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To my family

ABSTRACT

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Viral and bacterial interactions in respiratory tract infections in children

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Annales Universitas Turkuensis, Medica-Ontologica, Turku, Finland, 2019

Respiratory tract infections cause a great burden on children under 2 years of age. Most respiratory tract infections are caused by viruses, but co-infections with viruses and bacteria are common. The aims of these studies were to assess inhibiting and synergistic interactions between respiratory viruses and bacteria, to analyze the effects of genetic variants in the host innate immune system and to determine the effects of vaccination with a pneumococcal conjugate vaccine on viral and bacterial diseases and bacterial colonization in children.

We followed 923 children in a prospective cohort study for respiratory tract infections during the years 2008-2012 by symptom diaries, nasal swab samples and clinical examinations. Viruses and bacteria were analyzed during respiratory tract infections and at certain ages when children were healthy. Genetic polymorphisms of mannose-binding lectin and Toll-like receptors were determined in blood samples.

Infants with genetic polymorphisms of mannose-binding lectin were more prone to be colonized with *Streptococcus pneumoniae* during rhinovirus infection. Furthermore, rhinovirus infection increased the rate of community acquisition and transmission of pneumococcus in families with children. The rate of rhinovirus infections was significantly lower during respiratory syncytial virus infection compared to control children suggesting inhibiting interaction between these viruses. In a cluster randomized trial, 10-valent pneumococcal conjugate vaccine prevented not only acute otitis media but to some extent also all respiratory tract infections that are mainly caused by viruses.

These studies showed that viral infection predisposes children to bacterial colonization and synergism between viruses and bacteria is affected by genetic variations in the host immune system. However, also negative interactions between microbes occur. By affecting the microbiological environment in the nasopharynx, pneumococcal conjugate vaccines may have effect on both bacterial and viral infections.

Keywords: mannose-binding lectin, pneumococcal conjugate vaccine, respiratory tract infections, rhinovirus, *Streptococcus pneumoniae*, viral-bacterial interaction

TIIVISTELMÄ

LL Sinikka Karppinen

Virusten ja bakteerien interaktio lasten hengitystieinfektioissa

Turun yliopisto, Lääketieteellinen tiedekunta, Lastentautioppi, Turun kliininen tohtoriohjelma; Lasten ja nuorten klinikka, Turun yliopistollinen keskussairaala
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Hengitystieinfektioiden aiheuttama tautitaakka on suuri alle 2 vuoden ikäisillä lapsilla. Suurin osa tavallisista hengitystieinfektioista on virusten aiheuttamia, mutta virusten ja bakteerien yhteisinfektiot ovat yleisiä. Näiden tutkimusten tavoitteena oli saada lisää tietoa siitä, millaisia altistavia tai estäviä vaikutuksia mikrobeilla on toisiinsa ja miten synnynnäisen puolustusjärjestelmän geneettiset ominaisuudet ja pneumokokki-konjugaattirokote vaikuttavat lapsilla virusten ja bakteerien aiheuttamiin hengitystieinfektioihin ja bakteerikolonisaatioon.

Keräsimme vuosien 2008-2012 aikana tietoa alle 2-vuotiaiden lasten sairastetuista hengitystieinfektioista ja niiden aiheuttajista oirepäiväkirjojen sekä mikrobiologisten näytteiden avulla prospektiivisessa seurantatutkimuksessa, johon osallistui 923 lasta. Näytteet kerättiin hengitystieinfektioiden aikana sekä ennalta suunnitelluilla tervekäynneillä 2, 12 ja 24 kk:n iässä. Neljässä eri osatutkimuksessa selvitimme puolustusjärjestelmän geneettisen vaihtelun, ympäristön ja pneumokokkirokotteen vaikutusta mikrobien esiintyvyyteen ja interaktioon.

Synnynnäiseen immuniteettiin osallistuvan mannoosia sitovan lektiinin geneettinen vaihtelu altisti varhaislapsuuden pneumokokkikolonisaatiolle rinovirusinfektion aikana. Rinovirusinfektio lisäsi pneumokokkikolonisaatiota myös imeväisiän jälkeen ja myötävaikutti pneumokokin tarttumiseen perheenjäsenten välillä. Rinoviruksen ja pneumokokkibakteerin synergian lisäksi havaittiin negatiivinen yhteys respiratory syncytial -viruksen ja rinoviruksen aiheuttamien infektioiden välillä. Konjugoidun pneumokokkirokotteen todettiin vähentävän korvatulehdusepisodien lisäksi myös lieviä hengitystieinfektioita, mitkä tyypillisesti ovat virusten aiheuttamia.

Virusten ja bakteerien interaktio on monimuotoista ja siihen vaikuttavat yksilön puolustusjärjestelmän geneettiset ominaisuudet. Virusinfektiot altistavat bakteeri-infektioille, mutta osa mikrobeista myös estää toistensa ilmaantuvuutta. Konjugoitu pneumokokkirokote saattaa muokata hengitysteiden mikrobistoa ja näin vaikuttaa sekä virusten että bakteerien aiheuttamien hengitystieinfektioiden kehittymiseen.

Avainsanat: hengitystieinfektio, mannoosia sitova lektiini, pneumokokki, pneumokokkikonjugaattirokote, respiratory syncytial –virus, rinovirus, virusten ja bakteerien interaktio

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ABBREVIATIONS

AdV	adenovirus
AOM	acute otitis media
BoV	bocavirus
CI	confidence interval
CoV	coronavirus
EV	enterovirus
H2O2	hydrogen peroxidase
H. influenzae	*Haemophilus influenzae*
IAV	influenza A virus
IBV	influenza B virus
ICAM-1	intracellular adhesion molecule 1
LAIV	live attenuated influenza virus
LDLR	low density lipoprotein receptor
MBL	mannose-binding lectin
MPV	metapneumovirus
NLR	NOD-like receptor
PAFr	platelet activating factor receptor
PAMP	pathogen associated molecular pattern
PHiD-CV10	pneumococcal *Haemophilus influenzae* protein D conjugate vaccine, 10-valent
PIV	parainfluenzavirus
PRR	pattern-recognition receptor
RIG-I	retinoic acid-inducible gene-I
RLR	RIG-like receptor
RSV	respiratory syncytial virus
RTI	respiratory tract infection
RV	rhinovirus
SNP	single nucleotide polymorphisms
S. pneumoniae	*Streptococcus pneumoniae*
STEPS	Steps to the Healthy Development and Wellbeing of Children
TLR	Toll-like receptor
WHO	World Health Organization

LIST OF ORIGINAL PUBLICATIONS

Thesis is based on the following original publications, which are referred to in the text by the Roman numerals I-IV and on some supplementary unpublished data.

I Karppinen S*, Vuononvirta J*, He Q, Waris M, Peltola V. Effects of rhinovirus infection on nasopharyngeal bacterial colonization in infants with wild or variant types of mannose-binding lectin and Toll-like receptors 3 and 4. *J Pediatric Infect Dis Soc*. 2013; 2:240-247.

II Karppinen S, Toivonen L, Schuez-Havupalo L, Waris M, Peltola V. Interference between respiratory syncytial virus and rhinovirus in respiratory tract infections in children. *Clin Microbiol Infect*. 2016; 22:208.e1-208.e6.

III Karppinen S, Teräsjärvi J, Auranen K, Schuez-Havupalo L, Siira L, He Q, Waris M, Peltola V. Acquisition and transmission of *Streptococcus pneumoniae* are facilitated during rhinovirus infection in families with children. *Am J Respir Crit Care Med*. 2017; 196:1172-1180.

IV Karppinen S, Toivonen L, Schuez-Havupalo L, Teros-Jaakkola T, Waris M Auranen K, Palmu A, Peltola V. Effectiveness of the ten-valent pneumococcal *Haemophilus influenzae* protein D conjugate vaccine (PHiD-CV10) against all respiratory tract infections in children under two years of age. *Submitted.*

*Equally contributed

1 INTRODUCTION

Respiratory tract infections cause a great burden on children (Toivonen, *et al*. 2016a, Chonmaitree, *et al*. 2008). Most respiratory tract infections are caused by viruses, but these infections can be complicated by bacterial infection in diseases like acute otitis media (AOM), sinusitis, pneumonia and sepsis (McCullers and Rehg. 2002, Oliver, *et al*. 2008, Bosch, *et al*. 2013). Colonization of the nasopharynx with different bacterial species is needed for the development of bacterial respiratory diseases.

Interactions between different pathogens in the host are diverse. In addition to synergistic mechanisms also inhibiting interactions between microbes occur (Bogaert, *et al*. 2004, Greer, *et al*. 2009). Viral infection can predispose the host to bacterial colonization and infection by affecting the respiratory tract epithelium and by impairing ciliary function thus making the respiratory environment more favorable for bacterial attachment (Vareille, *et al*. 2011, Bogaert, *et al*. 2004). However, also bacteria may have effects on the development of viral infections (Almand, *et al*. 2017, Cebey-Lopez, *et al*. 2016). The host immune system is central in the clearance of pathogens and immunologic functions are affected by viral infections (Astry and Jakab. 1984, Oliver, *et al*. 2008). Also the living environment and the age of the child influences the colonization rates and development of diseases. Young children are most commonly colonized with pathogenic bacteria. The presence of siblings and attending daycare are known risk factors for bacterial colonization and respiratory tract infections, indicating that children are a great reservoir for carriage and transmission of viruses and bacteria (Regev-Yochay, *et al*. 2004, Garcia-Rodriguez and Fresnadillo Martinez. 2002).

It is common that more than one virus or bacterium are detected concurrently during respiratory infections, but co-detection of microbes is common also in healthy children (van den Bergh, *et al*. 2012a, Bosch, *et al*. 2013). In several studies, co-infections have been described to be more severe than single-microbe infections (Brealey, *et al*. 2018, Cebey-Lopez, *et al*. 2016). During the 1918 influenza A (H1N1) pandemic, mortality rates increased during and after influenza infections because of secondary bacterial diseases such as pneumonia and sepsis (McCullers. 2006, Morris, *et al*. 2017). Postmortem studies showed that influenza virus was often found concurrently with *Streptococcus pneumoniae (S. pneumoniae), Staphylococcus aureus (S. aureus), Haemophilus influenzae (H. influenzae)* or *Streptococcus pyogenes* showing synergism between these microbes (Brundage and Shanks. 2008, Morens, *et al*. 2008). By preventing influenza infections with vaccines also the rate of secondary bacterial diseases like acute otitis media and pneumonia have decreased (Tessmer, *et al*. 2011, Heikkinen, *et al*. 2013).

To prevent diseases that cause high morbidity and mortality in children, it is important to understand the course of interactions between different pathogens and the reasons that

may affect the development of disease. In this study, we focused on the interactions between commonly detected viruses and bacteria during respiratory tract infections in children and assessed the effects of host and environmental factors on the dynamics between these microbes and on the diseases that they cause.

2 REVIEW OF THE LITERATURE

2.1 Viral and bacterial respiratory tract infections in children

2.1.1 Epidemiology

Children under two years of age have on average 6 acute respiratory infections annually (Toivonen, *et al*. 2016b). Respiratory tract infections (RTIs) are a common cause for physician visits, antimicrobial treatments and hospitalizations. Most of the RTIs are caused by viruses. A high incidence of viral infections leads to economic losses and absenteeism from work and school, and recurrent RTIs have been associated with development of asthma (Sigurs, *et al*. 2000, Lemanske, *et al*. 2005, Fendrick, *et al*. 2003, Toivonen, *et al*. 2019). Viral respiratory tract infections are divided into upper and lower RTIs, and symptoms may vary from mild symptoms like rhinorrhea and cough to more severe symptoms that require hospitalization. Viral upper respiratory tract infections include the common cold with symptoms that mostly occur in the throat and nose. The lower respiratory tract infections include pneumonia and wheezing illnesses (e.g., bronchiolitis, obstructive bronchitis and exacerbation of asthma) and are the most common causes of hospitalizations. Viruses can be detected in over 90% of RTIs in children as a single or co-infection (MacIntyre, *et al*. 2012, Drews, *et al*. 1997, Ruohola, *et al*. 2009, Martin, *et al*. 2013, Chonmaitree, *et al*. 2015). Rhinovirus (RV) is the most common causative agent in RTIs (Arruda, *et al*. 1997, Chonmaitree, *et al*. 2015, Toivonen, *et al*. 2016b, Makela, *et al*. 1998). In children under 2 years of age, it causes a mean number of 3.5 RTIs annually and 50% of rhinovirus infections are related to acute otitis media and 41% to wheezing illnesses (Toivonen, *et al*. 2016b). In addition to rhinovirus, respiratory syncytial virus (RSV), influenza A and B viruses, parainfluenza viruses, enteroviruses, coronaviruses and adenovirus are frequently detected causative agents of RTIs (Toivonen, *et al*. 2016b, Rhedin, *et al*. 2015) (Figure 1). Rhinovirus, RSV and influenza viruses cause a substantial proportion of hospitalizations and physician visits in children (Heikkinen, *et al*. 2017, Nair, *et al*. 2010). RSV causes both upper and lower respiratory tract infections, and it is an important cause of pneumonia and bronchiolitis in small children (Mansbach, *et al*. 2012, Ruuskanen, *et al*. 2011, Jain, *et al*. 2015). Rhinovirus can be detected throughout the year while RSV and influenza viruses occur in biennial and annual cycles, respectively, with highest incidence during winter months (Figure 2).

Respiratory viruses are common findings also among asymptomatic individuals, especially when sensitive PCR methods are used for their detection. Up to 27-40% of asymptomatic children are positive for at least one virus in their nasopharynx, and 6% are

positive for 2 or more viruses (Chonmaitree, *et al*. 2015, van der Zalm, *et al*. 2009). It is unclear whether all pathogens detected during RTIs are the causative agents of symptoms or if some of them are just innocent bystanders in the nasopharynx. Pathogenicity of viruses and symptoms during RTIs depends on the host and environmental factors, and it has been speculated that not every infection with pathogen leads to symptomatic infection (van der Zalm, *et al*. 2009). Certain viruses like rhinoviruses and coronaviruses are detected often in asymptomatic subjects, while RSV and influenza viruses are predominantly detected only in symptomatic individuals. Young children are more often symptomatic during detection of virus compared to older children and adults (van der Zalm, *et al*. 2009, Peltola, *et al*. 2008).

