infections involving rhinoviruses were excluded from analyses of characteristics of rhinovirus infections. *Illness rates* per year were calculated from the duration of active follow-up for each child.

Data on children with a completed diary for at least one year were included in the Study II. The 10% of children with the highest number of days per year with symptoms of respiratory infection were defined to have *recurrent respiratory tract infections*.

Recurrent acute otitis media was defined as three or more episodes of acute otitis media during the follow-up. The 75th percentile of rhinovirus infections during the follow-up was used as the limit for *recurrent rhinovirus infections* (Study II). *Recurrent wheezing* was defined as two or more episodes of doctor-diagnosed bronchiolitis, obstructive bronchitis, or acute asthma.

Study IV

All vaccinations administered to the child during the 30 days preceding the MxA sample were included in the analysis in the Study IV.

4.4 Statistical analyses

Categorical data were compared by using the chi-square test or Fisher's exact test. Continuous data were described and compared by using either means, standard deviations, or 95% confidence intervals (CI), and Student's t-test, or medians, interquartile ranges, and Mann Whitney *U* test, as appropriate. In the boxplot presentations of the data, boxes show median and interquartile range (IQR), and whiskers show the lowest datum within 1.5 IQR of the lower quartile and the highest datum within 1.5 IQR of the upper quartile.

Studies I and III

Generalized linear models were used to describe and analyze rates of respiratory infections and rhinovirus-associated outcomes. Outcome counts were analyzed by using negative binomial distribution and log link with natural logarithm of follow-up time as an offset variable (Studies I and III). The data were described by using geometric means and 95% CI.

Pneumonia counts were low, and negative binomial regression failed, thus, for pneumonia, Poisson distribution was used instead (Study I). Binomial distribution with logit link was used to determine the proportion of rhinovirus-positive infections from infections with a nasal swab analyzed for viruses. The number of rhinovirus-associated acute respiratory infections was determined as a product of the proportion of rhinovirus infections and total infections. Confidence intervals for the product were determined using 500 bootstrap samples. The proportion of children positive for rhinovirus at 2, 13, and 24 months of age was compared with the Friedman test.

Rates of respiratory infections were analyzed first by using unadjusted negative binomial regression (Study III). Risks of recurrent rhinovirus infections, acute otitis media, recurrent acute otitis media, and rhinovirus-associated acute otitis media were analyzed first by unadjusted binary logistic regression analysis. Background variables with P values < .10 in the univariate analyses were included in the final models (sex and siblings). Then, adjusted regression analyses were performed. Unadjusted and adjusted rate ratios (RR) and odds ratios (OR) with 95% confidence intervals were determined.

Study II

Children with recurrent respiratory tract infections were compared with the other study children. Risk for recurrent respiratory infections was analyzed by binary logistic regression analysis using first sex, siblings, maternal educational level, living environment, and breastfeeding as predictors. Then, the logistic regression analysis was performed for nasopharyngeal bacteria (*S. pneumoniae and M. catarrhalis*), adjusted for sex, siblings, maternal educational level, living environment, and breastfeeding. Parental smoking was inversely associated with recurrent respiratory

infections in univariate analysis and was not included in the adjusted analysis because of suspected reporting bias. Odds ratios (OR) with 95% confidence intervals (CI) were determined.

Study IV
A Mann–Whitney U test was used to compare blood MxA protein levels. A Kruskal–Wallis test was used for multiple comparisons followed by pairwise Mann–Whitney U test with Bonferroni correction. A decision threshold for MxA was calculated by receiver operating characteristic (ROC) analysis, and correlations between blood MxA and viral loads or duration of symptoms were calculated by Spearman's correlation.

P values < .05 were considered statistically significant in all analyses. Statistical analyses for original publications I-III were performed by using SPSS software, version 23.0 (IBM SPSS Statistics for Macintosh, IBM Corp., Armonk, NY, USA) and SAS version 9.4 (SAS Institute Inc., Cary, NC, USA), and for original publication IV, by SPSS software, version 21.0 (IBM SPSS Statistics for Macintosh, IBM Corp., Armonk, NY, USA).

4.5 Ethical aspects

The STEPS Study was found ethically acceptable by the Ministry of Social Affairs and Health (STM 1575/2008, STM 1838/2009) and the Ethics Committee of the Hospital District of Southwest Finland (19.2.2008 §63, 15.4.2008 §134, 19.4.2011 §113). The parents of participating children received written information about the study and gave their written informed consent. The participants were informed about their right to withdraw consent to participate at any time without reprisal or an effect on their medical care. The information collected in the study was handled in confidence and stored in locked cabinets and computers. Study visits were recorded in the hospital registry. The subjects were referred to health care centers or hospital if needed. The subjects were informed about the general results of the study via letters and the website of the research center. The study complied with the Declaration of Helsinki.

5 RESULTS

5.1 Study population

Families of 1827 children were recruited in the STEPS Study. Of these, 1570 children continued in the study after birth (Figure 3). A total of 982 children gave informed consent to participate in the intensive follow-up of respiratory infections. Fifty-nine children did not start the intensive follow-up resulting in the intensive follow-up group of 923 children and in the regular follow-up group of 647 children. Altogether 1303 (83%) children attended actively to the follow-up of respiratory infections by filling the study diary or visiting the study clinic during acute respiratory infections. In the intensive follow-up group, data on acute respiratory infections were received from 876 (95%) of the children. The median follow-up time was 1.99 (interquartile range [IQR], 1.31, 2.00) years. Children active at the follow-up during the study period are shown in Figure 4. Altogether 1089 children had completed the daily diary for at least one year and were included in the Study II.

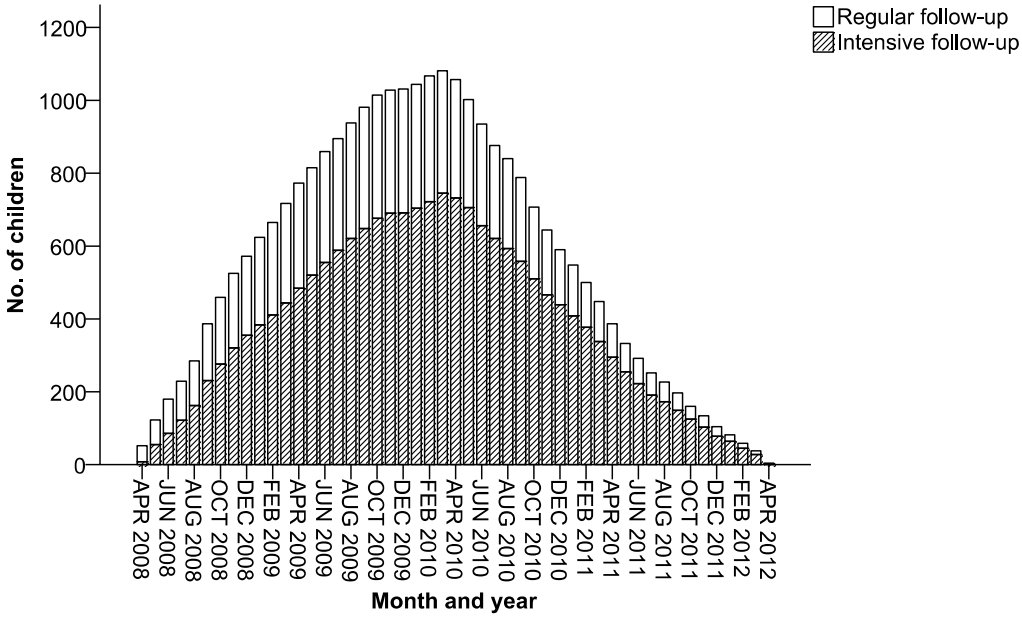

Figure 4. Children active in the follow-up according to the month and year. Altogether 1303 children attended actively to the follow-up of respiratory infections.

Based on data from the National Birth Registry, 55% of mothers in the eligible cohort (n = 9811) had one or more older children (Lagstrom *et al.* 2013). Thus, our study population with 46% of the children having one or more older siblings was

skewed towards including more firstborn children than the general child population. Participating children in the STEPS Study were more often than non-participating children from families with a higher occupational class, living in an urban area, and their parents were more often married (Lagstrom *et al.* 2013). Children in the intensive follow-up were more often first-borns, living in an urban area, from families with higher maternal educational level, and were less frequently exposed to parental smoking than children in the regular follow-up (Table 5). The pneumococcal conjugate vaccination coverage was higher among the children in the intensive follow-up.

Table 5. Characteristics of the study children.

Characteristic	All children (*n* = 1570)	Children in the intensive follow-up (*n* = 923)	Children in the regular follow-up (*n* = 647)	*P* [a]
Female	757 (48)	435 (47)	322 (50)	.26
Premature (gestational age < 37 wk)	75/1549 (5)	38/918 (4)	37/631 (6)	.12
Older siblings	725 (46)	376 (41)	349 (54)	< .001
Maternal educational level high	940/1517 (62)	574/892 (64)	366/625 (59)	.02
Living in the urban area	892/1527 (58)	544/895 (61)	348/632 (55)	.03
Maternal smoking during pregnancy	41/1065 (4)	25/540 (5)	16/525 (3)	.18
Parental smoking at the time of pregnancy or birth	269/1379 (19.5)	141/818 (17)	128/561 (23)	.01
Duration of breastfeeding, mean ± SD (mo)	7.3 ± 4.7	7.4 ± 4.9	7.0 ± 4.4	.11
Outside-home daycare				
At 13 mo of age	276/1264 (22)	185/784 (24)	91/480 (19)	.11
At 18 mo of age	467/1098 (43)	285/685 (42)	182/413 (44)	.42
At 24 mo of age	593/1079 (55)	370/681 (54)	223/398 (56)	.67
Vaccinated with pneumococcal conjugate vaccine[b]	435/1344 (32)	290/806 (36)	145/538 (27)	.001
Vaccinated against influenza[c]				
2008-2009	29/123 (24)	13/59 (22)	16/64 (25)	.70
2009-2010, with seasonal influenza vaccine	254/551 (46)	173/366 (47)	81/185 (44)	.44
2009-2010, with pandemic influenza vaccine	459/551 (83)	296/366 (81)	163/185 (88)	.03
2010-2011	171/486 (35)	130/371 (35)	41/115 (36)	.90
2011-2012	13/67 (19)	12/54 (22)	1/13 (8)	.23
Data on respiratory infections	1303 (83)	876 (95)	427 (66)	< .001
Duration of the follow-up, median (IQR), yrs	1.99 (1.31, 2.00)	1.99 (1.40, 2.00)	2.00 (1.16, 2.00)	.65

Values are *n* (%) unless otherwise specified. SD, standard deviation, IQR, interquartile range.

[a] Comparison between children in the intensive and regular follow-up.

[b] Administered before 12 months of age. All study children were born before the pneumococcal conjugate vaccine was introduced to the Vaccination Programme in Finland (before June 1st 2010).

[c] Calculated among children 6 months of age or older actively followed up at the beginning of each influenza season.

5.2 Acute respiratory infections (I)

5.2.1 Incidence and characteristics

Altogether 12 846 episodes of acute respiratory infection and 67 298 days with symptoms of respiratory infection were documented in all children. In the children in the intensive follow-up, a total of 8847 episodes of acute respiratory infection causing 4691 outpatient visits and 85 hospitalizations were documented. Of these outpatient visits, 2142 (46%) occurred at the study clinic, 2338 (50%) at other outpatient clinics, and 211 (4%) at the emergency department. A total of 1419 episodes of acute otitis media, 281 wheezing illnesses, and 1872 antibiotic treatments for acute respiratory infections were documented in the children in the intensive follow-up. Of the children in the intensive follow-up with data on respiratory infections, 520 (59%) had at least one episode of acute otitis media, 141 (16%) were diagnosed with a wheezing illness, 567 (65%) were treated at least once with antibiotics for an acute respiratory infection, and 62 (7%) were hospitalized for an acute respiratory infection.

A total of 6961 nasal swab samples were collected, of which 4878 (70%) samples were taken during an acute respiratory infection. A nasal swab sample was collected during 4728 (53%) of the total of 8847 acute respiratory infections documented in the children in the intensive follow-up. Of the swabs taken during acute respiratory infections, 3415 (70%) were taken at home by parents. The mean time for home samples to be delivered to the study clinic via standard mail was 2.8 (SD, 2.1) days. A total of 2270 samples were collected at scheduled visits. At the time of the scheduled visit, 227 children had an episode of an acute respiratory infection and eight had fever only. All episodes of fever only were excluded from the analyses (n = 48).

Characteristics of acute respiratory infections with and without a nasal sample are compared in Supplementary Table in the Study I. Because of the routine sample collection, study clinic visits were more frequent during acute respiratory infections with nasal samples. However, visits to other outpatient clinics or an emergency department were more frequent during episodes without a nasal sample. Parents reported less symptoms in the diary during episodes without a sample, but there was no difference in the rates of acute otitis media or pneumonia and slightly more antibiotic treatments during episodes without a sample.

Rhinovirus was detected in 59% of acute respiratory infections with a specimen analyzed for viruses (Table 6). RSV was detected in 6%, other respiratory viruses in 5%, and coinfections were confirmed in 2% of acute respiratory infections. A total of 737 (89%) of the children had at least one detected symptomatic rhinovirus infection during the first two years of life (range, 0-16).

Table 6. Detected viruses during acute respiratory infections.

Detected virus	Acute respiratory infections, No. (%) (*n* = 4728)
Any	3340 (70.6)
Rhinovirus	2775 (58.7)
RSV	262 (5.5)
Enterovirus	69 (1.5)
Parainfluenza virus 1, 2 or 3	66/4308 (1.5)
Influenza virus A or B	45 (1.0)
Human metapneumovirus	41/4308 (1.0)
Adenovirus	10/4308 (0.2)
More than one virus detected[a]	72 (1.5)

RSV, respiratory syncytial virus.
[a] Rhinovirus was co-detected with RSV (*n* = 25), enterovirus (*n* = 15), parainfluenza virus (PIV, *n* = 9), influenza virus (*n* = 6), adenovirus (*n* = 8), human metapneumovirus (hMPV, *n* = 5), or with influenza virus and PIV (*n* = 1), RSV was co-detected with hMPV (*n* = 2), and enterovirus with influenza virus (*n* = 1).

Characteristics of acute respiratory infections positive or negative for rhinovirus are compared in Table 7. Rhinorrhea was present in almost all rhinovirus positive infections. Children with an acute respiratory infection positive for rhinovirus experienced fever and cough less frequently than those with an acute respiratory infection who were negative for rhinovirus, but there was no difference in duration of the symptoms. Acute otitis media complicated 13% of all documented rhinovirus-positive acute respiratory infections, and antibiotic treatment was prescribed in 15%. Children visited a physician during 36% of rhinovirus infections, and acute otitis media was diagnosed in 351 (35%) of 1011 rhinovirus infections during which a child was evaluated by a physician. Over half of the children attending day-care stayed at home during a rhinovirus infection thereby also often necessitating parental absenteeism from work. Physician visits, diagnoses of acute otitis media, wheezing illness, and pneumonia, antibiotic treatments, and use of over-the-counter pain or fever medications were more frequent in infections negative for rhinovirus, but they were common also in rhinovirus infections.

Table 7. Characteristics of rhinovirus-positive and rhinovirus-negative acute respiratory infections.

	Acute respiratory infections[a]		
Characteristic	**Rhinovirus-positive** (*n* = 2775)	**Rhinovirus-negative** (*n* = 1884)	***P***
Age, mean ± SD, y	0.97 ± 0.50	0.96 ± 0.51	.60
Symptoms			
Duration of respiratory symptoms, median (IQR), d	9.0 (6.0, 13.0)	9.0 (5.0, 13.0)	.16
Rhinorrhea	2358/2383 (99.0)	1488/1623 (91.7)	<.001
Cough	1449/2383 (60.8)	1173/1623 (72.3)	<.001
Fever ≥38.0°C	715/2383 (30.0)	784/1623 (48.3)	<.001
Outcomes			
Child absent from day-care[b]	197/384 (51.3)	171/273 (62.6)	.004
Days, median (IQR)	2.0 (1.0, 4.0)	3.0 (2.0, 4.0)	.14
Parent absent from work[b,c]	163/384 (42.4)	130/273 (47.6)	.19
Days, median (IQR)	2.0 (1.0, 3.0)	2.0 (1.0, 4.0)	.26
Outpatient clinic visit	999 (36.0)	1030 (54.7)	<.001
Hospitalization	16 (0.6)	24 (1.3)	.01
Days, median (IQR)	1.0 (1.0, 2.0)	1.0 (1.0, 2.0)	.39
Diagnoses (other than ARI)			
Acute otitis media	351 (12.6)	333 (17.7)	<.001
Wheezing illness	71 (2.6)	91 (4.8)	<.001
Laryngitis	33 (1.2)	59 (3.1)	<.001
Pneumonia	5 (0.2)	12 (0.6)	.01
Treatment			
Antibiotic treatment	408 (14.7)	392 (20.8)	<.001
Systemic corticosteroids	20 (0.7)	18 (1.0)	.38
Analgesics or antipyretics	992/2197 (45.2)	749/1341 (55.9)	<.001

Values are *n* (%) unless otherwise specified. ARI; acute respiratory infection; IQR, interquartile range.

[a] Episodes positive for rhinovirus and another virus were excluded from the analysis (*n* = 69). Of 1884 rhinovirus-negative acute respiratory infections, 262 were positive for respiratory syncytial virus (RSV); 69 for enterovirus; 66 for parainfluenza virus type 1, 2 or 3; 45 for influenza virus A or B; 41 for human metapneumovirus; 10 for adenovirus; 2 for RSV and hMPV; 1 for enterovirus and influenza virus A; and 1388 were virus-negative.

[b] Calculated among children attending outside-home day-care at the time of acute respiratory infection.

[c] Parent absent from work because of child's respiratory illness.

5.2.2 The disease burden associated with rhinovirus infections

The mean annual rate of all acute respiratory infections, including those lacking a specimen for virologic analysis, was 5.9 per child (Table 8). The rate of documented rhinovirus infections was 2.0 per child per year, and the estimated annual rate of acute respiratory infections caused by rhinovirus was 3.5 per child in children under 2 years of age. The rate of rhinovirus infections was highest among children 6 to 23 months of age, but noticeably high already before 6 months of age (Figure 5). Rhinovirus was associated with half of the episodes of acute otitis media and with almost half of antibiotic treatments and outpatients visits for acute respiratory infection (Figure 6). Rhinoviruses were associated with 41% of acute wheezing illnesses, and the estimated rate of rhinovirus-associated wheezing illnesses was 7.8 per 100 children per year (95% CI, 5.8-10.2). Rhinovirus infections were associated with 715 (46%) of 1540 febrile acute respiratory infections. Rhinovirus infections were associated with over half of the missed work-days of the parents due to child's respiratory infections, and the estimated rate of lost workdays because of child's rhinovirus infections was 3.4 per child per year (95% CI, 2.9-4.0) during the daycare.

Table 8. Rates of acute respiratory infections and associated outcomes. Modified from the Study I.

	Rhinovirus-positive ARIs	**Proportion of rhinovirus-positive ARIs[a]**	**All ARIs**	**Estimated rhinovirus-associated ARIs[b]**
	mean/child/y (95% CI)	*proportion (95% CI)*	*mean/child/y (95% CI)*	
Episodes	2.0 (1.9-2.1)	0.59 (.57-.60)	5.9 (5.7-6.1)	3.5 (3.3-3.6)
Days with respiratory symptoms	18.8 (17.1-20.6)	0.61 (.60-.61)	50.4 (47.8-53.2)	30.7 (29.0-32.5)
Outpatient visits	0.9 (0.8-0.9)	0.48 (.46-.50)	3.1 (2.9-3.3)	1.5 (1.4-1.6)
	mean/100 children/y (95% CI)		*mean/100 children/y (95% CI)*	
Hospitalizations	1.2 (0.7-2.0)	0.39 (.25-.55)	5.9 (4.4-7.9)	2.3 (1.2-3.9)
AOM	24.9 (22.0-28.2)	0.50 (.46-.54)	94.2 (86.9-102.3)	47.2 (41.9-52.2)
Wheezing	5.1 (3.8-6.8)	0.41 (.34-.49)	18.8 (15.4-23.0)	7.8 (5.8-10.2)
Pneumonia	0.4 (0.2-0.9)	0.29 (.13-.54)	2.4 (1.8-3.4)	0.7 (0.2-1.3)
Antibiotic treatments	30.8 (27.3-34.7)	0.49 (.46-.53)	123.5 (114.2-133.6)	61.2 (54.5-68.2)

ARI, acute respiratory infection; AOM, acute otitis media; CI, confidence interval.

[a] Proportion of rhinovirus-positive infections from all infections analyzed for viruses.

[b] Number of rhinovirus-associated acute respiratory infections was determined as the product of the proportion of rhinovirus infections and all infections.

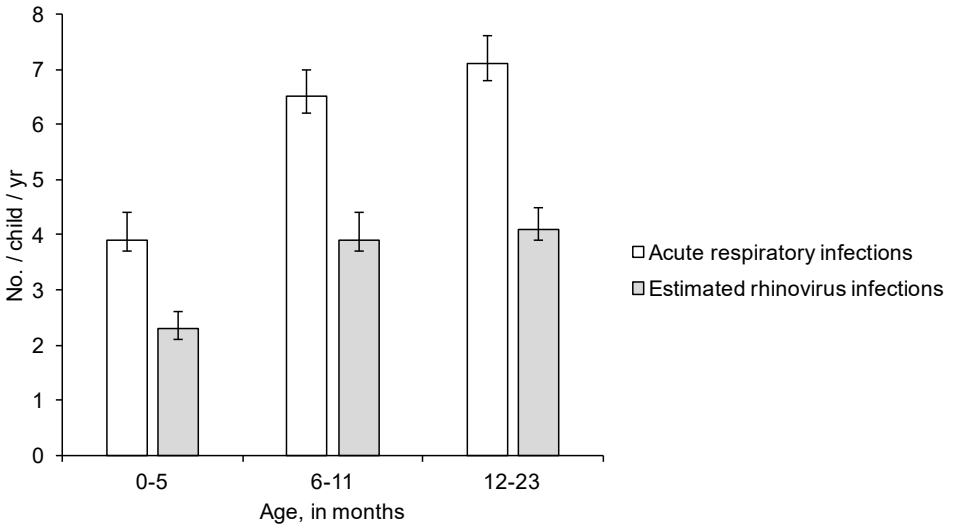

Figure 5. Mean rates of observed acute respiratory infections and estimated rhinovirus infections in children during the first two years of life. Error bars represent 95% CI for mean.

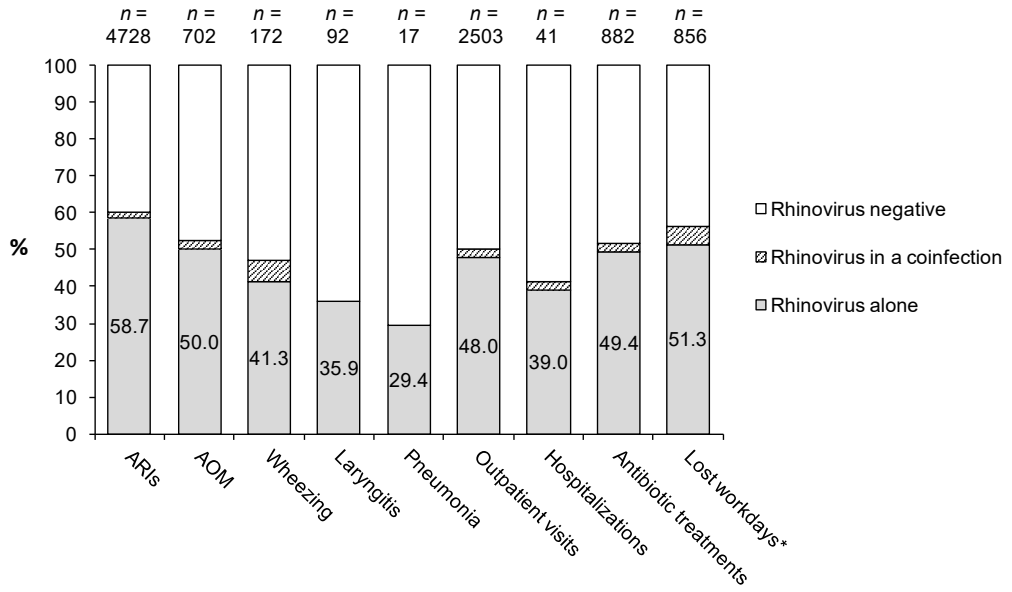

Figure 6. Proportion of detected rhinoviruses in nasal swabs collected during all acute respiratory infections and during infections associated with specified diagnoses and outcomes. Total numbers of observed outcomes are presented above each column. *Lost parental workdays because of child's acute respiratory infection. AOM, acute otitis media; ARI, acute respiratory infection.

5.2.3 Seasonality of rhinovirus infections

Rhinoviruses were detected throughout the year with high-incidence periods from August to November and from April to May (Figure 7). From August to November, rhinovirus was found in 1245 (73%) of 1713 acute respiratory infections and in 154 (68%) of 228 acute respiratory infections complicated by acute otitis media. From April to May, rhinovirus was found in 504 (58%) of 866 acute respiratory infections and 69 (54%) of 127 infections complicated by acute otitis media. Rhinovirus detections were similar in each of the four years covered by the study period, and the circulation of rhinoviruses in this cohort seemed not to be largely affected by influenza pandemic in 2009, which was mild in this cohort with a high vaccination coverage (88%).

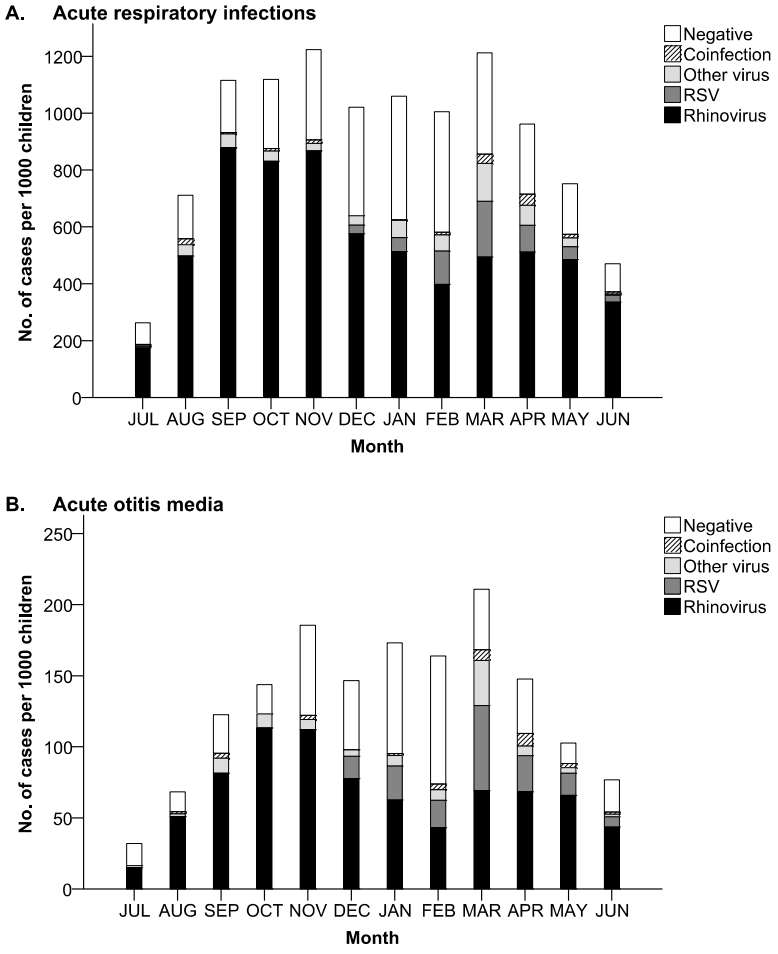

Figure 7. Seasonality of rhinovirus infections. Monthly distribution and viral etiology of acute respiratory infections (panel A) and acute respiratory infections with an acute otitis media (panel B) are shown by bars that indicate monthly numbers of detected viruses in nasal samples per 1000 children in the active follow-up from the four-year study period. RSV, respiratory syncytial virus. Modified from the Study I.

5.2.4 Prevalence of rhinovirus at 2, 13, and 24 months of age

The proportion of children positive for rhinovirus regardless of symptoms increased from 14% at 2 months of age to 18% at 13 months of age and to 24% at 24 months of age ($P < .001$), Figure 8. When specifically asked by the study nurse at scheduled visits, 653 of 2316 children (28%) reported respiratory symptoms. Rhinovirus was detected in 252 (41%) of 619 children with respiratory symptoms at the scheduled visit and in 140 (9%) of 1606 asymptomatic children ($P < .001$).