Viral infections can be complicated by bacterial diseases (Rhedin, *et al*. 2015, Chonmaitree, *et al*. 2016). The most important bacteria causing respiratory tract infections in children are *S. pneumoniae, H. influenzae* and *Moraxella catarrhalis (M. catarrhalis*) (Del Beccaro, *et al*. 1992). Bacterial pneumonia is a common and potentially serious bacterial infection in children. In 2016, lower respiratory tract infections caused 13.1% of all deaths in children under five years of age (GBD 2016 Lower Respiratory Infections Collaborators. 2018). Of milder respiratory diseases, AOM is highly important, because it often leads to antibiotic use and, in recurrent cases, to surgical interventions such as tympanostomy tube replacement. AOM can be caused by viruses, bacteria or by co-infection with both of these pathogens (Ruohola, *et al*. 2006) *S. pneumoniae, H. influenzae and M. catarrhalis* being most common causative agents. AOM complicates viral RTIs usually within the first week after the onset of symptoms, and it develops in up to one-third of acute respiratory tract infections caused by any virus in children and even more frequently in RSV infections (Nokso-Koivisto, *et al*. 2015, Chonmaitree, *et al*. 2016, Chonmaitree, *et al*. 2008, Heikkinen, *et al*. 1999). The mean rate of AOM has been reported to be 1.7 episodes annually, and 80% of children have had at least one AOM infection by three years of age (Vergison, *et al*. 2010).

Bacterial pneumonia and AOM can be partly prevented with vaccines that are targeted against bacteria (most importantly *S. pneumoniae*) (Palmu, *et al*. 2017a, Tessmer, *et al*. 2011, Eskola, *et al*. 2001). However, prevention of influenza by vaccines also prevents pneumonia and AOM demonstrating the role that both viruses and bacteria play in the pathogenesis of these diseases.

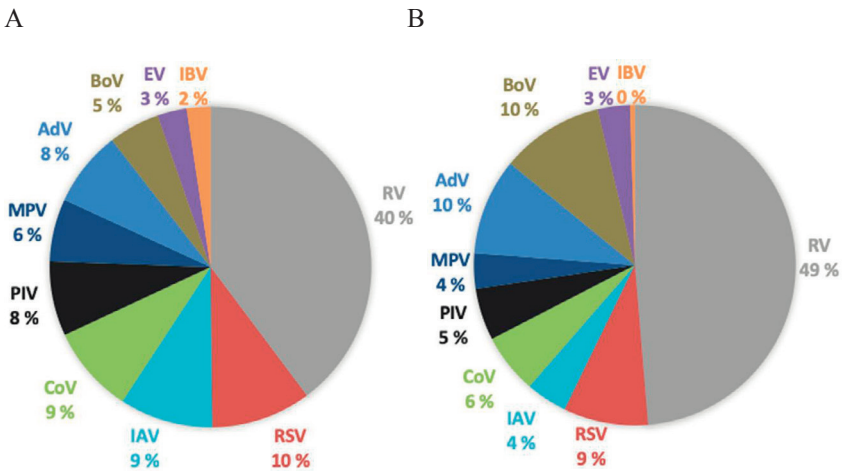


Figure 1. Data from the Diagnostic Virology Unit of University of Turku (2013–2014) and Turku University Hospital (2015-) by Anyplex RV16 (2013–2015) and the Allplex Respiratory Panel (2016) PCR assays (Seegene, Seoul, Korea) from respiratory tract specimens.

A) Distribution of virus findings from adults and children. The number of positive samples was 2706 (41%) of the total of 6557. The number of co-detections of 2 or more viruses was 489 (18% of positive samples).

B) Distribution of virus findings from children <2 years of age. The number of positive samples was 533 (56%) of the total of 954.

The number of co-detections of 2 or more viruses was 107 (20% of positive samples).

AdV, adenovirus; BoV, bocavirus; CoV, coronavirus; EV, enterovirus; IAV, influenza A virus; IBV, influenza B virus; MPV, metapneumovirus; PIV, parainfluezavirus types 1–4; RSV, respiratory syncytial virus A and B; RV, rhinovirus.

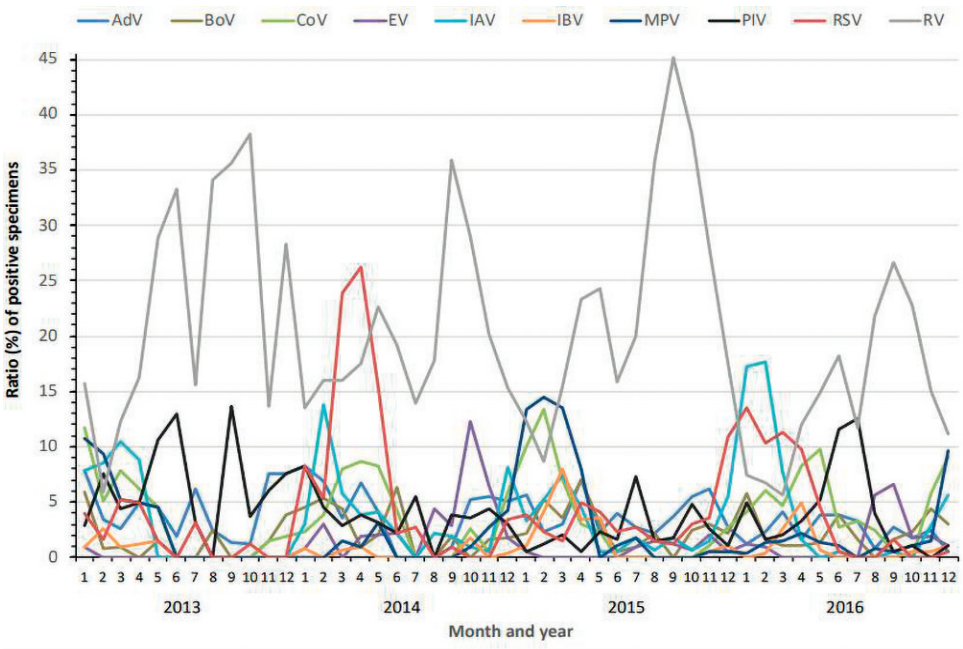


Figure 2. Viral findings during the years 2013-2016 in the Hospital District of Southwest Finland. Data and figure from Matti Waris, the Diagnostic Virology Unit of the University of Turku (2013–2014) and Turku University Hospital (2015-) by the Anyplex RV16 (2013–2015) and the Allplex Respiratory Panel (2016) PCR assays (Seegene, Seoul, Korea). Samples were collected during respiratory tract infections from pediatric and adult patients (n=6557). Monthly percentages of specimens positive for each virus are reported. AdV, adenovirus; BoV, bocavirus; CoV, coronavirus; EV, enterovirus; IAV, influenza A virus; IBV, influenza B virus; MPV, metapneumovirus; PIV, parainfluezavirus types 1–4; RSV, respiratory syncytial virus A and B; RV, rhinovirus.

2.1.2 Microbiological etiology

The following viruses and bacteria are reviewed below: rhinovirus, RSV, *S. pneumoniae*, *H. influenzae*, and *M. catarrhalis*. These microbes are common and important causative agents of respiratory tract infections, and they were studied in this thesis.

2.1.2.1 Rhinovirus

Rhinoviruses belong to the *Picornaviridae* family and to the genus *Enterovirus.* There are three different species of rhinoviruses that have been identified, groups A, B and C, together with over 160 different types (Jacobs, *et al*. 2013). Rhinoviruses can be detected throughout the year with the highest incidence from August to November and from April to May (Toivonen, *et al*. 2016b). Rhinovirus is easily transmitted from person-to-person in direct contacts via droplets, by handshake or indirectly from a contaminated environment that contains rhinovirus. Transmission via aerosols may happen, even though it has not been considered as important route for transmission as contact and droplets. (Hendley, *et al*. 1973, Dick, *et al*. 1987). Several rhinovirus types can circulate simultaneously in communities and even within families with children (Peltola, *et al*. 2008). Most rhinovirus types attach and enter epithelial cells via intercellular adhesion molecule 1 (ICAM-1), while part of species A rhinovirus types enter cells via the low-density lipoprotein receptor (LDLR) (Bochkov and Gern. 2016).

Rhinovirus infections range from asymptomatic to severe infections requiring hospitalization, and rhinovirus is the second most common cause of wheezing illnesses in children (Papadopoulos, *et al*. 2002). Rhinovirus has been detected in over 70% of all RTIs in children and in 9-30% of asymptomatic children (van der Zalm, *et al*. 2009, Toivonen, *et al*. 2016b, van Benten, *et al*. 2003, Jartti, *et al*. 2004, Johnston, *et al*. 1993). The meaning of a viral finding in asymptomatic children is unclear. It may be a truly asymptomatic infection (i.e., subclinical infection), or symptoms can be so mild that they are not reported by the individual or by parents. It may also be possible that a symptomatic episode preceded sampling, or that a virus is found during the incubation period, which is on average 2 days in duration (Lessler, *et al*. 2009, van Benten, *et al*. 2003). Rhinovirus can be detected in respiratory secretions for several days after the symptoms of RTI have resolved (Winther, *et al*. 2006, Nokso-Koivisto, *et al*. 2002, van Benten, *et al*. 2003). In immunocompromised children, rhinovirus infection can be prolonged, and the virus can be detected even for 2 months or longer with or without chronic symptoms (Peltola, *et al*. 2013).

The economic effect of rhinovirus is remarkable. In children under 2 years of age, the annual rate of RTI caused by rhinovirus has been estimated to be 3.5 infections per child, and rhinovirus is the most common cause of recurrent respiratory tract infections.

Rhinovirus has been associated with a high incidence of outpatient visits and prescription of antibiotics, and in children attending daycare, the rate of lost work days related to rhinovirus infections has been estimated to be 3.4 per child per year (Toivonen, *et al*. 2016b).

2.1.2.2 Respiratory syncytial virus

RSV belongs to the family *Paramyxoviridae* and is a member of the genus *Pneumovirus* with two antigenic groups, A and B. It causes typically RTIs during the winter season with a peak prevalence yearly between late fall and early spring. In Finland, epidemics follow a regular 2-year cycle pattern with larger epidemics in winters that begin during odd-numbered years (Waris. 1991). Up to 90% of children are affected by RSV before the age of 2 years (Simoes. 1999a, Glezen, *et al*. 1986). RSV causes both upper and lower respiratory tract infections, and although infant bronchiolitis is the classical manifestation of RSV infection, the majority of infections are mild, common cold like diseases (Jain, *et al*. 2015, Ruuskanen, *et al*. 2011). AOM is a common complication during RSV infection, and it has been detected in more than half of the episodes (Heikkinen, *et al*. 2015). RSV is the most important causative agent of bronchiolitis under 12 months of age with a highest incidence at the age of 1-6 months. In infants, RSV bronchiolitis often causes hospitalization because of dyspnea and feeding difficulties (Borchers, *et al*. 2013). RSV infection of bronchial epithelial cells leads to mucosal inflammation and edema of the peribronchial region. Epithelial cell necrosis and mucus cause airway obstruction leading to hyperinflation of distal airways. Cellular immunity plays an important role in recovery. Prematurely born children and those with immunodeficiency may have more severe and long-lasting infections. Treatment of RSV infection is symptomatic and supportive (Caballero, *et al*. 2017, Simoes. 1999b). Prevention with a humanized monoclonal antibody can be used in preterm children and in children with certain underlying diseases that predispose them at risk of more severe disease, but passive immunization by currently available products is not suitable for large-scale use. (Homaira, *et al*. 2014).

2.1.2.3 Streptococcus pneumoniae

Streptococcus pneumoniae, or pneumococcus, is a gram-positive alpha-hemolytic encapsulated bacterium that appears in cultures as pairs (diplococcus) or small groups or chains. Over 90 different serotypes have been identified based on antigenic differences in their capsular polysaccharides. The major immune mechanism that protects the host against pneumococcal infections is thought to be antibody-initiated complement-dependent opsonization, where the classic complement pathway is activated (Paton, *et al*. 1993). Acquisition and colonization are needed for development of disease, but

pneumococcus can be detected in the nasopharynx of asymptomatic subjects as well. Most children acquire *S. pneumoniae* into their nasopharynx during early childhood (Syrjanen, *et al*. 2001, Regev-Yochay, *et al*. 2004). The rate of pneumococcal carriage increases during the first months of life and decreases after 3 years of age (Aniansson, *et al*. 1992, Dagan, *et al*. 1996, Hendley, *et al*. 1975). In Finland, up to 87% of children have been colonized with *S. pneumoniae* at some point before 2 years of age (Syrjanen, *et al*. 2001). In addition to age, one of the most important risk factors for pneumococcal colonization is the presence of siblings (Dagan, *et al*. 1996, Leino, *et al*. 2001, Givon-Lavi, *et al*. 2002). Because of high rate of pneumococcal colonization, small children are the main source for pneumococcal transmission that occurs via respiratory droplets (Musher. 2003). Pneumococcus is an important cause of AOM, sinusitis, pneumonia and invasive diseases (sepsis and meningitis), and it causes 700 000 to 1 million deaths every year in children worldwide (O'Brien, *et al*. 2009). The burden of pneumococcal diseases is highest in small children and in the elderly (Morrow, *et al*. 2007). It has been shown that the risk for development of disease is greatest soon after acquisition of the organism in the nasopharynx, but it is also possible after months of carriage (Gray and Dillon. 1986, Austrian, *et al*. 1977, Syrjanen, *et al*. 2005a).

Vaccination with conjugate and polysaccharide vaccines has led to the reduction of pneumococcal diseases. The 23-valent polysaccharide vaccine gives protection to adults and children over 2 years of age against invasive pneumococcal diseases. Because polysaccharide vaccines do not cause a sufficient immune response in children under 2 years of age, pneumococcal conjugate vaccines are used in small children (Daniels, *et al*. 2016). Pneumococcal conjugate vaccines give protection not only against invasive pneumococcal diseases but also against acute otitis media, pneumonia and carriage of serotypes included in the vaccine (Palmu, *et al*. 2015, Eskola, *et al*. 2001, Grijalva, *et al*. 2007).