Of 1278 children who were asymptomatic at the scheduled visit, 307 (24%) had respiratory symptoms in the period from 14 days preceding to 7 days following the visit. At the time of the scheduled visit, there were respiratory symptoms in 290 (19%) families of 1505 asymptomatic children. Respiratory symptoms occurring within 14 days before to 7 days after sampling were documented in 37 (32%) of 115 asymptomatic rhinovirus-positive children and in 270 (23%) of 1163 asymptomatic rhinovirus-negative children ($P = .03$). Respiratory symptoms were documented in 45 (36%) families of 125 asymptomatic rhinovirus-positive children and in 245 (18%) of 1380 asymptomatic rhinovirus-negative children ($P < .001$).

Asymptomatic rhinovirus-positive children had lower numbers of rhinovirus copies in nasal swabs than children with respiratory symptoms (mean [SD] log of copies per swab, 4.5 [1.3] vs. 5.7 [1.5], P < .001).

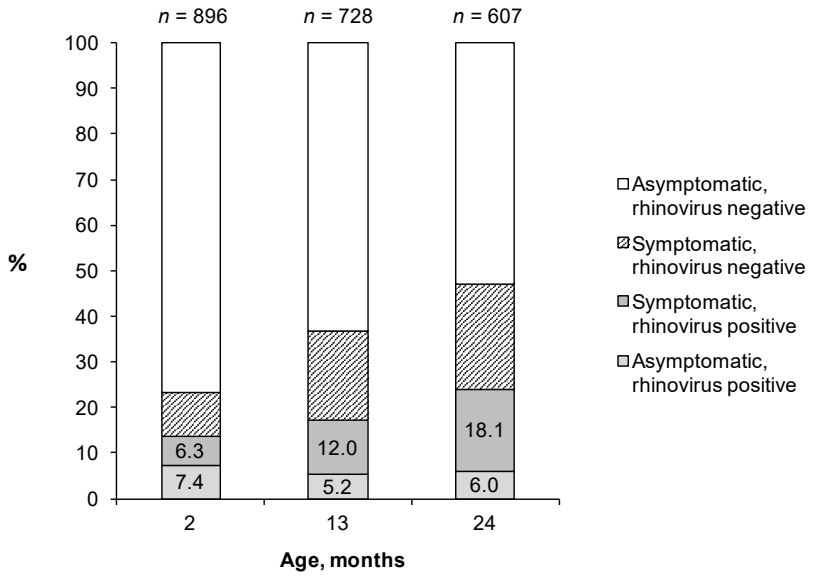

Figure 8. Prevalence of rhinovirus and respiratory symptoms at scheduled visits. Modified from the Study I.

5.3 Recurrent respiratory tract infections (II)

5.3.1 The disease burden

The median number of annual days with symptoms of respiratory infection was 44.2 (IQR, 24.7, 69.0) per child, and the 90th percentile was 98.0. Children above this limit (n = 109) were defined as having recurrent respiratory tract infections and were compared with other children (n = 980). The children with recurrent respiratory tract infections had a mean rate of 9.5 acute respiratory infection episodes and 6.2 physician visits for respiratory infection per child per year (Table 9). They had their first acute respiratory infection at a younger age than children without recurrent infections (mean [SD] age 2.2 [1.7] vs. 4.0 [2.9] months; P < .001), and the first episode of acute otitis media occurred at younger age in these children (mean [SD] age 8.7 [4.6] vs. 10.7 [5.2] months; P < .001). The rate of respiratory infections in these children was higher than that of the other children from the first months of life and stayed constantly higher during the first two years of life (Figure 9).

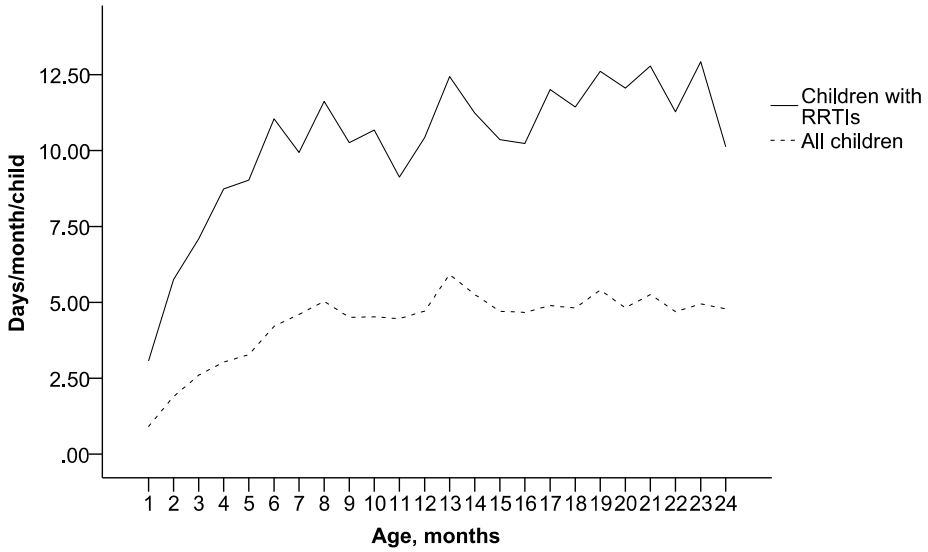

Figure 9. Mean rate of days with symptoms of respiratory infection in children with recurrent respiratory tract infections (RRTI) and in all children calculated among children at active follow-up at each age.

Table 9. Clustering of acute respiratory infections and associated outcomes in children with and without recurrent respiratory tract infections (RRTI). Modified from the Study II.

Variable	Children with RRTIs (*n* = 109)	Children without RRTIs (*n* = 980)	RR (95% CI)	*P*
	mean/child/yr (95% CI)			
Days with symptoms of ARI	123.6 (109.8-139.0)	42.5 (40.8-44.2)	2.91 (2.57-3.29)	< .001
ARI episodes	9.5 (8.8-10.2)	5.7 (5.6-5.9)	1.66 (1.54-1.80)	< .001
Rhinovirus infections	3.1 (2.7-3.6)	1.9 (1.8-2.0)	1.67 (1.43-1.95)	< .001
Outpatient clinic visits	6.2 (5.3-7.3)	2.6 (2.5-2.8)	2.36 (2.00-2.79)	< .001
	No. (%)		OR (95% CI)	*P*
ARI before 3 mo of age	84 (77.1)	437 (44.6)	4.18 (2.63-6.64)	< .001
AOM before 6 mo of age	35 (32.1)	104 (10.6)	3.98 (2.54-6.25)	< .001
Detected viruses[a]				
Rhinovirus	73/73 (100.0)	552/607 (90.9)	10.35 (2.31-∞)	.003
Recurrent rhinovirus infections[b]	49/73 (67.1)	186/607 (30.6)	4.62 (2.75-7.76)	< .001
RSV	26/73 (35.6)	183/607 (30.1)	1.28 (.77-2.13)	.34
Other virus	23/73 (31.5)	159/607 (26.2)	1.30 (.77-2.19)	.33
Coinfection	15/73 (20.5)	49/607 (8.1)	2.95 (1.56-5.58)	.001
Diagnoses, n (%)				
AOM	101 (92.7)	596 (60.8)	8.13 (3.92-16.90)	< .001
Recurrent AOM[c]	65 (59.6)	243 (24.8)	4.48 (2.98-6.75)	< .001
Laryngitis	20 (18.3)	130 (13.3)	1.47 (.87-2.47)	.15
Wheezing illness	36 (33.0)	117 (11.9)	3.64 (2.34-5.67)	< .001
Recurrent wheezing[d]	17 (15.6)	42 (4.3)	4.13 (2.26-7.54)	< .001
Pneumonia	8 (7.3)	34 (3.5)	2.20 (.99-4.89)	.05
Hospitalization	15/73 (20.5)	29/641 (4.5)	5.46 (2.77-10.76)	< .001
Treatment				
Antibiotic treatment	101 (92.7)	663 (67.7)	6.04 (2.90-12.55)	< .001
Recurrent use of antibiotics	80 (73.4)	333 (34.0)	5.36 (3.44-8.36)	< .001
Systemic corticosteroids	14 (12.8)	55 (5.6)	2.48 (1.33-4.62)	.004
Inhaled bronchodilators	33 (30.3)	102 (10.4)	3.74 (2.37-5.90)	< .001
Inhaled corticosteroids	15 (13.8)	37 (3.8)	4.07 (2.15-7.68)	< .001
Tympanostomy tubes	35/99 (35.4)	115/903 (12.7)	3.75 (2.38-5.91)	< .001
Adenoidectomy	13/99 (13.1)	25/903 (2.8)	5.31 (2.62-10.75)	< .001
Asthma at 24 mo of age	10/84 (11.9)	33/747 (4.4)	2.92 (1.39-6.17)	.005

AOM, acute otitis media; ARI, acute respiratory infection; CI, confidence interval; OR, odds ratio; RR, rate ratio; RRTI, recurrent respiratory tract infection; RSV, respiratory syncytial virus.

[a] Calculated among children in the intensive follow-up who returned nasal swabs or were healthy.

[b] ≥ 5 rhinovirus infections during the follow-up.

[c] ≥ 3 episodes during the follow-up.

[d] ≥ 2 episodes during the follow-up.

All documented diagnoses except laryngitis, treatments, outpatient visits and hospitalizations for acute respiratory infections were more frequent in children with recurrent respiratory tract infections than in other children (Table 9). For pneumonia, the difference was not statistically significant. The median number of administered antibiotics for respiratory infections in children with recurrent infections was 4.0 (IQR, 2.0, 7.0) during the follow-up, while the median number of antibiotics for respiratory infections in children without recurrent infections was 1.0 (IQR 0.0, 3.0) ($P < .001$).

The use of asthma medications was more frequent in these children. The children with recurrent respiratory tract infections were at an increased risk for asthma by 24 months of age and the risk was increased after adjustment for child's atopy and maternal asthma (OR 2.87; 95% CI, 1.33-6.23, P = .007). These children had over a threefold risk for tympanostomy tube placement and over a fivefold risk for adenoidectomy during the follow-up as compared to other children.

Of the overall respiratory disease burden, 25% of all respiratory illness days, 21% of physician visits, and 32% of hospitalizations occurred in the children with recurrent respiratory infections; 19% of acute otitis media episodes and 28% of wheezing illnesses were diagnosed in these children (Supplemental Digital Content 3 in the Study II). Of all antibiotic treatments prescribed for respiratory infections, 20% were prescribed to children with recurrent infections, and 58 (25%) of all otorhinolaryngological procedures (n = 229) were made to these children.

5.3.2 Viral etiology

Rhinovirus alone was detected in 427 (58%) of the total of 733 acute respiratory infections in children with recurrent respiratory tract infections, and in 2113 (59%) of 3591 acute respiratory infections in children without recurrent infections (P = .34). Other viruses were found in 73 (10%) and in 444 (12%) of the infections in children with and without recurrent respiratory tract infections, respectively. Recurrent rhinovirus infections and coinfections were detected more often in children with recurrent infections, but RSV and other virus infections were found at a similar rate (Table 9).

At the scheduled visits, rhinovirus was not detected in any of the children with recurrent respiratory tract infections at asymptomatic state at the age of 2 or 13 months, but in 8 (27%) children at the age of 24 months. In contrast, rhinovirus was constantly found in 8 to 9% of asymptomatic children at 2, 13, or 24 months of age among children without recurrent infections (P = .05, P = .06, and P = .001, respectively. Table 5 in the Study II).

5.3.3 Risk factors

Of the children with recurrent respiratory tract infections, 26 (24%) of 108 were at outside-home daycare at 13 months of age as compared to 201 (21%) of 959 children without recurrent infections (P = .45). Older siblings and living in the rural area were associated with an increased risk for recurrent respiratory infections based on univariate analysis, and parental smoking was inversely associated with recurrent respiratory infections (Table 3 in the Study II). Reporting bias was suspected for the effect of smoking, and it was not included in the final model. After adjustment, only

older siblings remained as a significant risk factor for recurrent respiratory tract infections (Table 10).

Table 10. Adjusted analysis of risk factors of recurrent respiratory tract infections (RRTI). Modified from the Study II.

Characteristic	Children with RRTIs, No. (%) (*n* = 109)	Children without RRTIs, No. (%) (*n* = 980)	Adjusted OR (95% CI)	*P*
Male sex	62 (56.9)	502 (51.2)	1.17 (.77-1.77)	.46
Older siblings	76 (69.7)	422 (43.1)	3.03 (1.94-4.74)	< .001
Maternal educational level high	73 (67.0)	612/955 (64.1)	1.33 (.85-2.06)	.21
Living in the non-urban area	57 (52.3)	389/957 (40.6)	1.34 (.88-2.04)	.17
Breastfed at least for 6 mo	70/106 (66.0)	566/937 (60.4)	1.20 (.78-1.85)	.41

CI, confidence interval; OR, odds ratio; RRTI, recurrent respiratory tract infection.
Binary logistic regression analysis was performed using sex, siblings, maternal educational level, living environment, and breastfeeding as predictors.

5.3.4 Nasopharyngeal bacterial colonization

A nasopharyngeal sample was cultured for bacteria from 312 children at two months of age. *S. pneumoniae* was found in 38 (12%), *M. catarrhalis* in 72 (23%), *H. influenzae* in 3 (1%), and other bacteria in 265 (85%) children. Early nasopharyngeal colonization with *S. pneumoniae* was more frequent in children with recurrent respiratory tract infections, but the association was not significant after adjustment (Figure 10).