2.1.2.4 Haemophilus influenzae

Haemophilus influenzae is a gram-negative coccobacillus that is divided into encapsulated and non-encapsulated forms based on the presence of a polysaccharide capsule. Of several encapsulated *H. influenzae* types, the type B is an important causative agent of meningitis and other invasive infections. It can be efficiently prevented by a conjugated vaccine, which is included in the child immunization programmes of most countries. Non-typeable, non-encapsulated *H. influenzae* (NTHi) is a common finding in the nasopharynx of asymptomatic children. During the first year of life, approximately 20% of infants, and by the ages of 5-6 years over 50% of children are colonized with NTHi (Aniansson, *et al*. 1992). NTHi is a causative agent in one-third of AOM, and it is the most common cause of recurrent otitis media (Murphy, *et al*. 2009, Leibovitz, *et al*.

2004). In addition to AOM it is a common cause of sinusitis and a rare cause of pneumonia or invasive diseases in children (Faden, *et al*. 1995). There are no vaccines available against NTHi.

2.1.2.5 Moraxella catarrhalis

Moraxella (Branhamella) catarrhalis (M. catarrhalis) is a gram-negative diplococcus that was first described in 1896. It can be found as a part of normal microbiota in the nasopharynx. Carriage rates of *M. catarrhalis* are higher in small children compared to adults (Ejlertsen, *et al*. 1994). Up to 78% of children are colonized with *M. catarrhalis* by the age of two years at least once. In adults, the carriage rates are low, and 1-5% of adults carry *M. catarrhalis* in their nasopharynx (Faden, *et al*. 1997, Bisgaard, *et al*. 2007, DiGiovanni, *et al*. 1987). In addition to the age, seasonality, living conditions, smoking and hygiene have effects on the colonization rates (Verduin, *et al*. 2002, Van Hare, *et al*. 1987). The mean duration of *M. catarrhalis* colonization is one month. Colonization with *M. catarrhalis* in neonates may increase susceptibility to respiratory tract infections such as bronchiolitis and pneumonia during later childhood (Vissing, *et al*. 2013). In addition to lower respiratory tract infections, *M. catarrhalis* is a common cause of AOM being responsible for of up to 20% of AOMs diagnosed in children (Verduin, *et al*. 2002). Most *M. catarrhalis* strains produce *beta*-lactamase, which is important to take into account when choosing antibiotics (Aguilar, *et al*. 2009). In adults, *M. catarrhalis* is a common cause for the exacerbations, i.e., intermitted worsening of symptoms, in chronic obstructive pulmonary disease (Leung, *et al*. 2017).

2.2 Innate and adaptive immune system

The human immune system can be divided into innate and adaptive immunity. Innate immunity consists of physical and chemical barriers and immune responses that are activated within minutes in response to pathogen recognition (Kumar, *et al*. 2011). Innate immunity is particularly important in young children under one year of age, when the adaptive immune responses are still immature and antibodies transported from the mother through the placenta gradually fade away. The responses to the innate immune system instruct the development of adaptive immune system. Adaptive immunity is pathogen-specific and can be divided into antibody-mediated (humoral) and cell-mediated immunity, which are both carried out by lymphocytes. Receptors that are expressed on the surface of B and T lymphocytes are able to recognize antigen epitopes. Recognition of these epitopes leads to elimination of the invading pathogen and generation of immunological memory. B lymphocytes produce pathogen-specific antibodies. In cell-mediated response, T lymphocytes recognize foreign antigens on the surface of infected

cells and are responsible for killing and eliminating the infected cell (Iwasaki and Medzhitov. 2010, Akira, *et al*. 2006). T lymphocytes are classified into two major types, helper T cells and cytotoxic T cells. Helper T cells support other white blood cells in immunologic responses and can differentiate into several subtypes, i.e., Th1, Th2, Th17, Th9, Tfh, and Tregs, which secrete different cytokines. Cytotoxic T helper cells are focused on the elimination of pathogen-infected host cells (Andersen, *et al*. 2006, Evans and Jenner. 2013).

The innate immune system includes chemical, physical and cellular barriers against pathogens. The airway epithelium serves as a first line defense against respiratory tract pathogens. When encountering pathogens, airway cells increase the production of cytokines, chemokines and antimicrobial peptides that take part in eradication of pathogens and regulate inflammation and immunity. Epithelial cells secrete mucins forming a mucus layer where pathogens can trap to, and ciliary movement on the epithelial cells transports pathogens away from the airways (Ryu, *et al*. 2010).

In addition to the epithelial barrier, the innate immune response has an important role in defense against microbes at all ages but especially in infants, whose adaptive immune system is still immature. The innate immune system is able to recognize structures on pathogens by pattern-recognition molecules. Pattern-recognition molecules like mannose-binding lectin (MBL), the family of Toll-like receptors (TLRs), retinoic acid-inducible gene-I receptors (RIG-I), and NOD-like receptors (NLRs) are able to recognize structures of the pathogens called pathogen-associated molecular patterns (PAMPs) (Akira, *et al*. 2006, Medzhitov, *et al*. 1997). These receptors that are called pattern-recognition receptors (PRRs) recognize pathogens or pathogen-derived products in the plasma membrane or inside the cells on the endosomes or in the cytoplasm (Kumar, *et al*. 2009). In response to pathogens, the innate immune system is able to produce cytokines and activate the complement system to target microorganisms for phagocytosis by macrophages and neutrophils. Viral infection of the cells leads to production of interferons, which inhibit viral replication and activate natural killer cells and T lymphocytes (Brandstadter and Yang. 2011, Hoffmann, *et al*. 2015). Congenital deficiencies in the complement or other parts of the immune system have been associated with risk of pneumococcal infections and single nucleotide polymorphisms (SNPs) in *MBL* or *TLR* genes may alter the risk of respiratory tract infections (Koch, *et al*. 2001, Mittal, *et al*. 2014).

2.2.1 Mannose-binding lectin

Mannose-binding lectin (MBL) is a pattern recognition molecule of the collectin family, which also includes surfactant proteins A and D (Ohtani, *et al*. 2012). MBL is able to recognize viruses, bacteria, yeasts, fungi and protozoa and initiate complement lectin

pathway and phagocytosis of pathogens (Daha. 2010, Takahashi, *et al*. 2006, Schweinle, *et al*. 1989). MBL is especially important in the first line defense between 6 and 18 months of age, when the adaptive immunity has not yet been developed. Production of this protein starts already in the first trimester of pregnancy. MBL is produced in the liver, and it presents as free PRR-protein in the plasma. It is able to recognize carbohydrates, like mannose and N-acetylglucosamine, which pathogens have on their surface. By binding to these carbohydrates, it activates the complement system and leads to killing of the pathogenic microbes (Kuhlman, *et al*. 1989, Takahashi, *et al*. 2006).

MBL is able to recognize different pathogenic bacteria including *S. aureus, S. pneumoniae, H. influenzae*, *Mycoplasma pneumoniae*, *Mycobacterium tuberculosis, Mycobacterium avium, Legionella pneumophila, Nocardia farcinica* and *Neisseria meningitidis* (Eisen. 2010). In addition to respiratory bacteria, MBL is able to protect against influenza viruses, and genetic polymorphisms of MBL have been related to more severe influenza virus infections (Levy, *et al*. 2018).

In humans, the MBL protein is encoded by the *MBL2* gene. Three different polymorphisms have been found in the *MBL2* gene at codons 52, 54 and 57, which encode variant alleles D, B and C. Variant-type alleles are commonly called O and the wild type is A. Thus, O/O refers to either homozygous variant at one of the codons or a combination of heterozygous variants. Polymorphisms in the *MBL2* gene cause reduction of biological activity and serum concentration of MBL protein (Lipscombe, *et al*. 1995).

Those O/O individuals with a homozygous variant at one of the three codons, or combined heterozygous variants, have MBL plasma concentrations less than 1% of normal levels and have an increased risk of serious bacterial infections. Subjects with one heterozygote variant have an MBL concentration about 10% of normal, but there is considerable variation in the serum MBL levels between individuals (Summerfield. 2003). Prevalence of MBL deficiency due to the mutations of *MBL2* in the Finnish population has been estimated to be 5% for O/O and 30% for heterozygotes (Aittoniemi, *et al*. 2008, Rantala, *et al*. 2008, Huttunen, *et al*. 2008). Clinical studies have linked heterozygous MBL polymorphisms and low MBL levels to susceptibility to viral and bacterial respiratory infections and AOM in children (Koch, *et al*. 2001, Summerfield, *et al*. 1997, Turner. 2003, Wiertsema, *et al*. 2006, Cedzynski, *et al*. 2004, Levy, *et al*. 2018, Toivonen, *et al*. 2017).

2.2.2 Toll-like receptors

TLRs are glycoproteins that recognize microbial structures (PAMPs) and lead to induction of interferons and cytokines, which take part in clearance of pathogens. The *Toll* gene that encodes Toll receptors was originally discovered by Christiane Nüsslein-

Volhard and colleagues in fruit fly *Drosophila melanogaster* in 1985 (Anderson, *et al*. 1985). The importance of Toll as a part of innate immunity was discovered in 1996 and one year later, the first TLR was identified in mammals (Medzhitov, *et al*. 1997, Lemaitre, *et al*. 1996). TLRs can be located either on the outer membrane of cell (TLRs 1, 2, 4, 5, 6, 10) or on the surface of endosomes (TLRs 3, 7, 8, 9) (Akira, *et al*. 2006, Akira. 2006). Human TLRs are numbered from 1 to 10. SNPs in *TLR* genes may alter the host's susceptibility to infections (Siebert, *et al*. 2018).

TLR1, TLR2 and TLR6 are able to recognize peptidoglycan and lipoproteins on mycobacteria and gram-positive bacteria. The *TLR2* variant (G/A) is associated with an increased risk of recurrent AOM (Toivonen, *et al*. 2017). Polymorphisms of the *TLR3* gene L412 have been associated with bronchiolitis during early infancy (Nuolivirta, *et al*. 2012). TLR4 is able to detect lipopolysaccharide from the outer membrane of gram-negative bacteria, and polymorphism of TLR4 is associated with early colonization by *M. catarrhalis* in infants and with gram-negative sepsis (Grissell, *et al*. 2007, Vuononvirta, *et al*. 2011). TLR5 is specific for bacterial flagellin on gram-negative bacteria. TLR7 and 8 recognize single-stranded RNA, and TLR9 recognizes CpG DNA of viruses and bacteria (Medzhitov. 2001) (Figure 2).

2.2.3 Interferons

The interferons (IFNs) are secreted cytokines that have antiviral effects (Richard, *et al*. 2008). Interferons are divided into three groups based on their amino acid sequence. The type I interferon system plays a major role in antiviral defense, while type II interferon is mainly secreted by T cells, natural killer (NK) cells and macrophages (Le Page, *et al*. 2000). Infected cells start to synthesize and secrete type I interferons in response to viral infection and circulating IFNs, and proteins that are induced by IFNs, for example, myxovirus resistance protein A (MxA), cause an antiviral state within the cells to prevent further viral growth. However, many viruses have evolved to produce IFN-antagonistic proteins, which can partly counteract the IFN effects. Viral interference protects an infected cell from a new infection, when infection by one virus makes the cell resistant to other viruses, largely due to the actions of the interferon system.

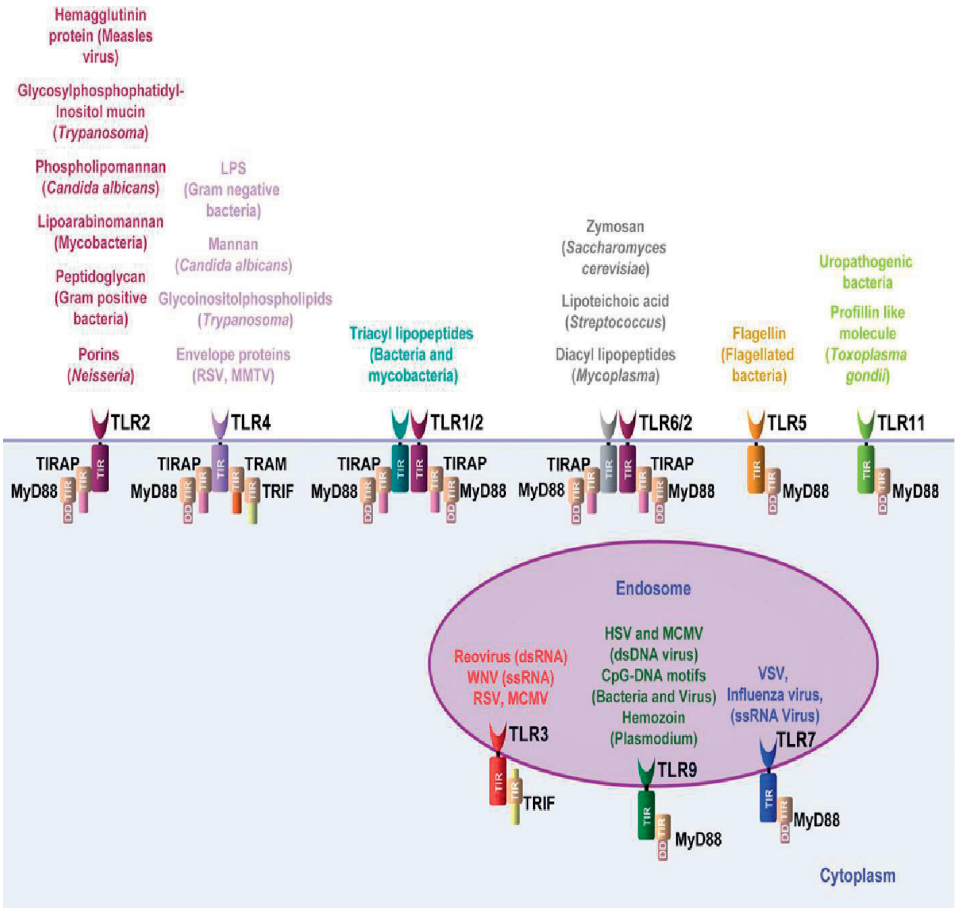


Figure 3. PAMPs (pathogen-associated molecular patterns) recognized by TLRs (Toll-like receptors) and their adaptors.
Plasma-membrane-localized TLRs (TLR2, TLR4, TLR5, alone and TLR2 in association with TLR1 or TLR6) and endosomal localized TLRs (TLR3, TLR7 and TLR9) recognize the indicated ligands. TLR2 is specific for peptidoglycan (bacterial) and lipoproteins, TLR3 recognizes double-stranded RNA, TLR4 is specific for lipopolysaccharide (LPS) of gram negative bacteria, TLR5 is specific for bacterial flagellin and TLR9 is specific for CpG-DNA. RSV, respiratory syncytial virus; MMTV, mouse mammary tumor virus; WNV, West Nile virus; HSV, herpes simplex virus; MCMV, murine cytomegalo virus; VSV, vesicular stomatitis virus. Reprinted from (Kumar, *et al.* 2009). Used with the permission of Biochemical Society, Portland Press.