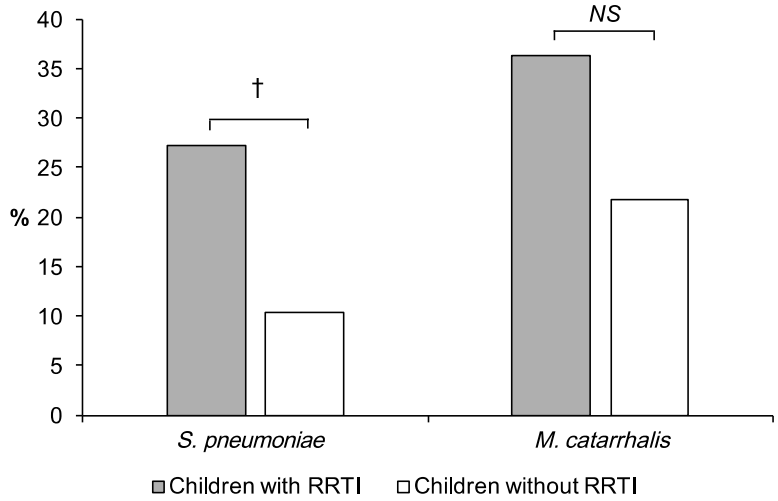

Figure 10. Bacterial colonization at 2 months of age in children with and without recurrent respiratory tract infections (RRTI). † For *S. pneumoniae*, unadjusted OR, 3.26 (95% CI, 1.38-7.68), *P* = .007; adjusted OR (aOR) 2.44 (95% CI, .93-6.39), *P* = .07. For *M. catarrhalis*; unadjusted OR, 2.06 (95% CI, .96-4.42), *P* = .06; aOR 1.71 (95% CI, .75-3.88), *P* = .20. *NS*, not significant.

5.4 Genetic susceptibility (III)

Genotypes for MBL, TLR2, TLR3, TLR4, TLR7, and TLR8 are presented in Table 11. Minor allele frequencies (MAFs) in the STEPS cohort and European and global reference populations are shown in Table 12. MAFs of MBL, TLR2 and TLR3 in the STEPS cohort were similar to those detected in the European population, and SNPs in TLR4, TLR7, and TLR8 were found more frequently in the STEPS population. All studied SNPs in TLRs were detected more frequently in the STEPS than in the global default population. The most frequent MBL polymorphism, in codon 54, was found at a similar frequency in the STEPS and global populations, and SNP in codon 52 was more frequent and SNP in codon 57 was more rare in the STEPS cohort.

Table 11. Genotypes of the study children for mannose-binding lectin (MBL) and toll-like receptors (TLR) 2, 3, 4, 7, and 8. Modified from the Study III.

Genotype	No. (%) (*N* = 381)
MBL	
A/A (wild-type)	256 (67)
A/B (variant type A/O)	79 (21)
A/C (variant type A/O)	2 (1)
A/D (variant type A/O)	35 (9)
C/B (variant type O/O)	2 (1)
B/D (variant type O/O)	1 (0)
B/B (variant type O/O)	5 (1)
D/D (variant type O/O)	1 (0)
TLR2 Arg753Gln	
G/G (wild-type)	363 (95)
G/A	18 (5)
A/A	0 (0)
TLR3 Leu412Phe	
C/C (wild-type)	136/285 (48)
C/T	120/285 (42)
T/T	29/285 (10)
TLR4 Asp299Gly	
A/A (wild-type)	317 (83)
A/G	62 (16)
G/G	2 (1)
TLR7 Gln11Leu	
A/A (girls) or A (boys) (wild-type)	175/290 (60)
A/T (girls)	53/290 (18)
T/T (girls) or T (boys)	62/290 (21)
TLR8 Leu651Leu	
G/G (girls) or G (boys) (wild-type)	121/292 (41)
G/C (girls)	68/292 (23)
C/C (girls) or C (boys)	103/292 (35)

MBL, mannose-binding lectin; TLR, toll-like receptor.

Table 12. Comparison of minor allele frequencies (MAFs) in the STEPS Study cohort of Finnish children and European and global sample populations.

Gene	Reference SNP[a]	MAF in the STEPS cohort	MAF in the European population[b]	*P* (STEPS vs. European)	Global MAF[c]	*P* (STEPS vs. global)
MBL2	rs5030737	5.0%	6.0%	.37	2.7%	.001
MBL2	rs1800450	11.9%	14.1%	.18	12.2%	.84
MBL2	rs1800451	0.5%	1.2%	.14	8.1%	< .001
TLR2	rs5743708	2.4%	2.4%	.97	0.7%	< .001
TLR3	rs3775291	31.2%	32.4%	.63	23.2%	< .001
TLR4	rs4986790	8.7%	5.7%	.01	6.0%	.005
TLR7	rs179008	21.7%	17.6%	.05	11.8%	< .001
TLR8	rs2407992	33.9%	28.9%	.04	27.7%	.002

MAF, minor allele frequency; MBL, mannose-binding lectin; SNP, single nucleotide polymorphism; TLR, toll-like receptor.

[a] For MBL, SNP in codon 52 (rs5030737) referred as allele D, in codon 54 (rs1800450) as allele B, and in codon 57 (rs1800451) as allele C.

[b] Based on the sample of 1006 chromosomes from a European population reported in the SNP database by the National Center for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov/snp/).

[c] Reported in the SNP database in a default global population (1000Genome phase 3 genotype data from 2500 worldwide individuals, released in 2013).

5.4.1 Respiratory infections

Children with the MBL variant genotype had a mean annual rate of 59 days with symptoms of respiratory infection as compared to 49 days in the children with wild-type (P = .01). The children with the MBL variant genotype had a mean rate of 6.2 episodes of acute respiratory infection per child per year as compared to 5.9 episodes in the children with wild-type, but the difference was not significant when assessed by episodes (aRR, 1.06; 95% CI, .95-1.17; P = .29). Other studied genetic variants had no significant effects on the rate of days with respiratory symptoms or on the rate of episodes of acute respiratory infections (Table 13; data for episodes not shown).

5.4.2 Rhinovirus infections

The median number of symptomatic rhinovirus infections was 3.0 (IQR, 1.0, 5.0) during the follow-up, and recurrent rhinovirus infections were defined as 5 or more rhinovirus infections during the follow-up (the 75th percentile of rhinovirus infections). The associations between MBL and TLR genotypes and the mean rate of rhinovirus infections and recurrent rhinovirus infections are shown in Table 13. The TLR8 variant type was associated with an increased rate of rhinovirus infections and with an increased risk of recurrent rhinovirus infections. Children with the TLR7 variant genotype had a decreased risk of recurrent rhinovirus infections. The TLR7 variant genotype was associated with a decreased rate of rhinovirus infections in univariate analysis, but the difference was not significant after adjustment.

Because *TLR7* and *TLR8* genes are located on the X chromosome, we performed analyses separately for males and females (Supplementary Tables 2 and 3 in the Study III). Between wild-type and variant genotypes, the differences in rates of rhinovirus infections were parallel in boys and girls, but they were not significant, possibly because of a lack of statistical power. Genetic polymorphisms in MBL, TLR2, TLR3, or TLR4 had no effect on the rate of rhinovirus infections. Genetic polymorphisms in MBL, TLR2, TLR3, TLR4, TLR7, or TLR8 were not associated with the prevalence of rhinovirus or with asymptomatic rhinovirus infection at the age of 2, 13, or 24 months (Supplementary Table 5 in the Study III).

5.4.3 Acute otitis media

Children with the TLR2 variant genotype had an increased overall risk of acute otitis media and an increased risk of recurrent acute otitis media (Table 14). The MBL variant type was specifically associated with an increased risk of rhinovirus-associated acute otitis media, and the TLR7 variant type was associated with a decreased risk of rhinovirus-associated acute otitis media. When analyzed separately for males and females, children of both sexes with the TLR7 variant type had a low risk of rhinovirus-associated acute otitis media, but the difference was significant only in females ($P = .03$) (Supplementary Table 4 in the Study III). TLR3, TLR4, and TLR8 polymorphisms were not significantly associated with acute otitis media.

Table 13. Association between genotypes of mannose-binding lectin and toll-like receptors 2, 3, 4, 7, and 8 and respiratory infections and rhinovirus infections. Modified from the Study III.

	Days with symptoms of respiratory infection[a]			Rhinovirus infections[a]			Recurrent rhinovirus infections[b]		
Genotypes	Mean/child/year (95% CI)	Adjusted RR (95% CI)	*P*	Mean/child/year (95% CI)	Adjusted RR (95% CI)	*P*	No. (%)	Adjusted OR (95% CI)	*P*
MBL									
A/A (wt)	48.6 (44.1-53.7)	1		2.0 (1.8-2.2)	1		65/233 (28)	1	
A/O or O/O	59.4 (51.6-68.4)	**1.24 (1.05-1.47)**	**.01**	2.2 (1.9-2.5)	1.11 (.95-1.30)	.19	43/116 (37)	1.53 (.95-2.47)	.08
TLR2 Arg753Gln									
G/G (wt)	51.6 (47.5-56.1)	1		2.0 (1.9-2.2)	1		101/332 (30)	1	
G/A	61.2 (43.5-86.2)	1.17 (.82-1.66)	.38	2.1 (1.5-3.0)	1.03 (.74-1.44)	.85	7/17 (41)	1.55 (.57-4.21)	.39
TLR3 Leu412Phe									
C/C (wt)	55.2 (48.2-63.2)	1		2.2 (1.9-2.5)	1		42/120 (35)	1	
C/T or T/T	49.5 (43.5-56.3)	0.90 (.75-1.08)	.25	2.0 (1.8-2.3)	0.96 (.81-1.14)	.63	41/141 (29)	0.77 (.46-1.31)	.33
TLR4 Asp299Gly									
A/A (wt)	51.0 (46.6-55.8)	1		2.0 (1.8-2.2)	1		88/286 (31)	1	
A/G or G/G	57.6 (47.4-69.9)	1.15 (.93-1.41)	.20	2.2 (1.9-2.6)	1.13 (.94-1.36)	.20	20/63 (32)	1.06 (.59-1.91)	.85
TLR7 Gln11Leu									
A/A or A (wt)	55.6 (49.3-62.6)	1		2.2 (2.0-2.5)	1		59/159 (37)	1	
A/T, T/T, or T	48.8 (42.1-56.4)	0.89 (.74-1.07)	.22	1.9 (1.6-2.1)	0.84 (.70-1.01)	.06	25/106 (24)	**0.53 (.30-.92)**	**.02**
TLR8 Leu651Leu									
G/G or G (wt)	53.2 (46.0-61.4)	1		1.8 (1.6-2.1)	1		28/114 (25)	1	
G/C, C/C, or C	51.1 (45.4-57.6)	0.99 (.82-1.20)	.94	2.2 (2.0-2.5)	**1.21 (1.01-1.45)**	**.04**	57/153 (37)	**1.93 (1.10-3.36)**	**.02**

CI, confidence interval; MBL, mannose-binding lectin; OR, odds ratio; RR, rate ratio; TLR, toll-like receptor; wt, wild-type.
Statistically significant associations are shown in bold.
[a] Rates of days with symptoms of respiratory infection and rhinovirus infections were compared by using negative binomial regression analysis adjusted for sex and siblings.
[b] Risk of recurrent rhinovirus infections ($n \geq 5$ during the follow-up) was compared by using binomial logistic regression analysis adjusted for sex and siblings.

Table 14. Association between genotypes of mannose-binding lectin and toll-like receptors 2, 3, 4, 7, and 8 and acute otitis media. Modified from the Study III.

	Acute otitis media			Recurrent acute otitis media			Rhinovirus-associated acute otitis media		
Genotypes	No. (%)	Adjusted OR (95% CI)	*P*	No. (%)	Adjusted OR (95% CI)	*P*	No. (%)	Adjusted OR (95% CI)	*P*
MBL									
A/A (wt)	143/248 (58)	1		64/248 (26)	1		63/233 (27)	1	
A/O or O/O	75/121 (62)	1.21 (.77-1.89)	.41	39/121 (32)	1.40 (.86-2.26)	.17	45/116 (39)	**1.72 (1.07-2.76)**	**.03**
TLR2 Arg753Gln									
G/G (wt)	202/351 (58)	1		93/351 (26)	1		100/332 (30)	1	
G/A	16/18 (89)	**5.91 (1.33-26.18)**	**.02**	10/18 (56)	**3.37 (1.27-8.94)**	**.02**	8/17 (47)	2.02 (.75-5.41)	.16
TLR3 Leu412Phe									
C/C (wt)	74/130 (57)	1		37/130 (28)	1		39/120 (33)	1	
C/T or T/T	88/145 (61)	1.18 (.73-1.92)	.50	45/145 (31)	1.14 (.67-1.92)	.63	38/141 (27)	0.78 (.46-1.34)	.37
TLR4 Asp299Gly									
A/A (wt)	181/306 (59)	1		87/306 (28)	1		88/286 (31)	1	
A/G or G/G	37/63 (59)	0.99 (.57-1.72)	.97	16/63 (25)	0.87 (.47-1.64)	.67	20/63 (32)	1.06 (.59-1.90)	.86
TLR7 Gln11Leu									
A/A or A (wt)	109/169 (64)	1		55/169 (33)	1		60/169 (38)	1	
A/T, T/T, or T	60/111 (54)	0.66 (.40-1.08)	.10	30/111 (27)	0.80 (.47-1.36)	.41	23/106 (22)	**0.45 (.26-.80)**	**.006**
TLR8 Leu651Leu									
G/G or G (wt)	67/117 (57)	1		33/117 (28)	1		33/114 (29)	1	
G/C, C/C, or C	102/165 (62)	1.25 (.76-2.06)	.39	51/165 (31)	1.21 (.70-2.07)	.49	48/153 (31)	1.11 (.64-1.92)	.71

CI, confidence interval; MBL, mannose-binding lectin; OR, odds ratio; TLR, toll-like receptor; wt, wild-type.

Risk of acute otitis media and recurrent acute otitis media ($n \geq 3$ during the follow-up) was calculated by using binomial logistic regression analysis adjusted for sex and siblings. Statistically significant associations are shown in bold.

5.5 Blood MxA response to respiratory virus infections (IV)

Blood MxA protein, CRP, and WBC count were measured from 120 children visiting the study clinic during an acute respiratory infection (n = 115) or during an acute febrile infection diagnosed as a suspected virus infection (n = 5). Children with bacterial infections (n = 3, two with culture-positive pyelonephritis and one with orbital cellulitis) were excluded from the study. Blood MxA protein was also measured from 110 children at scheduled visits at the age of 2 (n = 67) and 13 months (n = 43). Of the children at scheduled visits, 33 (30%) had respiratory symptoms and 77 were asymptomatic when specifically asked by the study personnel. The children with respiratory symptoms at the scheduled visit were included in the group of symptomatic infections resulting in a total of 153 samples taken during symptoms of respiratory or virus infection. Blood MxA levels were also analyzed in 44 asymptomatic adults (mean age 32.9 ± 5.6 (SD) years; 59% females).