2.3 Bacterial colonization of the respiratory tract and development of disease

The nasopharynx is colonized with commensal and pathogenic bacterial species soon after birth (Kwambana, *et al*. 2011). Carriage of bacteria is usually asymptomatic but mild symptoms like rhinorrhea may occur (Bogaert, *et al*. 2004, Brogden, *et al*. 2005). Colonization with bacteria is needed for development of bacterial disease. The three main bacterial pathogens causing respiratory tract infections in children are *S. pneumoniae, M. catarrhalis* and *H. influenzae*. The polymicrobial colonization with these bacteria is a common finding during AOM and pneumonia (Del Beccaro, *et al*. 1992, Kilpi, *et al*. 2001, Ruohola, *et al*. 2006).

The rates of bacterial colonization vary depending on age. Colonization with pneumococcus is most common in small children and starts to decrease by the age of 3 to 5 years (Murphy, *et al*. 2009). In a recent Finnish vaccine trial, the carriage rates with these pathogens were 16% for *S. pneumoniae*, 22% for *M. catarrhalis* and 3% for *H. influenzae* at the age of 3 months and increased up to 40%, 33% and 5%, respectively, by the age of 12 months (Vesikari, *et al*. 2016). By the age of 2 years, up to 87% of children have been reported to be colonized at least once with pneumococcus, 80% with *M. catarrhalis* and 44% with *H. influenzae* (Faden, *et al*. 1997, Faden, *et al*. 1995, Syrjänen, *et al*. 2001). In contrast, the rate of *S. aureus* colonization is highest during the first two months of life and decreases significantly by the age of 1 year. The co-colonization by these pathogens (*S. pneumoniae, M. catarrhalis* and *H. influenzae)* has been reported to occur in 18% of healthy children (Xu, *et al*. 2012).

In different studies, the reported rates of bacterial acquisition and carriage are largely variable. In addition to the age, viral infections, season, socioeconomic status, vaccinations, medications, other colonizing bacteria and characters of the host immune system affect the rate of colonization (Garcia-Rodriguez and Fresnadillo Martinez. 2002). The presence of siblings and daycare attendance has been associated with increased rates of colonization. As the colonization rates are highest in small children, they are an important reservoir for bacterial transmission in families and other settings with close contacts among individuals (Bosch, *et al*. 2013).

During colonization, bacteria interact in the nasopharynx maintaining the balance among different bacterial species. Both antagonistic and synergistic interactions among bacterial species have been described. Positive associations between *S. pneumoniae*, *H. influenzae* and *M. catarrhalis* are well-documented as well as negative associations with *S. aureus* and the three most common colonizing bacteria in children, *S. pneumoniae. H. influenzae* and *M. catarrhalis* (Regev-Yochay, *et al*. 2004, Garcia-Rodriguez and Fresnadillo Martinez. 2002).

In the majority of individuals, the nasopharyngeal colonization by pathogenic bacteria does not lead to development of disease. The epithelial surface with mucus and ciliated cells prevents bacterial invasion to surrounding tissues and inhibits spreading of bacteria into the lower respiratory tract or blood. However, in different studies, it has been shown that preceding carriage is related to development of bacterial disease. Especially newly acquired pneumococcal serotypes increase the risk for development of AOM, the most common disease caused by pneumococcus (Syrjanen, *et al*. 2005). The incidence of invasive pneumococcal disease, upper respiratory tract infections and AOM are highest at the ages of 6-18 months when the pneumococcal colonization rates are high (Harrisn, *et al*. 1999). Nasopharygeal colonization with bacteria during RTIs may also have effect on the course of disease. The symptoms of RTIs have been reported to be longer-lasting and more severe in children colonized with bacteria (Brealey, *et al*. 2018).

2.3.1 Impact of viral infection on bacterial colonization and disease

Viral infection plays an important role in bacterial acquisition and in the development of bacterial diseases (Nokso-Koivisto, *et al*. 2015). The relationship between influenza virus and bacterial pneumonia has been well documented during influenza pandemics (Dingle and Abernethy. 1948, Schwarzmann, *et al*. 1971, Kim, *et al*. 1996, McCullers. 2006, Finland, *et al*. 1945, Petersdorf, *et al.* 1959). The first studies focusing on viral bacterial interactions during RTIs were conducted in the late 1920s after the Spanish flu epidemic (Noble W.C., *et al*. 1928, Webster and Clow. 1932). Ecological studies established the synergistic interactions between influenza virus and *S. aureus* or *S. pneumoniae,* and high mortality was detected to be caused by pneumococcal diseases 1 week after influenza infection (Schwarzmann, *et al*. 1971, Brundage and Shanks. 2008). More recently, seasonal associations have been demonstrated between diseases caused by different respiratory viruses and bacteria. Invasive pneumococcal infections in children are more common during autumn and spring months, when the circulation of rhinovirus in the population is at its highest (Peltola, *et al*. 2011, Dowell, *et al*. 2003).

The impact of viral infection on bacterial attachment, density and clearance may occur during the symptomatic infection but may be seen up to a week after symptoms of RTI have ended (Stark *et al*. 2016). In addition to increased acquisition rates, viral infection also affects the colonization density of existing bacteria (Thors *et al.* 2018). Nasopharyngeal colonization rates with different bacteria are higher during viral respiratory tract infection compared to the healthy state, and even higher at the onset of infection (Syrjanen, *et al*. 2001). During the healthy state, bacterial colonization is usually asymptomatic. When viral RTI occurs, the inflammatory response because of viral infection causes inflammation in the nasopharynx and increases the bacterial growth and may disturb bacterial clearance (Nokso-Koivisto, *et al*. 2015). By increasing the rate of

bacterial colonization and density, viral infection may play a role in increased intrathoracic dissemination and development of pneumonia (Diavatopoulos, *et al.* 2010, Wolter, *et al*. 2014). Viral infection also plays an important role in the development of bacterial AOM. The Eustachian tube normally drains secretions from the middle ear to the nasopharynx. During a viral respiratory tract infection, the inflammation process with edema obstructs the Eustachian tube and leads to impaired ventilation and negative middle ear pressure that may facilitate the entrance of pathogens into the middle ear (Winther, *et al*. 2006, Danishyar, Ashurst, 2018).

2.4 Viral and bacterial co-infections

Co-infections with viruses and bacteria are common (Heikkinen, *et al*. 1991, Brealey, *et al*. 2018, Brealey, *et al*. 2015, Gwaltney, *et al*. 1975, Jacoby, *et al*. 2007, Nichol and Cherry. 1967). In over 90% of upper respiratory bacterial infections, a concurrent viral infection can be documented (Marom, *et al.* 2012). During the 2009 influenza A/H1N1 pandemic, bacterial pneumonia was detected in 4-33% of hospitalized patients (Cilloniz, *et al*. 2012, Randolph, *et al*. 2011). In children with community-acquired pneumonia, both viruses and bacteria have been detected in 66% of sputum samples collected from children, rhinovirus – *S. pneumoniae* being the most common combination (16%) (Honkinen, *et al*. 2012).

Acute otitis media often develops within days after onset of RTI (Heikkinen and Chonmaitree. 2003). In children under 2 years of age, AOM has been detected during 40 to 50% of RTI episodes caused by rhinovirus and viruses (Blomqvist, *et al*. 2002, Toivonen, *et al*. 2016b). Co-detection with virus and bacteria has been found in 66% of collected middle ear fluid samples during AOM (Ruohola, *et al*. 2006).

The implications of viral-bacterial co-infections are partly unclear. In several studies, it has been shown that co-infections are more severe than single infections and that symptoms may last longer (Cebey-Lopez, *et al*. 2016). Secondary bacterial pneumonia during influenza is more severe with a higher mortality rate compared to primary influenza pneumonia (Louria, *et al.* 1959, Bisno, *et al.* 1971). In AOM, the co-infection by viruses and bacteria carries a higher risk of persistent infection compared to bacterial AOM, and viral infection has been associated with antibiotic treatment failure for AOM. Children with community-acquired pneumonia became more slowly afebrile during viral bacterial co-infection compared to sole viral or bacterial infection (Chonmaitree, *et al*. 1992, Juven, *et al*. 2004). Co-infection or co-colonization with virus and bacteria during the first wheezing episode during infancy has been related to a higher risk of relapses of wheezing compared to non-colonized individuals (Jartti, *et al*. 2011).

2.5 Mechanisms of viral-bacterial interactions

The interactions between pathogens and between pathogens and the host are diverse and occur by several different mechanisms (Hament, *et al*. 1999). Viruses and bacteria may have effects on the existence and functions of other microbes by damaging the epithelium, decreasing ciliary function, altering mucus viscosity, affecting immunologic signaling function or by up-regulating expression of the proteins that bacteria can attach to (Bosch, *et al*. 2013, Pittet, *et al*. 2010, Ishizuka, *et al*. 2003, McCullers, *et al*. 2010, Peltola, *et al*. 2005). These different mechanisms can be classified into direct and indirect interactions (Almand, *et al*. 2017) (Table 1). Viruses usually benefit from bacteria through direct interactions. Bacteria are able to stabilize viral capsids and stimulate viral infection with entzymes they synthesize. It has been shown that *S. aureus* produces proteases that influenza virus needs for proteolytic cleavage of hemagglutinin to become infectious and able to enter into cells (Tashiro, *et al*. 1987, Scheiblauer, *et al*. 1992, Bottcher-Friebertshauser, *et al*. 2013, Almand, *et al*. 2017).

Bacteria usually benefit from viral infection through indirect mechanisms. Viral infection may cause damage to the host cell by making the cells more vulnerable to bacterial colonization. Indirect mechanisms are often supported by one or more of the following mechanisms: viruses may increase the concentrations of receptors where bacteria can attach to, damage the epithelium, displace the commensal bacteria and suppress the immune system (Almand, *et al*. 2017). Increased temperature during viral infection because of inflammation may change the virulence of bacteria making them more pathogenic (Loh, *et al*. 2013). However, even though bacteria usually benefit from viral infection through indirect mechanisms, it has been shown in animal and in vitro studies that RSV may increase pneumococcal virulence gene expression and bacterial adherence to ciliated epithelial cells by binding to pneumococci (Smith, *et al*. 2014).

2.5.1 Modification of the host epithelium

The epithelial layer of the respiratory tract acts as a defense barrier against microbes. Viruses can predispose the host to bacterial adherence and colonization by affecting the epithelium (Fainstein, *et al*. 1980). Influenza A and B viruses are able to destroy the epithelium and expose elements like fibrinogen to which bacteria can bind (McCullers and Rehg. 2002, McCullers. 2014). Influenza viruses produce neuraminidases that may affect the host cells by cleaving sialic acid residues and in that way expose bacterial receptors on the epithelium of respiratory tract (Peltola, *et al*. 2005). Viral infection caused by influenza viruses, RSV, rhinovirus or parainfluenzavirus may also activate the local inflammatory factors and enhance the expression of epithelial receptors, like the platelet activating factor receptor (PAFr), that pneumococcus and *H. influ*enzae use for

binding (Avadhanula, *et al*. 2006, Ishizuka, *et al*. 2003, van der Sluijs, *et al*. 2006, McCullers. 2014, Tong, *et al*. 1999). In addition to increased adherence after viral or bacterial infection, viruses may also impair the clearance of bacteria by affecting ciliary function. Ciliary function of the epithelium may be impaired by different viruses like influenza virus, RSV and adenovirus leading to decreased clearance of bacteria such as *S. pneumoniae* and *H. influenzae* (Pittet, *et al.* 2010). However, as viral infection may increase the rate of bacterial infection, also bacterial colonization may increase susceptibility to viral infections by stimulating expression of viral host cell receptors like ICAM-1 and in that way, increase the attachment of viruses to the epithelial cells (Nguyen, *et al*. 2010, Sajjan, *et al*. 2006).

2.5.2 Impacts on the immune system

Viral or bacterial infection can alter functions of the immune system including cellular immunity, macrophage and neutrophil function, and thus increase the risk of simultaneous co-infections or secondary viral or bacterial infection (Jakab and Green. 1976, Kleinerman, *et al*. 1975, Nakamura, *et al*. 2011). Alveolar macrophages are located in lung alveoli. Macrophages are specialized cells that use TLRs to detect, phagocyte and destruct bacteria (Sibille and Reynolds. 1990, Rubins. 2003) as well as stimulate the production of chemokines and cytokines by macrophages, which activate neutrophil cells (Hippenstiel, *et al*. 2006, Werner and Steele. 2014). Dysfunction of alveolar macrophages and pattern-associated molecular patterns (PAMPs), like TLRs, leads to impaired recruitment of neutrophils. TLR activity has been shown to be downregulated after RSV and influenza virus infection in animal studies (Didierlaurent, *et al*. 2008). Rhinovirus has been shown to impair the macrophage antibacterial response to bacterial infection because of an impaired cytokine response (Oliver, *et al*. 2008). Also influenza virus and RSV are able to suppress macrophage function (Astry and Jakab. 1984, Raza, *et al*. 2000). Because neutrophils are important in defense towards *S. pneumoniae*, the impaired TLR function may result in development of pneumococcal diseases (Didierlaurent, *et al*. 2008).