Characteristics of the study children and detected viruses and laboratory values during respiratory infections and at an asymptomatic state are presented in Table 15. The mean duration of symptoms was 6.0 ± 6.2 (SD) days. Of the symptomatic children, 148 (97%) had respiratory symptoms and 107 (70%) had fever. Of the 120 children visiting a physician during an illness episode, 26 (22%) were diagnosed with acute otitis media, 17 (14%) with a wheezing illness, and 4 (3%) with laryngitis. The mean WBC counts and CRP levels were low.

Table 15. Characteristics and detected viruses in children with respiratory symptoms and at asymptomatic state. Modified from the Study IV.

	Symptomatic children (n = 153)	**Asymptomatic children** (n = 77)
Age	0.99 ± 0.46	0.55 ± 0.44
Female	61 (40%)	35 (46%)
Laboratory values		
Blood MxA protein (µg/l)	670 (290-1330)	110 (60-185)
White blood cell count (E9/l)	9.37 ± 4.07	ND
C-reactive protein (mg/l)	13.6 ± 19.8	ND
Detected viruses		
Any	124 (81%)	12 (16%)
Rhinovirus	46 (30%)	4 (5%)
RSV	16 (10%)	2 (3%)
Coronavirus OC43/HKU1 or 229E/NL63	13 (8%)	4 (5%)
Parainfluenza virus type 1, 2, or 3	9 (6%)	2 (3%)
Human metapneumovirus	6 (4%)	0 (0%)
Influenza virus A or B	5 (3%)	0 (0%)
Human bocavirus	5 (3%)	0 (0%)
Adenovirus	1 (1%)	0 (0%)
Enterovirus	0 (0%)	0 (0%)
Co-infection	23 (15%)	0 (0%)

Values are n (%), mean ± SD, and for blood MxA: median (interquartile range). MxA, myxovirus resistance protein A; RSV, respiratory syncytial virus.

The MxA levels of asymptomatic children negative for respiratory viruses were significantly higher than in asymptomatic adults (110 [60–185] µg/l vs. 10 [10–30] µg/l; $P < .001$; Figure 11). In asymptomatic children, the basal level of blood MxA protein was similar at the ages of 2 and 13 months (120 [60–215] µg/l vs. 90 [60–165] µg/l; $P = 0.55$).

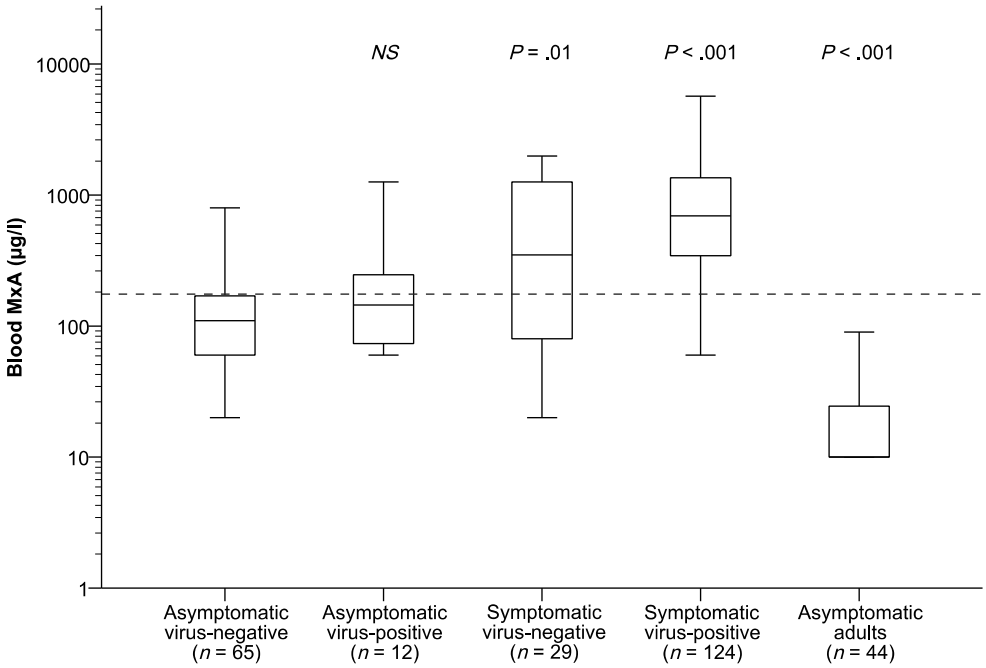

Figure 11. Blood MxA levels in children according to the presence of symptoms and detected respiratory viruses, and in asymptomatic adults. Values above different groups indicate statistical significance of the difference as compared to asymptomatic virus-negative children. The cut-off level of 175 µg/l from the ROC analysis is shown with a dotted reference line. Modified from the Study IV.

Blood MxA protein levels were elevated in symptomatic children when compared to asymptomatic children ($P < .001$, Table 15). The children with a symptomatic, confirmed respiratory virus infection had higher blood MxA protein levels than asymptomatic virus-negative children (695 [345-1370] µg/l vs. 110 [55-170] µg/l; $P < .001$; Figure 11). Symptomatic rhinovirus infections were associated with elevated MxA levels (490 [238-1128] µg/l) when compared to asymptomatic virus-negative children ($P < .001$), as were symptomatic RSV, parainfluenza virus, influenza virus, coronavirus, and human metapneumovirus infections (Figure 12). Only human bocavirus detections were not associated with increased MxA levels ($P = 1.00$). Febrile infections were associated with higher MxA levels than symptomatic respiratory infections without fever (930 [415–1440] µg/l vs. 240 [95–725] µg/l; $P < 0.001$). Within asymptomatic children, no significant difference was observed in MxA

responses between virus-positive children (145 [70–270] µg/l) and virus-negative children (P = .34). Children with confirmed bacterial infections who were excluded from the analyses (n = 3) had low MxA levels (50-90 µg/l).

Similar MxA responses were detected in children with coinfections and in those positive for only one virus (590 [320–1200] µg/l vs. 760 [345–1405] µg/l; P = .48). No significant correlation was found between rhinovirus or RSV copy numbers and the blood MxA levels (rhinovirus log of copies vs. MxA, Spearman's rho = -0.259, P = .064 and RSV log of copies vs. MxA, Spearman's rho = -0.112, P = 0.56).

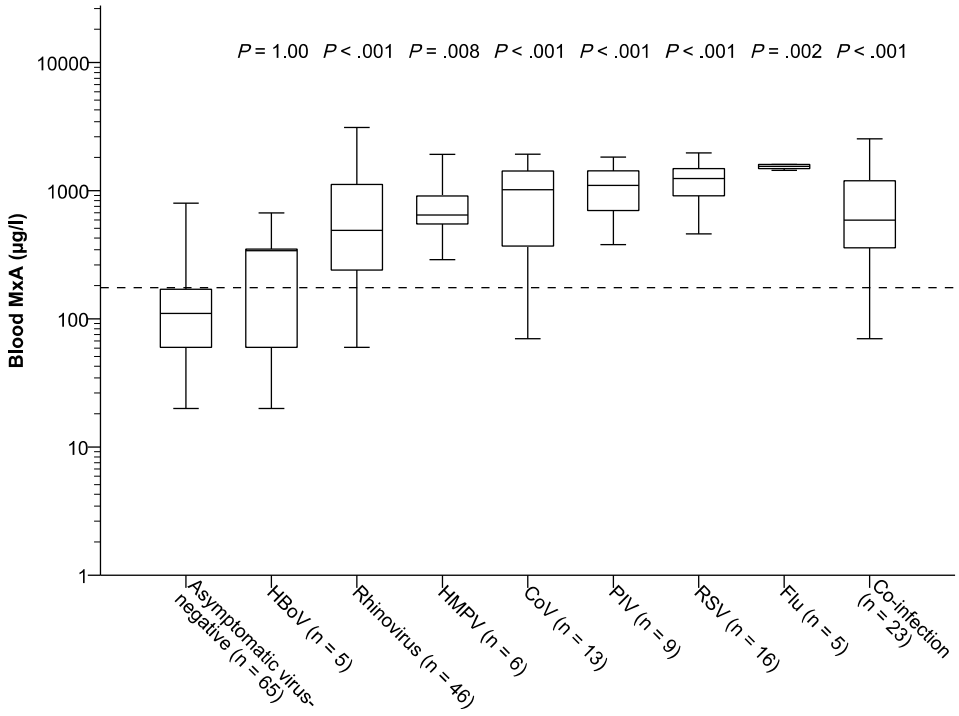

Figure 12. Blood MxA levels in symptomatic children according to detected viruses. A single case positive for adenovirus is not shown. Values above different groups indicate statistical significance of the difference as compared to asymptomatic virus-negative children. The cut-off level of 175 µg/l is shown with a dotted reference line. HBoV, human bocavirus; HMPV, human metapneumovirus; CoV, coronavirus; PIV, parainfluenza virus 1-3; RSV, respiratory syncytial virus; Flu, influenza virus A or B. Modified from the Study IV.

ROC analysis was used to estimate the ability of MxA to differentiate children with a confirmed, symptomatic respiratory virus infection from asymptomatic virus-negative children (Figure 13). With a cut-off level of 175 µg/l the greatest sum of sensitivity (92%) and specificity (77%) for a symptomatic respiratory virus infection was obtained. A weak negative correlation was observed between the MxA levels and duration of the symptoms preceding the sampling, but assay sensitivity was not affected (Figure 13).

Individual MxA responses were studied in 11 children with samples available both at healthy state and during an acute infection and showed a significant elevation of MxA level in response to virus infections (Figure 4 in the Study IV).

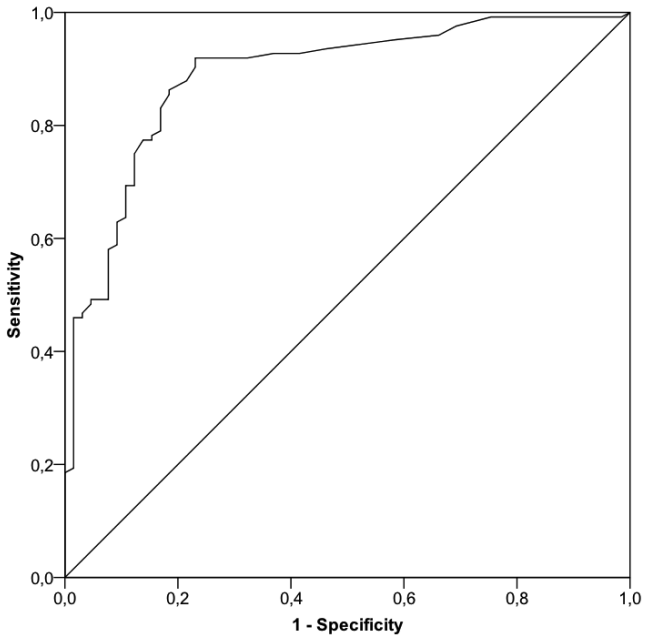

Figure 13. The ROC curve for predicting symptomatic virus-positive respiratory infection by elevated blood MxA protein level. The area under the curve (AUC) was 0.889, and the greatest sum of sensitivity (92%) and specificity (77%) was obtained with a cut-off level of 175 µg/l.

Blood MxA levels after live attenuated virus vaccines

The data on vaccinations were received from 207 cases (90%). Nineteen asymptomatic virus-negative children were vaccinated with a live virus vaccine (18 with rotavirus vaccine and one both with varicella zoster virus and measles, mumps, and rubella vaccines), eight received inactivated vaccines, and 32 were not vaccinated during the month preceding MxA sampling. MxA levels were elevated in asymptomatic virus-negative children who had received a live virus vaccine as compared to asymptomatic virus-negative children without preceding vaccinations (240 [120–540] µg/l vs. 85 [50–130] µg/l; $P = .001$), but not in children who had received inactivated vaccines (75 [50–135] µg/l; $P = .85$). The levels were significantly higher in children with an acute respiratory infection who had not received vaccinations (740 [350–1425] µg/l) than in asymptomatic virus-negative children who had received a live virus vaccine ($P < .001$; Figure 14).

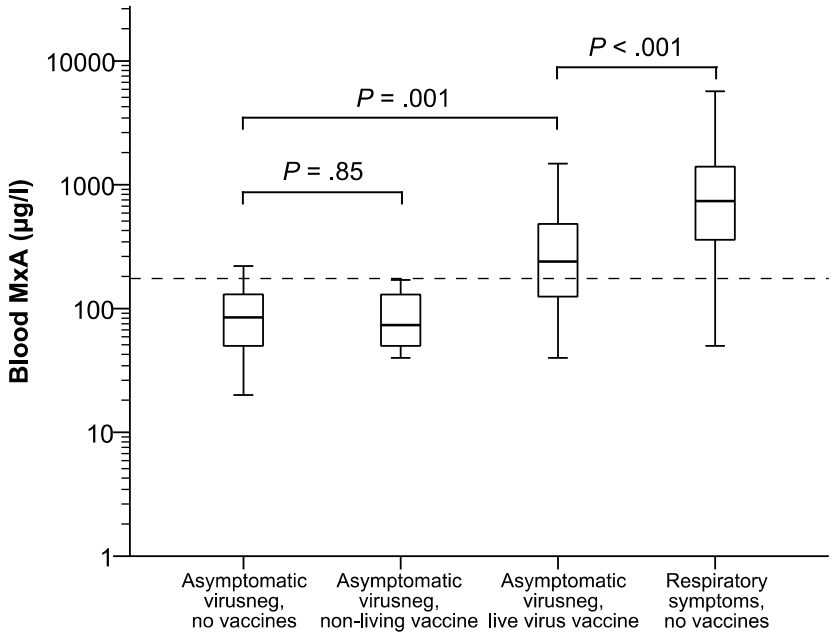

Figure 14. Blood MxA protein levels in asymptomatic virus-negative and symptomatic children with regard to received live-virus or other vaccinations during the 30 days before blood sampling. Of the children receiving live-virus vaccines, 18 received rotavirus vaccine and one both varicella zoster virus and measles, mumps, and rubella vaccines. The cut-off level of 175 µg/l is shown with a dotted reference line.

6 DISCUSSION

6.1 The disease burden

In this prospective cohort study, we showed that rhinovirus infections impose a major burden of respiratory infections and antibiotic use on young children. Rhinoviruses were detected in more than one-half of all acute respiratory infections resulting in an estimated annual rate of 3.5 infections per child. As a result of the high frequency of rhinovirus infections, rhinoviruses accounted for half of acute otitis media episodes and for almost half of antibiotic treatments and physician visits associating with acute respiratory infections. The disease burden was particularly seen in the number of outpatient visits for acute respiratory infections and in the use of antibiotics, with an annual rate of 1.5 outpatient visits and 0.6 antibiotic treatments per child associating with rhinovirus infections. Hospitalizations were infrequent in this outpatient setting, but still, the impact of rhinovirus infections was remarkable. Even mild home-treated infections that do not require a physician visit may be burdensome for the families, as we report a high annual number of days with respiratory symptoms, frequent use of over-the-counter medications, and more than one-half of all parental workdays lost because of child's acute respiratory infection to associate with rhinovirus infections.

Our results show, similar to earlier studies, that acute otitis media complicates rhinovirus infections more infrequently than respiratory infections caused by RSV or other viruses (Vesa *et al.* 2001, Chonmaitree *et al.* 2008), but the high frequency of rhinovirus infections makes it the most important virus associating with acute otitis media. This finding is supported by the results from another Finnish study where rhinoviruses were found in nasopharyngeal aspirates in 41% of the 458 acute otitis media episodes being the most frequent virus associating with acute otitis media (Blomqvist *et al.* 2002). In our out-patient setting that included an intensive follow-up on mild, home-treated, acute respiratory infections, the overall acute otitis media complication rate of rhinovirus infections was low at 13 percent. However, acute otitis media was detected in 35% of children who were examined by a physician during a rhinovirus-positive acute respiratory infection, which is consistent with previous data comprising only children examined by a physician (Chonmaitree *et al.* 2008). The role of respiratory viruses in the pathogenesis of acute otitis media is currently considered important (Nokso-Koivisto *et al.* 2015), and picornaviruses are the most frequent viruses found in middle ear effusion (Ruohola *et al.* 2006). Based on our results and the current data, rhinoviruses should be considered as important otopathogens and focus should be laid on developing prevention and treatment modalities for rhinovirus infections in order to efficiently prevent acute otitis media. Prevention of influenza infection has been shown to decrease the incidence of acute otitis media as a complication of influenza (Heikkinen *et al.* 1991, Whitley *et al.* 2001). Our results

suggest that a large proportion of acute otitis media and associated antibiotic use could be avoided by preventing rhinovirus infections.

We report that 41% of acute wheezing illnesses were associated with rhinovirus, which is in line with a previous birth cohort study detecting rhinovirus in 45% of wheezing illnesses (Kusel *et al.* 2006). Detection rates of rhinoviruses have varied from 3-34% in hospitalized bronchiolitis patients (Turunen *et al.* 2014), and rhinoviruses have been shown to dominate the etiology of wheezing illnesses after 9 to 12 months of age and that of recurrent wheeze (Rakes *et al.* 1999, Jartti *et al.* 2009).

Hospitalizations were infrequent in this setting, but almost 40% of hospitalizations for an acute respiratory infection were associated with rhinovirus. In previous studies, rhinoviruses have been shown to be an important cause of respiratory infections leading to hospitalization and have been found in up to 30% of children hospitalized for an acute respiratory infection (Cheuk *et al.* 2007, Miller *et al.* 2007, Peltola *et al.* 2009). We estimated the rate of rhinovirus-associated hospitalizations as 2.3 per 100 children per year.

Many studies have documented the dominating role of rhinoviruses in the etiology of respiratory tract infections. Monto and colleagues observed that even though the percentage of rhinovirus infections resulting in a physician consultation is lower than that of influenza or RSV, the total number of consultations was highest due to rhinovirus infections (Monto and Sullivan 1993). However, the burden of respiratory illnesses and associated outcomes caused by rhinoviruses has not been described in young children. Previous epidemiological studies assessing rhinovirus infections have been either small, conducted in selected populations, done before the era of PCR diagnostics, have not covered non-complicated upper respiratory tract infections that may contribute substantially to the disease burden because of high frequency, or respiratory samples have been collected only from a minority of the infections (Fox *et al.* 1975, Monto *et al.* 1987, Blomqvist *et al.* 2002, Kusel *et al.* 2006, Regamey *et al.* 2008, van der Zalm *et al.* 2009a, Fairchok *et al.* 2010, Budge *et al.* 2014, Anders *et al.* 2015). Intensive follow-up with home sampling and the use of sensitive PCR methods allow us to show that most rhinovirus infections are mild, but they are highly frequent and cause a substantial burden of disease in young children and their families. As rhinovirus infections are associated with half of health care visits and absenteeism of the parent from work due to a child's acute respiratory infection, they also cause a considerable burden on the healthcare system and the community.

By using home sampling in addition to sample collection at study clinic visits, we could assess also the burden of those respiratory infections that did not necessitate a physician visit. We were able to study the etiology of 53% of all acute respiratory infections from birth to two years of age in the children in the intensive follow-up. Home nasal sampling probably increased the rate of collected samples during mild,

home-treated infections as 70% of the samples taken during infections were collected by the parents. Importantly, home sampling might be even more efficient to detect viruses in nasal secretions than sampling at the study clinic, as it could be performed during the first days of infection when the virus copy numbers are highest (Douglas *et al.* 1966). Home sampling could also be performed during weekends or holidays when our study clinic was closed. Previously, we validated the home nasal sampling and sample delivery by standard mail for rhinovirus detection by PCR and showed that specimens are stable for at least four days at room temperature and survive mailing without loss of rhinovirus detectability (Waris *et al.* 2013). The diagnostic sensitivity was equal in flocked nasal swabs as compared to nasopharyngeal aspirate with no significant quantitative difference (Waris *et al.* 2013). Sampling of the anterior nose is easy and may be more readily accepted by the parents and the children than collection of nasopharyngeal aspirates, thus enabling parental sampling at home as well as repeated sampling of the children. Also in other studies, home nasal sampling for respiratory viruses has been found feasible (Lambert *et al.* 2007, Peltola *et al.* 2008). No adverse effects due to the nasal sampling other than highly infrequent minor bleeding of the nose were observed in this study.

Based on the frequency of complications and other outcomes in rhinovirus positive versus rhinovirus negative infections, we estimated the rhinovirus-associated morbidity. This estimation is subject to an assumption that rhinoviruses would occur with a similar frequency and severity in infections that were not analyzed for viruses as in those that were analyzed. This assumption is supported by the fact that physician visits were only moderately more frequent in virologically studied infections, which is caused by the study design of collecting samples during study clinic visits. Furthermore, the rates of acute otitis media or pneumonia did not differ between infections with or without nasal samples. Antibiotic prescriptions were even more frequent in infections without a virologic analysis.

6.2 Recurrent respiratory tract infections

We documented that recurrent respiratory tract infections cause a significant burden of illness and associated morbidity in young children. Rhinoviruses are the most common cause of recurrent respiratory infections. Children with recurrent respiratory infections are at an increased risk for asthma at two years of age.

We identified children with recurrent respiratory tract infections by using the 90^{th} percentile of days with respiratory symptoms (98 days with respiratory symptoms per year) as the limit. Clustering of different diagnoses of both upper and lower respiratory tract infections in these children was evident. The most common diagnoses were an upper respiratory tract infection and acute otitis media, but also bronchiolitis as well as recurrent wheezing were more common in these children and many had pneumonia.

Children with recurrent respiratory infections visited more frequently a physician office and emergency room, and they often used antibiotics. Many of these children were hospitalized for respiratory infections and a substantial proportion underwent surgical operations such as tympanostomy tube placements or adenoidectomy. These children carry a substantial part of the overall respiratory disease burden in young children, and the high morbidity in these children reflected in many ways also in the families of these children, which was documented by a higher number of missed workdays by the parents of these children.

The rate of respiratory infections in the children with recurrent infections was higher than that of the other children from the very first months of life and stayed constantly higher during the first two years of life. Almost eighty percent had their first infection already before three months of age and almost all of them had had at least one acute respiratory infection before age of six months. They also had their first acute otitis media early, one-third already before three months of age. These children at high risk for respiratory infections should be recognized early to focus possible interventions to them early enough.

Asthma was diagnosed in twelve percent of the children with recurrent respiratory infections by the age of two years as compared to four percent in other children. The children with recurrent respiratory infections were at increased risk for asthma independently from atopy of the child and maternal asthma. In this and other studies, rhinoviruses were the most frequent cause of recurrent respiratory infections. Rhinovirus-associated wheezing illnesses have been found to strongly associate with later asthma (Jartti *et al.* 2010, Rowe and Gill 2015). Atopy is an independent risk factor for asthma and may also contribute synergistically to the asthma risk with virus infections (Rowe and Gill 2015, Bochkov and Gern 2016). It has been proposed that early detection of rhinovirus as a cause of wheezing illness could be a marker for the proneness to develop asthma later in childhood (Jartti and Gern 2011). The susceptibility of these children to recurrent respiratory infections and asthma could reflect partly the common mechanisms, such as a defective interferon response as has previously been shown both in children with recurrent infections (Pitkaranta *et al.* 1999) and in asthma (Wark *et al.* 2005, Contoli *et al.* 2006, Message *et al.* 2008, Bartlett *et al.* 2012, Durrani *et al.* 2012). More detailed analyses of viral etiology and clinical manifestations of respiratory infections contributing to the asthma risk are needed but were beyond the scope of this study.

Setting the limit to the 90th percentile of days with symptoms of respiratory infection is a subjective choice. The substantial morbidity that we documented in the children identified by using this limit supports its use. Previous studies have defined recurrent respiratory infections based on a certain number of infection episodes or number of specific diagnoses (Alho *et al.* 1990, Nokso-Koivisto *et al.* 2002, Emonts *et al.* 2007). The principal argument against using the number of episodes to identify the children

with recurrent respiratory infections was that episodes of respiratory infections may overlap with each other, especially when documented in detail in daily diaries and particularly in children with recurrent infections. The duration of respiratory symptoms in rhinovirus infections, the most frequent cause of recurrent respiratory infections, is one to two weeks, but symptoms may last longer (Peltola *et al.* 2013). As many rhinovirus types have been shown to circulate simultaneously in the community and little cross-protection is provided between the serotypes, frequent and overlapping rhinovirus infections occur (Jartti *et al.* 2008b, van der Zalm *et al.* 2011, Principi *et al.* 2015).

The use of certain number of days with respiratory symptoms in recognizing children with recurrent respiratory infections may however be difficult in clinical practice but also separating different episodes may be troublesome. The parents may neither remember exact days nor the exact number of episodes of respiratory infections the child has had especially in families with several children. However, we suggest that defining recurrent respiratory infections by approximately three months or 100 days of symptoms per year is useful in recognizing children with recurrent infections. In this study, we showed the clustering of different diagnoses in a small population of children, which may reflect possible underlying risk factors or immunological defects in these children. Defining recurrent infections based on annual days with symptoms could be useful especially in a research setting to assess immunological aberrancies in these children.

The presence of older siblings increased the risk for respiratory infections threefold. Nasopharyngeal colonization at two months of age with *S. pneumoniae* was more common in the children with recurrent respiratory infections, but the association did not remain significant after adjustment, probably due to older siblings who may transmit *S. pneumoniae* early to the infant (Principi *et al.* 1999). This finding could nevertheless reflect the role that *S. pneumoniae* plays in the increased rate of acute otitis media and other infections in young children with close child contacts. Previously, early nasopharyngeal bacterial colonization has been linked with acute otitis media, recurrent wheezing, and asthma (Faden *et al.* 1997, Bisgaard *et al.* 2007). Although not associated with the risk of recurrent infections in this study, breastfeeding has been found to protect against respiratory infections (Alho *et al.* 1990, Chantry *et al.* 2006, Duijts *et al.* 2010, Chonmaitree *et al.* 2016), with the highest impact occurring in developing countries. In our child-cohort, the mean duration of breastfeeding was long, over seven months. In this study, children attending daycare at the age of 13 months were not overrepresented in the group with recurrent respiratory infections. This cross-sectional analysis may not show accurately the effect of daycare on the risk of infections, because the children started the daycare at a highly variable age, and our definition of recurrent respiratory infections was based on the number of respiratory illness during the whole follow-up period. Although it may be transient, the

effect of daycare has been shown in previous studies (Ståhlberg 1980, Wald *et al.* 1988, Alho *et al.* 1990, Louhiala *et al.* 1995, Ball *et al.* 2002, Côté *et al.* 2010). However, these children seemed to have recurrent respiratory illness days from the very first months of their life, which would suggest other factors than daycare laying behind this condition.

Parental smoking seemed to decrease the risk for recurrent respiratory infections, but based on previous data, we suspected a reporting bias and hypothesized that the parents who did not smoke reported respiratory symptoms in the children more precisely. Previously, smoking has been found to be a significant risk factor for respiratory infections (Haberg *et al.* 2007). Nevertheless, another recent study did not confirm the association of parental smoking to the risk of otitis media or respiratory infections (Chonmaitree *et al.* 2016). It could be hypothesized that the increasing knowledge about the harmful effects of smoking as well as tightening legislation of consuming and marketing cigarette products may have changed the smoking habits so that the children would not be exposed to smoke indoors as much as before even though the parent would smoke.

An unexpected yet interesting finding was that none of the children with recurrent respiratory infections was positive for rhinovirus or any other virus at an asymptomatic state at 2 or 13 months of age. In the comparison group, 8 to 9 percent of asymptomatic children were rhinovirus positive at these ages, and also other studies have reported high detection rates of rhinovirus in asymptomatic children (Jartti *et al.* 2008a). It could be hypothesized that the absence of asymptomatic rhinovirus infections at a young age in children with recurrent infections would be due to aberrant innate immune responses. At 24 months of age when acquired immune responses are more developed, asymptomatic rhinovirus detections were even more frequent in children with recurrent infections than in the other children.