2.5.3 Negative interactions

Antagonistic effects between microbes do occur as well. Different bacteria may inhibit other bacteria and also protect the host from viral infection (Ichinohe, *et al*. 2011). Bacteria can compete with each other by producing hydrogen peroxidase (H2O2), which is lethal to different bacteria. *S. pneumoniae* is more resistant to this product compared to other bacteria, and it is able to kill *S. aureus* and *H. influenzae* by remote-control bacteriophage induction and by producing H2O2 (Selva, *et al*. 2009, Regev-Yochay, *et al*. 2006).

In addition to excretion of bactericidal products, *S. pneumoniae* is able to inhibit attachment of *H. influenzae* to epithelial cells by removing sialic acid residues with neuraminidase (Shakhnovich, *et al*. 2002). These two bacteria also use the same receptor, PAFr, for adherence to the epithelium, which may lead to competition between these pathogens (Cundell, *et al*. 1995, Swords, *et al*. 2000).

2.6 Viral–viral interaction

Co-infections with different viruses are common. Viral co-infection, defined as simultaneous detection of two or more respiratory viruses in a sample collected from the respiratory tract, has been documented in 46% of children with RTI (Diaz, *et al*. 2015, Yoshida, *et al*. 2013, Chonmaitree, *et al*. 2015, Aberle, *et al*. 2005, Franz, *et al*. 2010, Martin, *et al*. 2013). In children with community-acquired pneumonia, viral co-infections with two viruses have been detected in 22% of samples and with three or more viruses in 8% (Honkinen, *et al*. 2012). Some viruses seem to increase the detection rate of another virus but also inhibiting interactions have been reported (Zheng, *et al*. 2017, Greer, *et al*. 2009, DaPalma, *et al*. 2010, van den Bergh, *et al*. 2012a). The presence of rhinovirus has a negative association with influenza A virus and adenovirus infections suggesting interference between these viruses (Casalegno, *et al*. 2010). Of environmental factors, overcrowding and smoking as well as young age have been regarded as risk factors for co-infections (Jackson, *et al*. 2013, Drews, *et al*. 1997). It is not clear why young children are more prone to co-infections compared to adults. It has been suggested that an immature immune system could predispose to co-infections or that children may have prolonged shedding of respiratory viruses (Drews, *et al*. 1997). The clinical significances of viral co-infections are controversial, and the presence of virus does not always indicate the causation of disease and clinical symptoms (Rhedin, *et al*. 2015). Some studies have revealed co-infections to be more severe, but in other studies, no such association was seen (Diaz, *et al*. 2015, Paranhos-Baccala, *et al*. 2008, Rhedin, *et al*. 2015). Increased severity in infections caused by specific combinations of viruses has been described. Bronchiolitis caused by RSV and rhinovirus together have been reported to be more severe than bronchiolitis caused by single RSV or rhinovirus (Paranhos-Baccala, *et al*. 2008).

Table 1. Viral-bacterial interactions and their significance. Modified from Almand *et al*. 2017.

Virus	Bacteria	Significance	Reference
Direct Interaction			
Poliovirus	N-acetyl glucosamine containing polysaccharides (lipopolysaccharides and peptidoglycan)	Enhanced cell association and viral replication; increased capsid stability and transmission	(Kuss, *et al*. 2011, Robinson, *et al*. 2014)
Influenza virus	*S. aureus*	Protease cleaves the hemagglutinin (HA) into HA1 and HA2 making the particles infectious	(Tashiro, *et al*. 1987, Scheiblauer, *et al*. 1992, Bottcher-Friebertshauser, *et al*. 2013)
Human immunodeficiency virus HIV	*Mycobacterium tuberculosis*	Increases HIV long terminal repeat-driven transcription and HIV production	(Goletti, *et al*. 1996, Pawlowski, *et al*. 2012)
Indirect Interaction			
Influenza virus	*S. pneumoniae, S. aureus, H. influenzae*, respiratory commensals	Viral neuraminidase cleaves epithelial cell sialic acid exposing bacterial receptors; damages epithelial cells	(Bosch, *et al*. 2013, Tashiro, *et al*. 1987, McCullers. 2006)
Measles virus	*Mycobacterium tuberculosis, S. aureus, Listeria monocytogenes*	Promotes a generalized state of immunosuppression leading to bacterial co infection	(Hahm, *et al*. 2004, Servet-Delprat, *et al*. 2000)
Respiratory syncytial virus	*S. pneumoniae, Pseudomonas aeruginosa, H. influenzae*	Increased bacterial invasiveness, increased host cell adhesion molecules	(Talbot, *et al*. 2005, Van Ewijk, *et al*. 2007, Avadhanula, *et al*. 2006)
Parainfluenza virus	Nasopharyngeal bacteria	Increased bacterial binding to the lower respiratory tract	(Korppi, *et al*. 1990, Ruohola, *et al*. 2013)
Rhinovirus	*S. pneumoniae, S. aureus, H. influenzae*	Increases host cell adhesion molecules	(Wang, *et al*. 2009a)
Adenovirus	*S. pneumoniae*	Increases host cell adhesion molecules	(Hakansson, *et al*. 1994, Murrah, *et al*. 2015)

2.7 Immunization and viral-bacterial interaction

It has been shown in several studies that by diminishing viral infections with medications and vaccines, the rate of bacterial diseases can be reduced. Despite the great disease burden on children, for most of the respiratory viruses, there are no prevention or treatment possibilities by specific medications or vaccines. Good hygiene is an important factor that can reduce the rate of viral infections and in that way also viral-bacterial co-infections.

The pneumococcal conjugate vaccines decrease the rate of pneumococcal diseases like AOM, pneumonia and invasive diseases caused by serotypes included in the vaccine (Palmu, *et al*. 2017b, Palmu, *et al*. 2013, Kilpi, *et al*. 2018, Palmu, *et al*. 2015). Effects of conjugated vaccines on colonization have led to indirect protection also in unvaccinated individuals by leading to reduced transmission and colonization rates of vaccine serotype (Lexau, *et al*. 2005). However, reduction of colonization by vaccine serotypes leads to increase of colonization by a non-vaccine-serotype, which is called a replacement effect (Pelton. 2004). This phenomenon has also been seen in increased incidence of non-vaccine-type diseases, although the replacement effect has been smaller in diseases than in colonization. The pneumococcal conjugate vaccine affects the viral-pneumococcal interaction by affecting the density of pneumococci and decreasing the prevalence of vaccine type serotype colonization. Pneumococcal vaccine reduces the severity of viral-bacterial co-infections, which may be explained by diminished microbiological interaction and reduced inflammatory response (Smith, *et al*. 2014, Cebey-Lopez, *et al*. 2016).

Interestingly, it has been shown that also the rate of all RTIs and the rate of virus-associated pneumonias has decreased in children vaccinated with pneumococcal vaccine (Madhi, *et al*. 2004). Furthermore, influenza vaccines prevent influenza-associated AOM and the severity of community-acquired pneumonias, both of which can be viral-bacterial infections (Heikkinen, *et al*. 1991, Fedson, *et al*. 1993). In one study, the nasopharyngeal density of pneumococcus was shown to increase after intranasal vaccination with the live attenuated influenza vaccine (LAIV) in mice, but clinical trials in children have not demonstrated any increase in bacterial respiratory tract disease following vaccination with LAIV (Mina *et al*. 2014, Block, *et al*. 2011, Belshe, *et al*. 2007).

3 AIMS OF THE STUDY

The aims of the thesis were to investigate interactions among the most common pathogens causing respiratory tract illnesses in children, to understand better the impact of genetic variations in the host innate immune system on the microbiological interaction and to clarify the effect of vaccination on viral-bacterial interactions.

The specific aims were:

1. To evaluate the impact of polymorphisms of the innate immune genes on early bacterial colonization in the nasopharynx during rhinovirus infection (I).

2. To assess the effect of respiratory syncytial virus on the presence of rhinovirus during respiratory tract infection (II).

3. To evaluate how rhinovirus infection affects the acquisition and transmission of *S. pneumoniae* in families with children (III).

4. To assess the effect of ten-valent pneumococcal *Haemophilus influenzae* protein D conjugate vaccine (PHiD-CV10) on respiratory tract infections that are mainly caused by viruses (IV).

4 MATERIALS AND METHODS

This thesis consists of four publications. A more detailed description of the methods used is presented in original publications I-IV.

4.1 Participants and study design

4.1.1 The STEPS study

A total of 1827 children were followed as a part of the prospective cohort study STEPS during the years 2008-2012 from birth to two years of age for respiratory tract infections (Lagstrom*, et al*. 2013). Recruitment was performed during the first trimester of pregnancy or during the first days after the birth from a cohort of children born in the Hospital District of Southwest Finland between January 2008 and April 2010. No other selection criteria than knowledge of Finnish or Swedish language by the parent(s) were applied. Of these children, 923 were recruited in a more intensive follow-up for RTIs. Parents reported all respiratory and other symptoms, physician visits, medications and diagnoses of the child in a detailed diary. Children were invited for prescheduled visits to the study clinic at the age of 2, 13 and 24 months. If there was a need for evaluation by a physician during the RTI, then the study doctor examined the child, and clinical findings were documented in a detailed and structured form. Microbiological samples were collected for viral diagnostics during the RTIs from both nostrils at a depth of 2-3 cm with flocked nylon swabs (Coban, Brescia, Italy) either by parents at home or by a physician at the study clinic, if an evaluation by physician was needed. Parents were trained by the study personnel at the first pre-scheduled study clinic visit to collect nasal swab samples. Samples collected at home were sent by mail to the study clinic. We have previously shown that samples collected at home and sent by standard mail to the laboratory for analysis are stable at least four days at room temperature, and there is no loss of virus detectability (Peltola*, et al*. 2008, Waris*, et al*. 2013). At the age of 2 months, blood samples for genomic DNA isolation and nasopharyngeal specimens for detection of viruses were collected, and nasopharygeal samples for culture of bacteria were collected from the children who visited the study clinic on Mondays to Tuesdays during the period between August 2008 and June 2010. Data of hospitalizations were collected from the electronic registry of Hospital District of Southwest Finland.

4.1.2 FinIP study

Finnish Invasive Pneumococcal disease (FinIP) was a cluster-randomized, double-blind field vaccine trial designed to assess the effectiveness of the 10-valent pneumococcal *H. influenzae* protein D conjugate vaccine (PHid-CV10) against invasive pneumococcal disease (Palmu*, et al*. 2013). The FinIP vaccine trial was conducted in Finnish healthcare centers during the years 2009-2012. Children at 6 weeks to 18 months of age were enrolled into this study by nurses in local well-baby clinics and healthcare centers in Finland during February 2009 through October 2010. The areas of participating study clinics were divided geographically into 78 clusters according to administrative structures of health care centers and the size of birth cohort. The southwest part of Finland (Turku area), where the STEPS study was conducted, was divided into eleven clusters. Children younger than 19 months were randomized to receive either PHiD-CV10 in 52 clusters or hepatitis B or A vaccine in 26 clusters as control. Hepatitis B vaccination was given to children under 12 months of age and hepatitis A vaccination for children over 12 months of age. Children received the first vaccination at different time points based on the age at the time of enrollment and were vaccinated according to the vaccine trial (infants under 7 months of age with either a 3+1 or a 2+1 vaccination schedule and children 7-18 months of age with catch-up schedules) (Palmu*, et al*. 2013).

4.2 Design of studies I-IV

Study I

The study population consisted of 337 infants, who participated in the STEPS study during the years 2008-2012. Nasal samples for bacterial and viral analyses and blood samples for the DNA isolation were collected during the first pre-scheduled visit at the age of 2 months from infants, who visited the study clinic on Mondays or Tuesdays during the period between August 2008 and June 2010. The association between rhinovirus infection and nasopharyngeal pneumococcal colonization was investigated between the infants with or without gene polymorphisms in MBL, TLR3 and TLR4.

Study II

The study population consisted of 923 children participating in the STEPS study. A total of 4810 nasal swab samples were collected during acute RTIs, and 2275 samples were obtained during the pre-scheduled visits. A total of 226 children with diagnosed RSV infection and 226 control children, whose samples were collected during prescheduled visits, were matched for age and for season of sample collection and included in the analyses. We compared the rates of rhinovirus infections in children with symptomatic RSV infection with children in their usual state of health.

Study III

The study population consisted of all children and parents of 29 families of a child in the STEPS study resulting in a total of 128 subjects. This study was conducted between July 2011 and March 2012. Parents collected nasal swab samples at home from all family members twice a week for three weeks, from the onset of symptoms of respiratory tract infection in any family member. Samples were sent to our study clinic for viral and bacterial analyses. Symptoms were reported in daily diaries. The impact of preceding or simultaneous rhinovirus infection on the acquisition and transmission of *S. pneumoniae* was analyzed in families with children. Serotyping and whole genome sequencing of part of the isolates were used for the analysis of clonality of transmitted pneumococcus between the family members.

Study IV

The study population consisted of 424 children, who participated in a cluster–randomized, double-blind Finnish Invasive Pneumococcal disease (FinIP) vaccine trial during the years 2009-2012 and were also followed in the STEPS study for respiratory tract infections from birth to two years of age. Children received the first PHiD-CV10 vaccine, or hepatitis A or B vaccine as control, at different time points based on the age at the time of enrollment (Palmu*, et al*. 2013). Data on respiratory tract infections were collected by symptom diaries, clinic visits and electronic registries. We compared the rate of all respiratory tract infection episodes between PHiD-CV10 vaccinated and control children.

4.3 Microbiological diagnostics and genetic analysis

4.3.1 Virology (I-IV)

Collected samples were stored at -80°C before analysis. The nasal swabs were eluted with phosphate buffered-saline, and nucleic acids were extracted from the specimens with NucliSense easyMag (BioMerieux, Boxtel, Netherlands) or MagnaPure 96 (Roche, Penzberg, *Germany*) automated extractor. Rhinoviruses, enteroviruses and RSV were detected by reverse transcription and triplex quantitative PCR assay with dual-label locked nucleic acid probes that were used to differentiate rhino- and enteroviruses (Osterback*, et al*. 2013, Toivonen*, et al*. 2015) (Studies I - IV). RNA copy number calibrations and a predetermined amplification efficiency was used to determinate the viral load for rhinovirus and RSV (Studies II and III). Gene sequencing at VP4/2 region was used for typing of rhinoviruses (Study III). Specimens that were collected during influenza seasons were analyzed with RT-PCR for influenza A and B (Jokela*, et al*. 2015).

Laboratory-developed antigen detection tests were performed for samples collected in January 2009 or later for influenza A and B viruses, parainfluenza type 1, 2 and 3 viruses, adenovirus, human metapneumovirus and RSV (Yliharsila, *et al*. 2015).

4.3.2 Bacteriology (I, III)

In Study I, the nasopharyngeal samples were taken by using flocked swabs (Coban, Brescia, Italy). Samples were transported to the laboratory within 3 hours after sampling. The swabs were immersed in 1 ml of 0.9% NaCl and homogenized by vortexing. Four different culture plates were used: a *S. pneumoniae* selective plate, a *H. influenzae* selective plate, a heated blood agar and a blood agar containing 5% sheep blood (Vuononvirta, *et al*. 2011).

In Study III, the nasal swab samples were first tested for pneumococcal antigen by a fully automated, multianalyte antigen detection test system mariPOC (ArcDia International Ltd, Turku, Finland), which is based on two-photon excited fluorescence detection (Gunell, *et al*. 2016, Hanninen, *et al*. 2000, Koskinen, *et al*. 2007). After antigen testing, the samples were stored in mariPOC test buffer (RTI) at -70ºC. Bacterial culture, identification and serotyping were performed for the samples from the subjects that had at least one antigen positive sample during the follow-up (Siira, *et al*. 2012, Terasjarvi, *et al*. 2016, Vuononvirta, *et al*. 2011). Identification and serotyping were performed by using multiplex PCR and Quellung reaction was used to confirm the results, if needed. We have previously shown that nasal swab samples collected at home, sent to the study clinic and stored in RTI buffer are suitable for bacterial culture (Terasjarvi, *et al*. 2016).

4.3.3 Whole genome sequencing (III)

Altogether 16 pneumococcal isolates were selected for whole genome sequencing of bacterial DNA. These 16 samples were selected from three families in which there were at least two family members colonized with *S. pneumoniae* at least once during the follow-up. DNA was extracted from the cultured bacteria according to the manufacturer's instructions with MagAttract HMW DNA Kit (Qiagen, Hilden, Germany). DNA samples were sent to Novogene Bioinformatics Technology Co., Beijing, China for whole genome sequencing by using HiSeq TM2000 (Illumina Inc., San Diego, CA). SAMTOOLS was used for detection of the individual SNPs. A phylogenetic tree was constructed based on SNPs of isolated strains.

4.3.4 Genetic polymorphism analysis (I)

DNA was first extracted from whole blood according to the manufacturer's instructions by QIAGEN QIAamp DNA Blood Min KIT 250 (Qiagen, Hilden, Germany). The gene polymorphisms in MBL structural gene at codons 52 [allele D, reference single nucleotide polymorphism (rs) 5030737], 54 (allele B, rs1800450), and 57 (allele C, rs1800451) and in TLR3 at Leu412Phe (rs3775291), and TLR4 at Asp299Gly (rs4986790) were detected by using pyrosequencing (PSQ™96MA Pyrosequencer, Biotage, Uppsala, Sweden) (Nuolivirta*, et al*. 2012, Vuononvirta*, et al*. 2011).

4.4 Definitions

4.4.1 Pneumococcal acquisition, colonization and transmission

The community acquisition of pneumococcus was defined as an event, when pneumococcal colonization was for the first time introduced into a family. Families in which pneumococci were already found on the first sampling date were excluded from the analyses. If there was a new detection of pneumococcus after two negative samples on at least two consecutive sampling days in each family member, the new community acquisition was recorded. Carriage of bacteria was defined as an ongoing colonization. Transmission was defined as the number of pneumococcal acquisition events that occur in the presence of a colonized family member. The rate of community acquisition and transmission was defined as a number of events divided by the total person time.

4.4.2 Episode of respiratory tract infection

An episode of RTI was defined as symptoms of rhinitis or cough with or without fever or as a diagnosis of an RTI by a physician. Symptoms were documented in the daily diary by parents. If there were more than one nasal sample collected during continuous symptoms, then the date of a nasal swab taken more than 14 days from the first one was considered as a start date of a new episode. The same definition was used for repeated diagnoses of bronchiolitis, AOM or pneumonia during continuous symptoms. Episodes were regarded as separate ones if there was at least one day without symptoms between the episodes.

4.5 Statistical analysis

Analyses were made by using SPSS version 16, 21.0 and 23.0 (IBM Corp., Armonk, NY), R 3.1.3 (R Foundation for Statistical Computing, Vienna Austria) and SAS version 9.1. *P* values < 0.05 were considered statistically significant.

Study I

Categorical variables were compared using a Chi-Square test or Fisher's exact test as appropriate. First, the associations between background variables and microbiological findings were analyzed by adjusted logistic regression. Then rhinovirus, gene polymorphisms and their interaction were included in adjusted logistic regression to analyze associations with bacterial colonization.

Study II

The rate of rhinovirus detection was first compared between cases and controls (children with RSV infections and matched controls). Then binary logistic regression analyses were performed to adjust for background variables that were not used for matching. A Chi-Square test was used to compare categorical variables, and the independent-samples t-test or Mann-Whitney U-test was used to compare continuous variables as appropriate.

Study III

Categorical variables were compared using a Chi-Square test and a Mann Whitney U test was used to compare continuous variables. Poisson regression analyses were used to estimate the rates of community acquisition and within-family transmission with 95% confidence intervals (CI). The rate of community acquisition and transmission was defined as a number of events divided by the total person time.

Study IV

Categorical analyses were compared by using a Chi-Square test and duration of infection episodes were compared by using Mann-Whitney U test between vaccinated and control children. The vaccine effectiveness (VE) was defined as: (1-relative rate) x 100. VE was estimated using negative binomial regression with log link and the follow-up time as the offset to account possible heterogeneity between children. We reported point estimates and 95% confidence intervals. Cluster identifier and the presence of siblings were included in the regression model.

4.6 Data management

The original, collected data was deidentified and transferred electronically to the Child and Youth Research Centre CYRI, University of Turku, Finland to ensure that the research community has long-term access to the data. Each participant has been given a code and the key connecting the code with identity is stored separately from the data in a secured server at the Child and Youth Research institute Centre CYRI, University of Turku, Finland.

4.7 Ethics

The STEPS study protocol and family study (III) were approved by the Ethics committee of the Hospital District of Southwest Finland. The FinIP vaccine trial was approved by ethical review boards and competent authorities and it was registered at ClinicalTrials.gov (number NCT00861380 and NCT00839254). Written informed consent was received from the parents of the participating children separately for the STEPS study, the FinIP study and the family study (III).

5 RESULTS

5.1 Characteristics of study population

Participant characteristics in four separate studies are shown in the original publications (Study I, Table 1, Study II, Table 1, Study III, Table E1, Study IV, Table 1).

Families of 923 children participated in an intensive follow-up of respiratory infections in the STEPS study. In these children, a total of 8847 episodes of RTI was documented with a total of 4728 nasal swab samples collected during symptomatic respiratory tract infections. A total of 2352 samples were collected during the pre-scheduled visits at the age of 2, 12 and 24 months (914, 771 and 667 samples, respectively). Virus was detected in 3709 (54%) of tested samples. Rhinovirus was detected in 58% of tested samples (Figure 4). Of the children in the follow-up, 47 % were female, and 46% had older siblings. More than half (58%) of families lived in an urban area, and 62% of mothers had a higher education (Table 2).

Table 2. Characteristics and clinical manifestations of 923 children participating in the follow-up for respiratory tract infections in the STEPS study.

Characteristic	Children (n = 923) Data are N (%) unless otherwise indicated
Female	435 (47)
Older siblings	376 (41)
Living in the urban area	544/895 (61)
<37 weeks of pregnancy	38/918 (4)
Maternal educational level high	574/892 (64)
Duration of the follow-up time, median (IQR) years	1.99 (1.4, 2.0)
Episodes of respiratory tract infection (total number)	8847
Samples collected during acute respiratory tract infection (total number)	4728
Samples collected at pre-scheduled visits (total number)	2352
AOM episodes (total number)	1419
Wheezing illnesses (total number)	281
Hospitalizations (total number)	85
Antibiotic treatments (total number)	1872

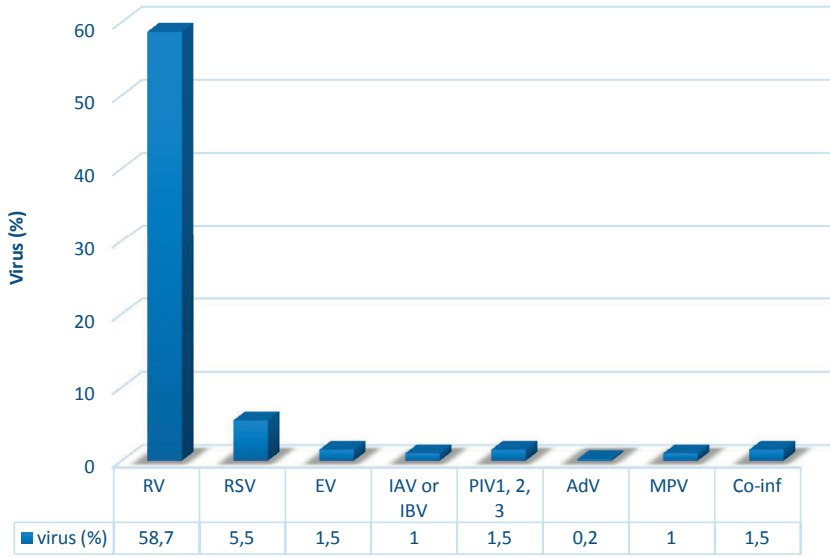


Figure 4. Proportions of detected viruses in 3340 of 4728 acute respiratory tract infections. The total number of tested samples for PIV 1, 2, 3, human metapneumovirus and adenovirus was 4308. AdV, adenovirus; EV, enterovirus; IAV or IBV, influenza A or B, respectively; MPV, metapneumovirus; PIV 1, 2 or 3, parainfluenzavirus 1, 2 or 3; RSV, respiratory syncytial virus; RV, rhinovirus; co-inf, co-infection with two or more viruses.

5.2 Innate immune system and viral-bacterial interaction (I)

5.2.1 Viral and bacterial findings at 2 months of age

Rhinovirus was detected in 61 of 337 (18%) infants at the age of 2 months (Table 3). Mild symptoms of RTI were documented on the day of sampling in 16% of children. Detection of rhinovirus was associated with symptoms of respiratory tract infection (43%), but 13% of rhinovirus infections were documented in asymptomatic children.

Bacterial colonization with *S. pneumoniae, M. catarrhalis, H. influenzae, S. aureus* or a combination with these bacteria was detected in 55% of infants. Co-detection of 2 or more bacterial species was found in 11% of samples with *S. pneumoniae* and *M. catarrhalis* being the most common combination (18 cases). *S. aureus* was the most frequent bacterium detected at 2 months of age (28%) followed by *M. catarrhalis* (25%), *S. pneumoniae* (14%) *and H. influenzae* (1%) (Table 3). Because of the small number of *H. influenzae* cases, these cases were excluded from the further analysis. Having one or more siblings was associated with *S. pneumoniae* and *M. catarrhalis* colonization but other background variables like breastfeeding,

sex, the use of antibiotics, socioeconomic status or mother's smoking habits did not associate with bacterial colonization. *S. pneumoniae* and *M. catarrhalis* were detected more often during symptoms of RTI than without symptoms. On the contrary, *S. aureus* was more common in asymptomatic infants.

Children with rhinovirus infection were more often colonized with *M. catarrhalis* and less often with *S. aureus* compared to rhinovirus-negative children. There was no significant association between rhinovirus infection and *S. pneumoniae* colonization. After adjustment for background variables, rhinovirus was not significantly associated with any of these bacteria.

5.2.2 Genotypes of mannose-binding lectin and Toll-like receptors 3 and 4

A variant type of MBL was detected in 32% (107) of 337 infants of whom 101 were heterozygotes, and six were homozygotes or compound-variant heterozygotes. A variant-type of TLR4 was detected in 18% of children (58 heterozygotes and two homozygotes), and variant TLR3 was detected in 51% (134) of children (113 heterozygotes and 21 homozygotes) (Table 3). Infants with wild- or variant-type of MBL, TLR3 or TLR4 did not differ with respect to background variables. The rate of rhinovirus infection did not differ between the infants with wild-type or variant-type MBL, TLR3 or TLR4.

Table 3. Rhinovirus infection, bacterial colonization and genetic variants in MBL and TLR3 and TLR4 in infants at age of 2 months (modified from Study I).

Variable		Children (n = 337)
Rhinovirus		
	Positive	61 (18)
	Negative	276 (82)
Bacterial colonization		
	S. pneumoniae	46 (14)
	M. catarrhalis	84 (25)
	H. influenzae	3 (1)
	S. aureus	96 (28)
MBL genotype		
	Wild type	230 (68)
	Variant type	107 (32)
TLR3 genotype		
	Wild type	128 (49)
	Variant type	134 (51)
TLR4 genotype		
	Wild type	277 (82)
	Variant type	60 (18)

Note: Data are number (%).

5.2.3 *Association between polymorphisms in MBL, TLR3 and TLR4 and rhinovirus-pneumococcal interaction*

MBL polymorphisms

The rates of *S. pneumoniae* colonization were higher during rhinovirus infection than without infection in infants with MBL polymorphisms (40% with rhinovirus vs. 10% without rhinovirus, $P = 0.003$) (Figure 5). In an adjusted logistic regression analysis, the association with *S. pneumoniae* was non-significant for rhinovirus, significant for the MBL variant ($P = 0.035$) and highly significant for the interaction between rhinovirus and the MBL variant ($P = 0.004$).