6.3 Asymptomatic rhinovirus infections

Rhinoviruses are commonly detected in asymptomatic children with the use of sensitive PCR methods, which has raised justified concern about the clinical relevance of positive rhinovirus detection. Rhinoviruses have been detected in 11-47% of the children (Kusel *et al.* 2006, Jartti *et al.* 2008a). In a recent study, rhinovirus infections were strikingly frequent in 88 young children who were followed and sampled weekly for four months from November to February. The rhinoviruses were typed, and the mean number of rhinovirus infections was 3.7 per child and 64% were asymptomatic (Principi *et al.* 2015). In our study, the prevalence of rhinoviruses, regardless of symptoms, was 14 to 24 percent increasing with age. However, rhinoviruses were detected in only nine percent of the asymptomatic children. Our results are in line with a previous study where asymptomatic rhinovirus infections were detected in 11

percent of the subjects who were asymptomatic around the sample collection (Kusel *et al.* 2006). Furthermore, in our study, asymptomatic rhinovirus detections were frequently associated with respiratory symptoms before or after sampling or respiratory symptoms in the family. Our results suggest that asymptomatic rhinovirus infections are more infrequent than previously thought, and rhinovirus detections in asymptomatic children may reflect a previous or an emerging infection. Mild rhinovirus infections are frequent in young children. Current data suggest that frequent rhinovirus detections result from highly frequent rhinovirus infections with different rhinovirus types (Jartti *et al.* 2008b, Principi *et al.* 2015). The mean duration of rhinovirus shedding in immunocompetent individuals is 11 days and longer shedding has been found only in immunocompromised patients (Peltola *et al.* 2013). Heinonen and colleagues showed that asymptomatic rhinovirus infections are not associated with significant systemic transcriptional immune responses, while symptomatic rhinovirus infections induce a robust transcriptional signature (Heinonen *et al.* 2015) providing evidence that symptomatic rhinovirus infections cause immunological responses and should be considered as true infections. In line with these observations, we documented that asymptomatic virus detections, most of which were rhinoviruses, were not associated with increased blood MxA levels. This is important with regard for the potential use of MxA in discriminating viral from bacterial infections as asymptomatic rhinovirus detections occur.

6.4 Genetic susceptibility to respiratory infections

In this prospective cohort study, certain polymorphisms in genes involved in the innate immunity promoted susceptibility to or protection against respiratory infections, rhinovirus infections, and acute otitis media in young children.

The MBL variant type was associated with an increased risk for respiratory infections defined as days with respiratory symptoms per year. MBL deficiency has been found to increase the risk for respiratory infections, but the results have not been conclusive. In a well-designed prospective study by Koch and colleagues, the number of acute respiratory infections was recorded by weekly visits to the families from six weeks to two years of age. An increased risk of respiratory infections was observed in MBL-insufficient children, but the risk was limited to children aged 6 to 17 months (Koch *et al.* 2001). Wiertsema et al found that MBL variant type was associated with an increased number of acute otitis media in children 12 to 24 months of age (Wiertsema *et al.* 2006). These results suggest that MBL could be especially important during the "vulnerable window" from approximately 6 to 24 months of age, as maternal antibodies wane during the first year of life and acquired immune responses are still immature. Other studies have also found an association between MBL polymorphisms and respiratory infections, but some studies have not been able to confirm these results (Table 4). Discrepant results may result from the small study size, cross-sectional

setting, or method of reporting respiratory infections in the studies with negative results (Ruskamp *et al.* 2008, Tao *et al.* 2012, Atan *et al.* 2016). Studies originally designed to detect asthma and allergy, recording symptoms on an annual basis, may not detect differences confidently (Muller *et al.* 2007, Ruskamp *et al.* 2008). Annual questionnaires on respiratory infections are retrospective in nature, based on the memory of the parents, and detailed data on infections may not become correctly documented. Our results from this prospective, unselected cohort study with intensive follow-up with daily diaries confirm the results of the study of Koch and colleagues. As MBL variant genotype was associated with significantly increased number of days with symptoms of respiratory infection, but the association was not significant for acute respiratory infections defined as episodes, our results suggest that these children either had prolonged or overlapping infections. It is possible that low MBL levels and a partially defective lectin pathway present as an increased number of respiratory infections only during early childhood, and this defect is later compensated by acquired immune responses. Even though MBL deficiency would increase the susceptibility only during the first years of life, it may be clinically important. During these years the children are at a high risk for infections and even a mild additive effect results in a substantial overall burden of infections, especially in children with several risk factors such as other immunological defects, presence of siblings, or other environmental risk factors.

In this study, MBL polymorphisms were not associated with rhinovirus infections. Either a larger study would be needed to show associations between the MBL variant type and rhinovirus infections, or these results suggest that MBL may be more important in host response to other virus infections than rhinovirus or to viral-bacterial infections. None of the studied TLR polymorphisms had an effect on the overall rate of respiratory infections. Previous studies have linked polymorphisms of TLR4 with RSV infection, otitis media, and wheezing illnesses, but the results have not been conclusive (Table 4). In a meta-analysis an association between TLR4 polymorphisms and severe RSV disease could not be confirmed (Zhou *et al.* 2016). In one study, TLR4 genotype was not found to associate with the risk of respiratory infections in children with history of bronchiolitis (Nuolivirta *et al.* 2009), which is in line with our results.

Earlier data on genetic susceptibility for rhinovirus infections are scarce. We found that the TLR7 variant type was protective against recurrent rhinovirus infections, and the TLR8 variant type was associated with a twofold increased risk for recurrent rhinovirus infections. As *TLR7* and *TLR8* genes are X chromosomal, we performed separate analyses in males and females. SNPs in these genes had similar effects in boys and girls, which suggests that effects on the outcomes that we studied are mostly dominant. Endosomal TLR7 and TLR8 have been shown to recognize rhinoviral ssRNA, which leads to the production of pro-inflammatory and antiviral cytokines (Triantafilou *et al.* 2011). It has been suggested that the TLR7 Gln11Leu polymorphism alters the

processing of TLR7 at the endoplasmic reticulum, and that TLR8 Leu651Leu would be associated with alternative splicing of TLR8, despite being a synonymous SNP (Moller-Larsen *et al.* 2008). TLR7 Gln11Leu and TLR8 Leu651Leu polymorphisms have been found to associate with an increased risk of asthma (Moller-Larsen *et al.* 2008), but there are no clinical data on their association with respiratory infections. TLR7 is an intriguing target for studying the immunological mechanisms of asthma and may play an important role in the pathogenesis of asthma. Data from mouse models show that allergic TLR7-deficient mice display impaired IFN response to a rhinovirus infection as well as increased virus replication, inflammation, and hyper-reactivity of the airways (Hatchwell *et al.* 2015). A synthetic TLR7/TLR8 ligand, resiquimod, has been found to be effective in preventing the development of asthma and inhibiting an inflammatory reaction to allergens in a mouse model (Moisan *et al.* 2006, Camateros *et al.* 2007, Sel *et al.* 2007, Nadeem *et al.* 2016). We did not study the association with asthma, and the children in this cohort study were mainly healthy. Our results suggest that defective TLR7 signaling could be protective to the host, whereas previous data show that defective TLR7 signaling is detrimental in an atopic/asthmatic phenotype. As the local inflammatory reaction to rhinovirus infection is important in the development of clinical symptoms of infection, it could be hypothesized that in a non-atopic host, functional TLR7 signaling and interferon response would lead to clinical symptoms during a rhinovirus infection, but impaired TLR7 signaling would protect the host from clinical symptoms and the rhinovirus infection would remain asymptomatic. Furthermore, in an atopic host, there could possibly be other underlying mechanisms or deficient compensatory mechanisms, and an intact TLR7 signaling could be more critical in developing a proper antiviral immune response in these individuals.

As TLR signaling leads to the induction of interferons and proinflammatory cytokines, an overwhelming reaction may be detrimental to the host. Thus, the SNPs in TLR genes may be protective to the host by inhibiting an excessive inflammatory reaction to the pathogens. This protective role of SNPs in TLR genes has been hypothesized for RSV (Rämet *et al.* 2011) as well as for TLR3 and virus infections (Perales-Linares and Navas-Martin 2013). Furthermore, the high prevalence of TLR7 SNP could suggest an advantageous role. SNPs with deleterious effects deteriorating pathogen recognition and leading to life-threatening infections are under a strong purifying selection, and on the contrary, those frequent in the population have either only mild deteriorating effect or even at least partly advantageous role for the host (Netea *et al.* 2012). Several studies have suggested that certain polymorphisms in genes encoding TLRs have resulted from positive selection through protection from infection (Netea *et al.* 2012).

TLR3 recognizes rhinoviral dsRNA in the endosome (Slater *et al.* 2010) and it has an important role in antiviral immunity (Zhang *et al.* 2007, Perales-Linares and Navas-Martin 2013). TLR2 recognizes the rhinovirus capsid on the cell surface (Triantafilou

et al. 2011). Polymorphisms in TLR3 may associate with susceptibility to bronchiolitis and post-bronchiolitis wheezing (Nuolivirta *et al.* 2012b). However, we found no associations between TLR3 or TLR2 polymorphisms and rhinovirus infections.

In the 1990s, studies in twins suggested a heritability for otitis media (Casselbrant *et al.* 1999), and since, many candidate genes have been studied (Mittal *et al.* 2014). Polymorphisms in TLR4 and MBL have been found to associate with the risk of (recurrent) otitis media, but the data are partly conflicting (Table 4). We found that the TLR2 variant type was associated with an almost sixfold increase in the overall risk for developing acute otitis media during the follow-up and with a more than threefold increased risk of recurrent acute otitis media. Previous studies have concentrated on TLR4 polymorphisms and the risk for otitis media, but few studies assessing the effect of TLR2 polymorphisms found no association with acute otitis media (Emonts *et al.* 2007) or tympanostomy tube placement for chronic or recurrent otitis media (Sale *et al.* 2011, Carroll *et al.* 2012). TLR2 is important in the recognition of Gram-positive bacteria (Skevaki *et al.* 2015) and also mediates immune responses to non-typeable *H. influenzae* infection (Shuto *et al.* 2002). Furthermore, TLR2 seems to be critical for bacterial clearance and the resolution time of otitis media caused by non-typeable *H. influenzae* in a mouse model (Leichtle *et al.* 2009). Higher mRNA levels of TLR2 and TLR4 have been found in the middle ear effusion of children with culture-positive versus culture-negative acute otitis media, albeit the difference was not statistically significant for TLR2 (Kaur *et al.* 2015). Our findings and the previous data suggest that the TLR2 polymorphism may increase the susceptibility to otitis media by attenuating the innate responses to *S. pneumoniae* and *H. influenzae.* This is the first study to identify an association between TLR2 polymorphisms and acute otitis media in young children.

In addition to protection against recurrent rhinovirus infections, the TLR7 variant type was protective against acute otitis media associating with rhinovirus infection. The finding that TLR7, which affects innate responses to virus infections, is associated with the risk for both recurrent rhinovirus infections and rhinovirus-associated acute otitis media is interesting. It provides further evidence for the important role that viruses play in the development of acute otitis media. Polymorphisms of MBL were not associated with an overall risk for acute otitis media or recurrent acute otitis media, but were specifically associated with the increased risk of rhinovirus-associated acute otitis media. Some studies have found an association between MBL variant type and risk of otitis media, but the data are controversial (Table 4). We have earlier shown that the MBL variant type is associated with an increased risk of nasopharyngeal colonization with *S. pneumoniae* in young children, and that in children with MBL variant genotype, rhinovirus infection further increases the rate of pneumococcal colonization (Vuononvirta *et al.* 2011, Karppinen *et al.* 2013). It seems that children with MBL

variant type are susceptible to colonization with *S. pneumoniae* and to develop acute otitis media as a complication of rhinovirus infections.

We found no additive effects among SNPs in the studied genes. The number of children with two or more SNPs was low. Several SNPs acting synergistically could confer an even higher susceptibility to respiratory infections, but larger cohort studies would be needed to study these synergistic effects of SNPs.

The overall presence of the studied SNPs in this healthy, unselected Finnish child cohort was close to that of the European prevalence although minor differences were detected. SNPs in TLR4, TLR7, and TLR8 were more frequently detected in our cohort as compared to a European reference population. All studied SNPs in TLRs were more frequent in our cohort than in a global reference population, and frequencies of MBL genotypes differed from that of the global population. Due to historical and geographical reasons, the human genotype is quite homologous and partly unique in Finland and certain genetic variants occur more frequently in Finnish than in other populations.

6.5 Blood MxA protein as a marker for respiratory virus infections

We showed that blood MxA protein levels are increased in young children with symptomatic respiratory virus infections, including infections caused by rhinovirus. MxA response has previously been shown in RS-, influenza-, parainfluenza-, and adenovirus infections in children, but the capability of rhinovirus to induce MxA expression has been controversial (Forster *et al.* 1996, Chieux *et al.* 1999, Mäkelä *et al.* 1999, Nakabayashi *et al.* 2006). As rhinoviruses have been shown to induce type I interferon response *in vitro* and in animal models (Bartlett *et al.* 2012), induced interferon response and elevated MxA levels would be expected in natural rhinovirus infections. However, only a weak MxA response was shown in adult patients with rhinovirus infections (Mäkelä *et al.* 1999). The severity of the symptoms or presence of fever in these patients was not specified, and blood MxA levels were defined by flow cytometric analysis from isolated blood lymphocytes. In our study, the patients were young children and most of the children had febrile infections that were associated with higher MxA levels, probably due to a stronger systemic immune response. In addition, we measured MxA from the whole blood lysates by EIA, which is less time-consuming and has the potential for a rapid diagnostic use. Our data indicate that in children, rhinoviruses are capable of inducing an efficient IFN response that correlates with elevated expression of MxA protein in blood cells.

By using 175 µg/L as the cut-off level, an elevated MxA level had 92% sensitivity and 77% specificity for a symptomatic respiratory virus infection. Blood MxA has previously been shown to discriminate between viral and bacterial infection in febrile children, but the number of investigated respiratory viruses has been low (Halminen *et*

al. 1997, Nakabayashi *et al.* 2006). In this study, 14 respiratory viruses were analyzed by PCR including human metapneumovirus, human bocavirus, and coronaviruses, which have not been included in previous clinical studies on MxA. MxA levels varied in different virus infections being higher in respiratory infections caused by RSV, parainfluenza, influenza, and human metapneumovirus and lower, but still elevated in rhinovirus and coronavirus infections. Recently, a study on MxA levels in diagnosing viral infections in children was published with consistent results (Engelmann *et al.* 2015). In that study, the sensitivity and specificity for virus infections was even higher being at 96% and 85%, respectively. However, studied virus infections were mainly bronchiolitis and gastroenteritis. Rhinovirus was not analyzed, and the most frequently detected viruses were RSV and rotavirus, with a few adenoviruses, enteroviruses and parainfluenzavirus. Shown in our study and in previous studies, RS-, rota-, adeno- and parainfluenzaviruses generally associate with high MxA responses (Halminen *et al.* 1997) and thus may influence the high sensitivity and specificity values from the ROC analysis. However, in clinical practice, rhinoviruses are common, and thus our results may reflect the usefulness of MxA especially in an out-patient setting.