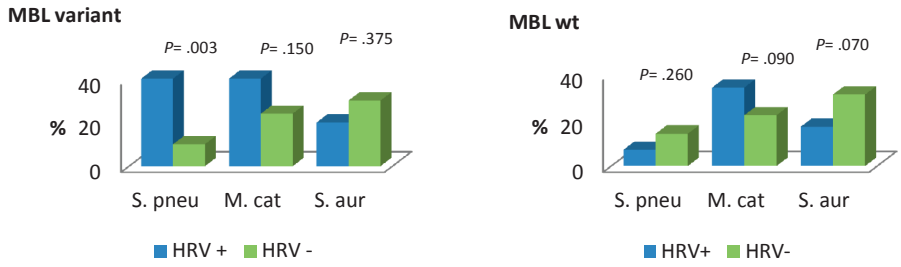


Figure 5. Bacterial colonization in infants positive or negative for rhinovirus, shown separately for infants with variant-type mannose-binding lectin (MBL) (n = 107) and wild-type MBL (n = 230). S. pneu, *Streptococcus pneumoniae*; M. cat, *Moraxella catarrhalis*; S. aur, *Staphylococcus aureus* (modified from Study I).

***TLR3* polymorphisms**

Infants with wild-type TLR3 were more often colonized with *M. catarrhalis* during rhinovirus infection than without rhinovirus infection ($P = 0.050$). The rate of *S. aureus* colonization was higher in children without rhinovirus than in those with rhinovirus infection ($P = 0.035$). In infants with variant-type TLR3, there was no difference in the bacterial colonization between infants with or without rhinovirus infection (Figure 6).

***TLR4* polymorphisms**

In infants with wild-type TLR4, the colonization rates with *M. catarrhalis* were higher during rhinovirus infection than without infection ($P = 0.014$). Colonization with *S. aureus* was significantly higher without rhinovirus infection than in those with infection ($P = 0.021$). In

infants with variant-type TLR4, there was no significant difference in the colonization rates with *S. pneumoniae, M. catarrhalis* or *S. aureus* between rhinovirus positive or negative infants (Figure 6).

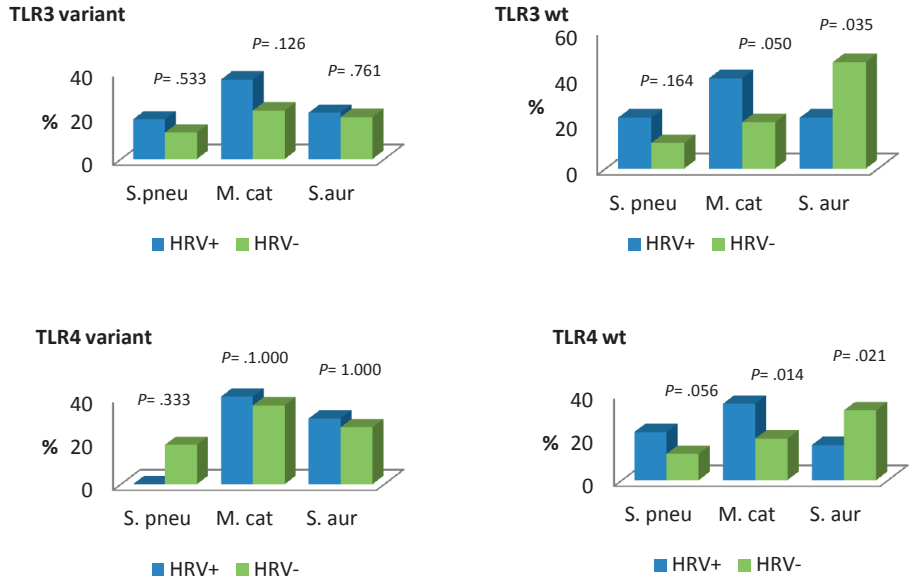


Figure 6. Bacterial colonization in infants positive or negative for rhinovirus, shown separately for infants with variant and wild type TLR3 and variant type and wild type TLR4. S. pneu, *Streptococcus pnemoniae*; M. cat, *Moraxella catarrhalis*; S. aur, *Staphylococcus aureus* (modified from Study I).

5.3 Viral interference in respiratory tract infections (II)

A total of 4810 nasal swab samples were obtained from children during RTIs during the years 2008-2010 from a total of 923 children. RSV was detected in 279 of these samples (5.8%). We compared the rate of rhinovirus infection between RSV-infected children and age- and season-matched control children, whose samples were collected at their usual states of health during the pre-scheduled visits at the age of 2, 12 and 24 months. From a total of 279 RSV-infected children, 226 were included into the final analyses. Rhinovirus infection was less common in children with RSV compared to control children. During the RSV infection, 18 (8.0%) children had a co-infection with rhinovirus, whereas 31 (14%) of the control children were infected with rhinovirus; $P = 0.002$ after adjustment for background variables (i.e., sex, number of siblings, socioeconomic status) (Table 4).

Table 4. Prevalence of rhinovirus (RV) in association with respiratory syncytial virus (RSV) and background variables (modified from Study II).

				Adjusted logistic regression		
	Total	RV neg.	RV pos.	OR	95% CI	p
	n = 452	no. (%)	no. (%)			
Sex						
Male	234	207 (88)	27 (12)	1.06	0.57-1.99	0.85
Female	218	196 (90)	22 (10)			
Siblings						
0	253	228 (90)	25 (10)	0.81	0.43-1.52	0.51
≥1	199	175 (88)	24 (12)			
Socio-economic status[1]						
Professional	329	297 (90)	32 (10)	0.78	0.39-1.56	0.48
Non-professional	99	86 (87)	13 (13)			
Group						
Case (RSV pos.)	226	208 (92)	18 (8.0)	0.46	0.24-0.90	0.02
Control	226	195 (86)	31 (14)			

[1] Information available from a total of 428 families.

5.3.1 Symptoms in co-infections

Duration of symptoms was longer in children with co-infection with RSV and rhinovirus compared to those with a single RSV infection (median 11 vs. 14 days; $P = 0.02$). The incidence of pneumonia, AOM or wheezing did not differ between these groups. Only one child with a single RSV infection needed hospitalization because of bronchiolitis.

Table 5. Severity of respiratory tract infection compared between single respiratory syncytial virus (RSV) infection and co-infection with RSV and rhinovirus (RV) (modified from Study II).

	Single RSV infection, n = 208	RSV/RV co-infection, n = 18	p
Duration of RTI symptoms, days; median (interquartile range)[1]	11 (8-14)	14 (12-21)	0.02
Wheezing, no. (%)	25 (12)	3 (17)	0.57
Acute otitis media, no. (%)	70 (34)	5 (28)	0.61
Pneumonia, no. (%)	3 (1.4)	0 (0)	0.61

[1] Data available from a total of 174 children (164 with single RSV and 10 with RSV-RV co-infection).

5.3.2 Viral copy number in single and co-infections

The copy number of RSV was higher in single infections than in co-infections with rhinovirus [7.3 (6.2-8.0) vs. 6.1 (4.8-7.8) log of copies / swab; $P = 0.04$)]. In co-infections, the copy number of RSV was higher than the copy number of rhinovirus [6.1 (4.8-7.8) vs. 4.6 (3.4-5.5) log of copies /swab; $P= 0.03$]. In the control group, the copy number of rhinovirus was higher in children with symptoms of RTI compared to asymptomatic children [5.5 (4.2-6.3) vs. 4.1 (2.7-5.2) log of copies / swab; $P = 0.01$]. The viral load of rhinovirus did not differ between single rhinovirus infection compared to rhinovirus and RSV co-infections [4.6 (3.4-5.5) vs. 5.0 (4.0-6.1) log of copies / swab; $P = 0.052$] (Figure 7).

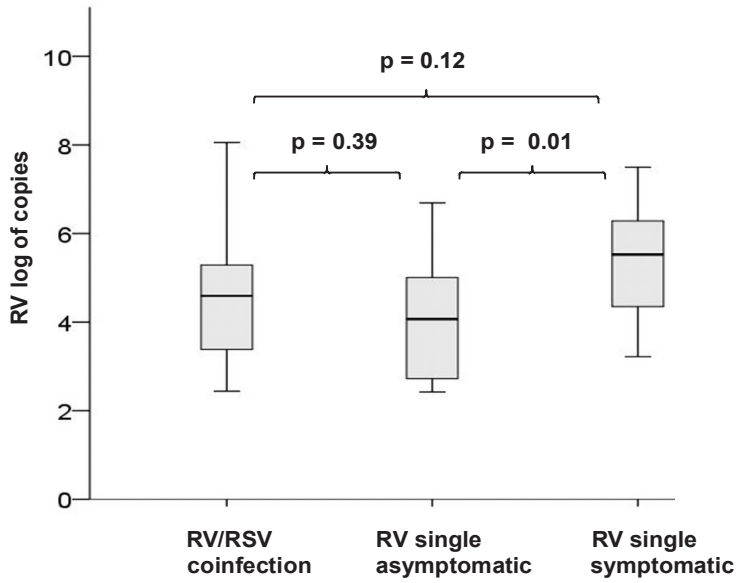


Figure 7. Viral copy number of rhinovirus (RV) in co-infections with respiratory syncytial virus (RSV) and in control children with single rhinovirus infections according to the presence of symptoms. Log-transformed data were analyzed with t-test (modified from Study II).

5.4 Association between rhinovirus infection and *Streptococcus pneumoniae* acquisition and transmission in families (III)

Altogether 29 families with 128 subjects were included in this study. A total of 742 nasal swab samples were collected during the three-week follow-up (mean 26 samples / family, range 15-36; and 5.8 samples / subject, range 3-7). Vaccination coverage with the 10-valent pneumococcal vaccine was 39% in the study children. The study was conducted from July 2011 through March 2012, and 78% of the samples were collected between September 2011 and October 2011. Symptoms of RTI (e.g., rhinorrhea, sore throat, and /or wheezing with or without fever) were detected in 81% of subjects during the three-week follow-up. Infections were mild, and no one needed hospitalization.

5.4.1 Rhinovirus infection

Rhinovirus was commonly detected in families with children during the autumn season. Altogether 67% (86/128) of the subjects were positive for rhinovirus at least once during the 3-week follow up period (84% of children and 47% of adults, $P < 0.001$). Rhinovirus was detected more frequently during respiratory tract symptoms in both children and adults compared to asymptomatic subjects (65% vs. 17% children, and 27% vs. 9% adults. Altogether 27 different

rhinovirus types within three rhinovirus species were detected in 146 of 205 rhinovirus positive samples; type A in 51%, type C in 43% and type B in 6% of positive samples. The median virus copy number was higher during the first week of the follow-up compared to samples collected during the last two weeks of follow-up [5.3 (3.8-6.6) vs. 4.5 (3.2-5.4) log of copies/swab, $P < 0.001$].

5.4.2 Pneumococcal colonization

Colonization with *S. pneumoniae* was detected in 30% (46% of children vs. 10% of adults) of subjects during the 3-week follow-up period. Colonization was more common in children attending daycare and families with three or more children compared with families with two children. Seven different serotypes of *S. pneumoniae* were detected (6B, 15A, 15B/C, 22F, 23F, 35B and 35F), with 6B being the most commonly detected serotype (in 18 samples).

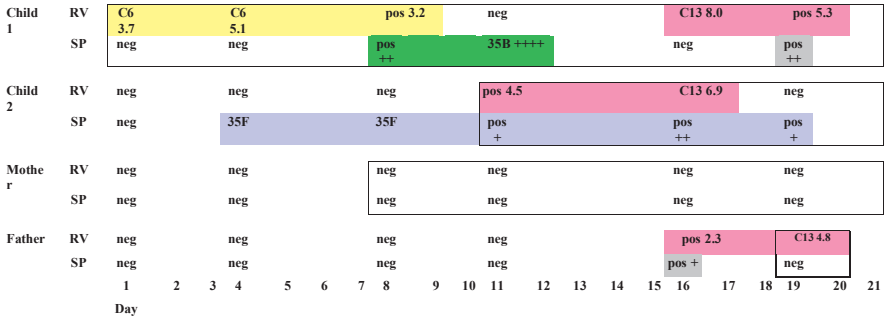
5.4.3 Co-detection of rhinovirus and Streptococcus pneumoniae

Of those subjects with rhinovirus infection, 40% were colonized with *S. pneumoniae* during the follow-up. Rhinovirus was detected before pneumococcal colonization in 23% (20/86) of the rhinovirus-infected subjects, concurrently in 13% and after that in three 3% subjects. The median time between the first detection of rhinovirus and pneumococcus was eight days. There was a clear chronologic association of rhinovirus and *S. pneumoniae* findings and clinical symptoms in families (Figure 8).

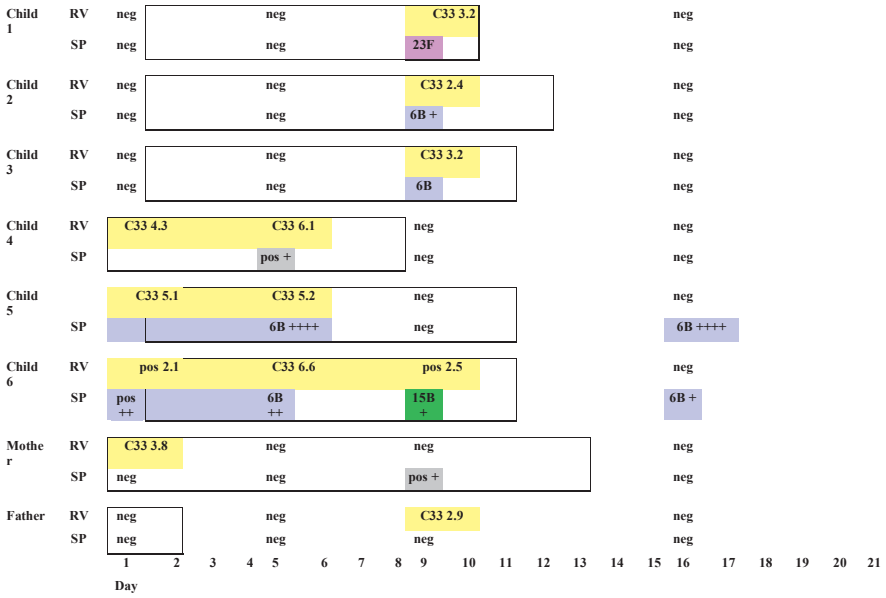
5.4.4 Acquisition and transmission of Streptococcus pneumoniae in families

Rhinovirus infection facilitated the acquisition and transmission of pneumococci in families with children. The rate of *S. pneumoniae* acquisition was 4.3 times more common in children with rhinovirus infection compared to children without rhinovirus infection. Within-family transmission of *S. pneumoniae* was 14-fold higher in children with rhinovirus infection compared to those without it. Within-family transmission was associated with recipient children's symptomatic infection. No such association was seen in the community acquisition of pneumococci.