As asymptomatic virus infections are common in young children (Jartti *et al.* 2008a), an important and novel finding was that asymptomatic virus infections were not associated with elevated MxA levels as compared to asymptomatic virus-negative children. Recent vaccinations with a live attenuated virus vaccine, which in these young children was most frequently a rotavirus vaccine, was associated with elevated MxA levels. However, acute respiratory infections were associated with a significantly higher MxA response. Inactivated vaccines had no influence on blood MxA levels. These data indicate that recent administration of live virus vaccines should be taken into account when interpreting the MxA results.

Blood MxA levels stayed elevated several days which reflects that the biological half-time of MxA during a natural infection is longer than that detected *in vitro* and suggests that the detection of elevated MxA levels in virus infections is not compromised due to a delay of one or more days from the onset of symptoms to the MxA sampling.

6.6 Limitations

Some limitations to the generalizability of our findings may be caused by the characteristics of the study population. The children participating in the study had less frequently older siblings and were more frequently from families with higher sosioeconomical status than the children not attending the study (Lagstrom *et al.* 2013). However, as respiratory tract infections are more common in children with siblings (Badger *et al.* 1953, Fox *et al.* 1985, Peltola *et al.* 2008, Anders *et al.* 2015, Chonmaitree *et al.* 2016), this difference might have led to an under-estimation of the

number and impact of rhinovirus infections. A considerable number of the children did not continue the active follow-up until two years of age, but the dropout rate was low during the first year of life. We took this into account by calculating the rates of respiratory infections and associated outcomes from the duration of active follow-up of each child. Our study design with the low-threshold study clinic for the children in the intensive follow-up might have increased the number of physician visits for respiratory infections and thus might have affected the number of diagnoses. Indeed, the children in the intensive follow-up visited a physician and were diagnosed with a wheezing illness more often than the children in the regular follow-up. Nevertheless, the numbers of other diagnoses or treatments did not differ.

Our virus diagnostics was not broad, as we performed multiplex PCR and bocavirus PCR only in the Study IV. However, rhinovirus was analyzed by quantitative PCR, which is the most sensitive method for detecting rhinovirus. RSV, enterovirus, and influenzavirus A and B were also analyzed by PCR, whereas an antigen test with a lower sensitivity than that of PCR was used for five respiratory viruses. Other viruses than rhinovirus and co-infections were found quite infrequently, which might be explained by the methodology used. Low detection rates of other viruses may also be explained by the study setting, as we investigated mostly mild, home-treated infections. A substantial proportion of the children were immunized against seasonal influenza each year. The vaccination coverage against pandemic influenza was high, and the influenza A/H1N1 pandemic in 2009 was mild in this population. The four-year study period probably evened the effects of epidemic variation in circulation of the respiratory viruses.

The low number of subjects with less frequent SNPs reduced the power in detecting their effects. Due to a rather low number of subjects in the Study III, we were not able to assess additive effects among SNPs. As in all genetic association studies, the findings from one population may not be directly generalizable to other populations that may harbor a different distribution of SNPs. However, the prevalence of minor alleles in studied genes in this Finnish unselected population was close to that of a European reference population. As multiple comparisons were made, any *P* values under but close to .05 should be interpreted with caution.

In the study IV, a low detected number of some viruses may affect the generalizability of interpreting the results. In addition, even though 14 respiratory viruses were included in the analyses, there are still some viruses, such as parainfluenzavirus 4, which were not analyzed. However, the virus detection rate was 81% in symptomatic children, and the number of rhinovirus infections (n = 46) was sufficient to show a reliable MxA response in symptomatic rhinovirus infections. We had no representative group of bacterial infections due to the study design, but the three children with bacterial infections had low MxA levels (<100 µg/L). We took the blood samples for MxA determination by a finger prick during acute respiratory infections, which is

convenient to the child and can be taken simultaneously with samples for the CRP level and the WBC count measurement. The samples at scheduled visits were taken by a venipuncture. An excellent correlation but 24% lower MxA levels were previously detected in capillary blood as compared to venous blood (Chieux *et al.* 1999). In this study, blood MxA levels were significantly higher during respiratory infections in capillary blood than those found at a healthy state in venous blood indicating that the strong MxA response induced by virus infections exceeds the modest difference caused by the sampling type.

6.7 Future considerations

By preventing or treating rhinovirus infections, a substantial part of the associated morbidity such as acute otitis media, antibiotic treatments, and the use of health-care services could be avoided. Previous studies show that otitis media associated with influenza can be effectively prevented by influenza vaccinations or antiviral treatment (Heikkinen *et al.* 1991, Whitley *et al.* 2001). However, there are currently no vaccines or treatment available for rhinovirus infections.

The children at the highest risk for respiratory infections due to both genetic and environmental risk factors should be identified in order to develop and focus novel intervention modalities. Efficient identification of children at a high risk is essential especially if potential preventive or treatment methods were expensive. Based on our results, by focusing on the children at the highest risk, a considerable burden of respiratory disease and surgical procedures could possibly be prevented. As rhinovirus is a major cause of wheezing illnesses and asthma exacerbations and is associated with the development of asthma, an effective vaccine against rhinovirus could possibly prevent many exacerbations and have an influence on the development of asthma. If certain rhinovirus genotypes were more strongly associated with wheezing, asthma, or acute otitis media, vaccine design and development could be targeted against these genotypes. More detailed epidemiological data on circulating rhinovirus types are needed from different countries to identify the most important rhinovirus types to be covered by a vaccine. As rhinovirus A and C are most frequent and seem to cause more severe infections (Iwane *et al.* 2011, Lee *et al.* 2012, Mackay *et al.* 2013, Lu *et al.* 2014), antirhinoviral strategies could focus on these species, but they still include over hundred types of rhinoviruses. However, as rhinovirus infections are associated with a substantial disease burden and antibiotic use in healthy children in the community, safe, effective, and affordable modalities are needed for both prevention and treatment of rhinovirus infections.

As the development of a rhinovirus vaccine and treatments has been difficult, mainly due to the wide number of different rhinovirus types, another approach is to develop treatments or prevention methods targeting immunological mechanisms. An interesting

subject of ongoing research is whether the clinical manifestations of rhinovirus infections are caused directly by viral pathogenesis or by the host immune response, and the current knowledge supports the latter notion (Jacobs *et al.* 2013). More clinical and translational data on immunological mechanisms and aberrancies of the host in rhinovirus infections, recurrent infections, and rhinovirus-associated wheezing illnesses are needed.

Our clinical study in healthy children brings new data about the effect of TLR7 and TLR8 on the clinical presentation of rhinovirus infections. Our results are the first to indicate that polymorphisms in TLR7 and TLR8 affect the susceptibility to rhinovirus infections in a clinical setting. Combined to the previous data, it seems that TLR7 signaling in rhinovirus infections would lead to different outcomes in atopic and non-atopic hosts. We found that TLR7 polymorphisms protect the healthy host from rhinovirus infections, while previously, TLR7-deficient allergic mice have shown an impaired IFN response to rhinovirus, an increased rhinoviral replication, inflammation, and hyper-reactivity of the airways (Hatchwell *et al.* 2015). In asthmatic individuals innate and antiviral mechanisms could be impaired due to still partly unknown mechanisms, and thus TLR7 signaling would be more crucial. In a non-atopic host, a TLR7 SNP would be beneficial if the symptoms did not develop due to the defective TLR7-mediated interferon signaling, and instead the rhinovirus infection would remain asymptomatic in an otherwise immunocompetent host. This data could be important with regard to the potential development of new treatment modalities targeting TLR7 signaling, as the possible treatments could work differently in non-atopic and atopic individuals. The antiviral and proinflammatory signaling pathways are complex and due to several receptors and pathways, compensatory mechanisms may replace a deficiently working pathway. Susceptibility to rhinovirus infections in atopic individuals with a TLR7 variant type would be an interesting topic of research covering the underlying mechanisms of interactions of rhinovirus infections and allergic phenotype in the development of asthma. The current data suggest that suppression of TLR3 or MDA5 and the induction of TLR7 and their downstream signaling pathway could be novel targets for prevention and treatment of rhinovirus induced wheezing illnesses (Moller-Larsen *et al.* 2008, Wang *et al.* 2011). In mouse models, a synthetic TLR7/TLR8 ligand, resiquimod, has been found effective in attenuating airway reactivity and inflammation and preventing the development of asthmatic phenotype (Moisan *et al.* 2006, Camateros *et al.* 2007, Sel *et al.* 2007, Nadeem *et al.* 2016). In the future, new immunomodulatory treatments or even genetically tailored treatments for wheezing illnesses and respiratory infections may become available.

Future studies are needed to further clarify the usefulness of blood MxA protein in discriminating respiratory viral from bacterial infections both in the out-patient and in-patient setting. It is of interest whether the diagnostic value of MxA could be improved

by combining the determination of blood MxA, CRP level, WBC count, and/or procalcitonin to new markers such as TRAIL or IP-10 (Wark *et al.* 2007, Oved *et al.* 2015). To have an impact on clinical decisions in the out-patient care and emergency departments, there is need for a diagnostic test or combination of tests that would be easy to use, be available at the site of the patient care, and provide results rapidly. Whole blood MxA determination based on EIA has potential for rapid diagnostic use.

Current knowledge of the high frequency and remarkable disease burden caused by rhinovirus infections in form of acute otitis media, wheezing illnesses, antibiotic treatments, and health-care use calls for development of effective treatment or prevention modalities as well as quick diagnostic methods of rhinovirus infections.

7 SUMMARY AND CONCLUSIONS

This study provides new information about the major role of rhinoviruses in acute respiratory infections in young children. Rhinovirus infections are generally mild, but because of their high frequency, the impact of rhinoviruses on the total number of acute otitis media and antibiotic treatments in young children is greater than that of any other respiratory virus. Rhinovirus infections are detected in more than half of acute respiratory infections in young children and are associated with one-half of all acute otitis media cases, antibiotic treatments, and outpatient visits for acute respiratory infections, and a substantial part of wheezing illnesses. We report here that rhinovirus infections occur at an estimated rate of 3.5 per child per year. Genetic polymorphisms in MBL and TLRs promote the susceptibility to or the protection against respiratory infections, rhinovirus infections, and acute otitis media and may explain why some children are more prone to respiratory infections. Blood MxA protein is an informative marker of respiratory virus infections including rhinovirus infections.

With an intensive, prospective follow-up with daily diaries, study clinic visits, and by collecting data from the hospital district medical records in a birth cohort without selection criteria, we documented the remarkably high occurrence and impact of rhinovirus infections in young children in the community. Using home nasal swab sampling and RT-PCR diagnostics in this setting, we were able to document rhinoviruses in respiratory infections at all severities from mild colds not necessitating a physician visit to severe infections treated at the hospital. Even though rhinovirus infections are mostly mild, the high frequency of rhinovirus infections results in a heavy respiratory disease burden in young healthy children and a substantial proportional effect of the use of health-care services and antibiotics related to respiratory infections. Earlier studies have not properly addressed the disease burden caused by rhinovirus, and rhinovirus-associated antibiotic use has not been previously reported. We also report that rhinoviruses are more infrequently detected in completely asymptomatic children than previously thought.

While several annual episodes of uncomplicated respiratory tract infections at young age are typical, particular attention should be paid to children who have unusually frequent or prolonged infections as they and their families carry a substantial burden of respiratory infections and associated outcomes. Children with recurrent respiratory infections frequently use healthcare services and antibiotics, undergo surgical procedures, and are at risk for asthma in early life. Having older siblings increases the risk of recurrent infections. Respiratory infections occur early in children who develop recurrent respiratory infections. These children carry a high burden of respiratory illness and should be recognized in order to develop and focus possible intervention.

Genetic polymorphisms in mannose-binding lectin (MBL) promote the susceptibility to respiratory infections and variants of toll-like receptor (TLR) 2 associate with an increased risk of acute otitis media. Variants in TLR7 protect against, and variants in TLR8 promote the susceptibility to recurrent rhinovirus infections. With an intensive follow-up in a Finnish birth cohort without selection criteria, we documented that genetic polymorphisms of MBL and TLRs associate with the risk of respiratory infections and may explain why some children are more prone to respiratory infections.

Blood MxA protein levels are increased in young children with symptomatic respiratory virus infections including infections caused by rhinovirus. The MxA response in rhinovirus infections has not been shown before and is crucial regarding the use of MxA as a general diagnostic marker of respiratory virus infections as rhinoviruses account for the majority of respiratory infections. As also asymptomatic virus infections are relatively common in young children, an important and novel finding is that asymptomatic virus infections were not associated with elevated MxA levels. Our data indicate that in children, rhinoviruses are capable of inducing an efficient interferon response seen as elevated blood levels of MxA protein. Blood MxA can be used as a general diagnostic marker of common respiratory virus infections and could possibly help to reduce unnecessary use of antibiotics in young febrile children.

The development of new molecular techniques has led to the increasing recognition of rhinoviruses as a cause of severe infections, hospitalizations, and infections in immunocompromised hosts, and the role of rhinovirus infections in both the development and exacerbations of asthma is under intensive research. Sensitive PCR techniques have also led to the discovery of novel species C rhinoviruses, and the clinical importance of rhinovirus infections is now undeniable. Improving our knowledge about the epidemiology, associated morbidity, and susceptibility to rhinovirus infections is crucial to the further development and targeting of novel antiviral treatments and prophylaxis modalities. This translational study adds important data on the current knowledge about rhinovirus infections in young children at the community level. Our study brings new information about the burden, considerable associated morbidity, and susceptibility to rhinovirus infections in young healthy children as well as novel insights into diagnostics of rhinovirus infections with a general marker of virus infections, the blood MxA protein. Rhinovirus infections impose a major burden of respiratory disease and antibiotic use in children during the first years of life. Novel prevention or treatment modalities of rhinovirus infections could substantially decrease the use of antibiotics for respiratory tract infections in children.

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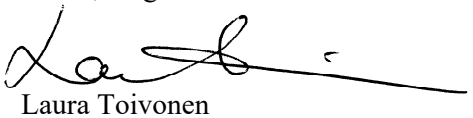

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