Family 1



Family 2



Family 3

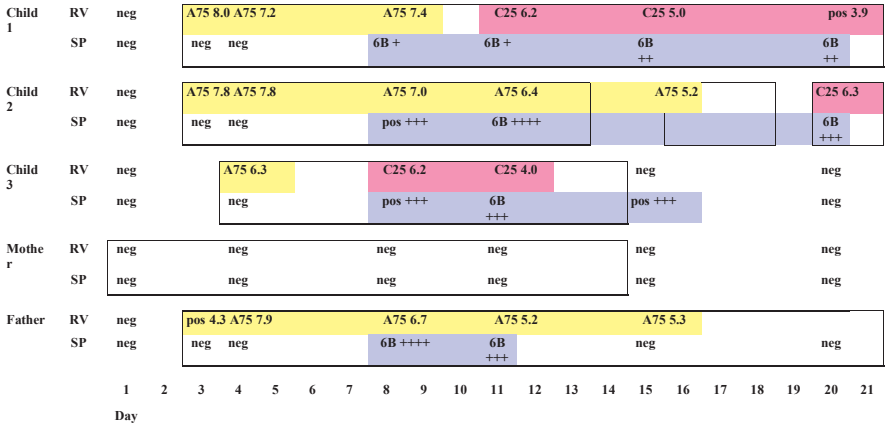


Figure 8 (previous page). Rhinovirus (RV) and pneumococcal findings and symptoms (shown in boxes) of respiratory tract infection in three representative families during the 3-week follow-up. The first detected RV is highlighted with yellow and the second detected RV with pink color. First, pneumococcal colonization is marked with blue, second with green and third with purple. Copy numbers and RV types are shown (modified from Study III).

5.4.5 Whole genome sequencing of pneumococcal isolates

We were able to identify, based on conventional serotyping, that one (or two) pneumococcal serotype(s) prevailed in each family throughout the follow-up. The whole genome sequencing revealed only minor differences in isolates of the same serotype supporting the hypothesis that the same strain is transmitted among the family members instead of a new acquisition from the community or that previously acquired bacteria becomes detectable during viral infection because of bacterial growth (Figure 9).

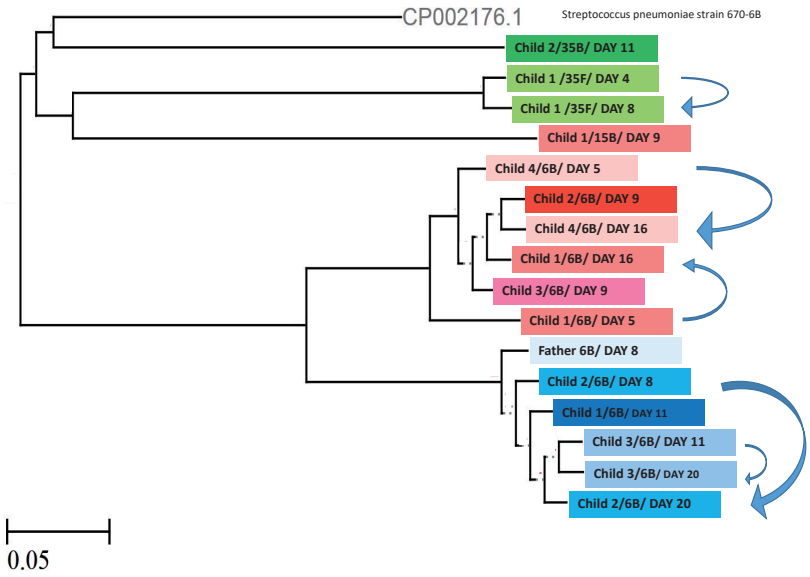


Figure 9. Phylogenetic tree of *S. pneumoniae* isolates from three families. Person number, serotype and sampling date are shown. Samples collected from the same person at different time points are shown in the same colors and arrows indicate the changes in each individual in pneumococcal strains during the follow up. A scale for the differences among the strains is shown with a bar (modified from Study III).

5.5 The effect of ten-valent pneumococcal *Haemophilus influenzae* protein D conjugate vaccine (PHiD-CV10) on respiratory tract infections

A total of 368 children were included in this study. Of these children, 236 were vaccinated with the PHid-CV10 pneumococcal vaccine, and 132 children received the control vaccine (hepatitis A or B vaccine). The first vaccine was given at different ages based on the time of the recruitment to the vaccine study; 266 children received their first vaccine at the age of 0-6 months, 38 at the age of 7-11 months and 64 at the age of 12-18 months. The median follow-up time for RTIs after the first vaccination was 1.4 IQR (1.0-1.7) years.

5.5.1 Clinical characteristics and viral findings in vaccinated and control children

A total of 3193 episodes of RTI were documented in the 368 children. Eight children (2%) did not have respiratory tract infections during the follow-up. Infections were mostly mild with only 12 children needing hospitalization in 26 episodes and, wheezing illness being the most common cause for hospital treatment. AOM was detected in 521 (16%) episodes, wheezing in 93 (3%) episodes and pneumonia in 12 (0.4%) episodes. The duration of the RTI episodes did not differ between the PHid-CV10 vaccinated and control children with median of 8 (IQR [5-12] vs. 7 IQR [4-11]) days respectively (P = 0.078).

Nasal swab samples were collected during 53% of episodes, and virus was detected in 73% of tested episodes with rhinovirus being the most common virus. There was no difference between vaccinated and control children regarding viral findings (Figure 10).

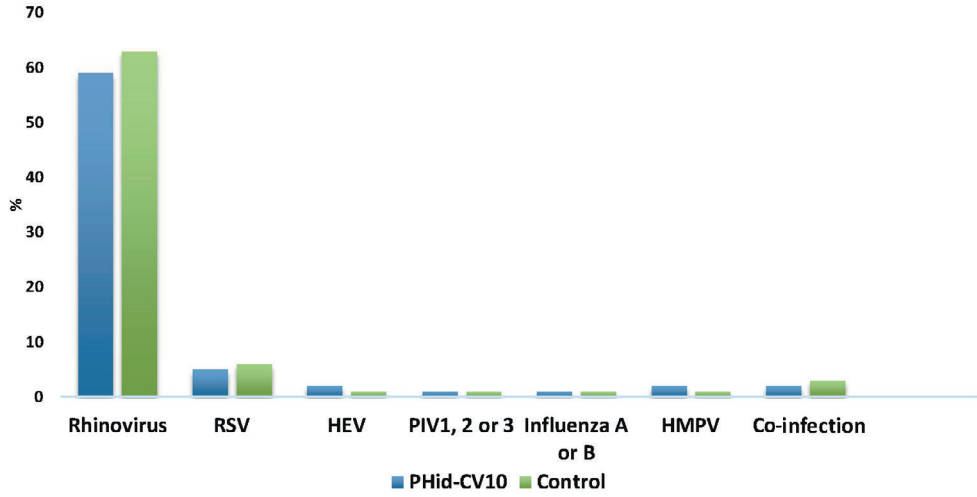


Figure 10. Viral findings during the follow-up in children vaccinated with PHid-CV10 vaccine or control vaccine. RSV, respiratory syncytial virus; PIV1, 2 or 3, parainfluenza virus 1, 2, and 3; HEV, human enterovirus; HMPV, human metapneumovirus (modified from Study IV).

5.5.2 The effect of pneumococcal vaccination on RTI episodes

RTI episodes were less common in children who were vaccinated with PHid-CV10 vaccine compared to control children. The incidence rates (95% CI) were 6.4 (6.0-6.8) and 7.4 (6.8-8.0) per person-year, respectively; $P = 0.004$ (Table 6). The VE was 12% [95% CI, (2-22%)] against all RTI episodes.

5.5.3 The effect of pneumococcal vaccination on RTI episodes with or without acute otitis media

The incidence rates (95% CI) of AOM episodes in PHid-CV10 vaccinated and control children were 1.0 (0.9-1.2) and 1.3 (1.1-1.6). The rate of non-AOM episodes were 5.4 (5.0-5.7) and 6.1 (5.6-6.6) in PHid-CV10 vaccinated and control children, respectively. VE against RTI episodes with AOM was 23% (95% CI, 0-40%) and 10% (95% CI, 0-19) against RTI episodes without AOM.

Table 6. Vaccine effectiveness on all RTI episodes, RTIs with or without AOM and antibiotic descriptions (modified from Study IV).

	Incidence rate of RTI episodes per person-year (95% CI)		Vaccine effectiveness, % (95% CI)
	PHiD-CV10-vaccinated children	Control children	
All RTI episodes	6.4 (6.0 − 6.8)	7.4 (6.8 − 8.0)	12 (2 − 22)
RTIs with AOM	1.0 (0.9 − 1.2)	1.3 (1.1 − 1.6)	23 (0 − 40)
RTIs without AOM	5.4 (5.0 − 5.7)	6.1 (5.6 − 6.6)	10 (0 − 19)
RTIs with antibiotic use	1.2 (1.0 – 1.4)	1.6 (1.3 – 2.0)	24 (3– 40)

6 DISCUSSION

6.1 Variations in the host innate immune system affecting viral-bacterial interaction (Study I)

The impact of viral infection on bacterial colonization and development of bacterial diseases is a well-recognized phenomenon (Brealey, *et al*. 2015, Pettigrew, *et al*. 2011, Nokso-Koivisto, *et al*. 2015). It has been shown in *in vitro* studies that the presence of a viral infection affects the epithelium making it more favorable to bacterial attachment (Ishizuka, *et al*. 2003). Co-infections with viruses and bacteria are common, which suggests synergistic association among these pathogens (Heikkinen, *et al*. 1991, Brealey, *et al*. 2018, Brealey, *et al*. 2015, Gwaltney, *et al*. 1975, Jacoby, *et al*. 2007, Nichol and Cherry. 1967). The importance of bacterial complications after viral infection was first established during influenza pandemics, when the rate of bacterial pneumonias and deaths due to bacterial infections increased (Brundage and Shanks. 2008). The importance of viral infection has also been documented in the development of AOM, which clearly associates with preceding viral infection. The reasons why some children are more prone to get bacterial complications after a viral infection are unclear, but variations in innate immune genetics may play role here.

In this study, we showed that *S. pneumoniae* colonization was strongly associated (Odds Ratio of 5.8) with rhinovirus infection in infants with a polymorphism in the *MBL* gene but not in those with wild-type MBL. Such heterozygous polymorphisms were found in one-third of our study population, in line with previous reports concerning the Finnish population. MBL is part of the innate immune system, and it plays an important role, especially during early childhood, when the adaptive immunity has not developed yet. It has been shown that polymorphisms in this gene lead to lower levels of MBL protein and increased susceptibility to severe infections, respiratory infections and AOM (Eisen. 2010, Wiertsema, *et al*. 2006, Koch, *et al*. 2001, Frakking, *et al*. 2007, Ruskamp, *et al*. 2006, Toivonen, *et al*. 2017, Figueiredo, *et al*. 2016). In the STEPS study cohort, it has been shown earlier that MBL polymorphisms are associated with a higher susceptibility to rhinovirus-associated acute otitis media in children under two years of age, indicating that the MBL polymorphism has an effect on virus-related bacterial colonization and disease also after early infancy (Toivonen, *et al*. 2017). A connection between variant-type MBL and co-infection caused by viruses in community-acquired pneumonia has been described in adults (Endeman, *et al*. 2008). The frequency of variant-type MBL was also higher among patients with pneumonia caused by pneumococcal-viral co-infections.

The association between MBL polymorphism and bacterial colonization during viral infection supports the hypothesis that infants with defects in their innate immune system have an impaired capacity to defend against bacterial infection especially during viral

infection. In our study, we did not analyze possible mechanisms behind this association. It was interesting that the rate of rhinovirus infection did not differ between infants with wild-type or variant-type MBL, suggesting that MBL is not highly important in prevention of upper respiratory tract rhinovirus infections. Viral infection itself alters susceptibility to bacterial colonization and disease by different mechanisms. A virus may damage the epithelium and predispose it to bacterial attachment. Induced inflammation leads to impaired clearance of pathogen because of impaired ciliary function and suppressed immune response. It seems that viral infection and defects in the innate immune system potentiate the impact of each other and together lead to increased rates of bacterial colonization. This synergism might be seen by increased rates of virus-induced bacterial complications in genetically susceptible individuals.

We did not find associations between TLR3 or TLR4 polymorphisms and viral-bacterial interactions (co-detection of rhinovirus and pneumococcus). Variations in *TLR3* and *TLR4* genes did not alter children's susceptibility to rhinovirus infection at 2 months of age. Polymorphisms in TLRs affect the receptor function but mechanism at molecular level is unclear (Medvedev. 2013). TLR3 binds to double-stranded RNA, which is produced by rhinovirus and other single-stranded RNA viruses in the process of viral replication within epithelial cells (Wang*, et al*. 2009b). In previous studies, it has been associated with bronchiolitis during infancy and recurrent wheezing. Our study children were mostly healthy, when they visited the study clinic at 2 months of age and had no RSV infections. Polymorphism of TLR4 has been linked to infection with gram-negative bacteria, because TLR4 is able to recognize LPS on the surface of these bacteria. In our study population, colonization rates with *M. catarrhalis*, which is a gram-negative bacterium, were significantly higher in subjects with variant-type TLR4.

Even though there were no statistically significant interactions between variant types TLR3 or TLR4 and co-infection with rhinovirus and *S. pneumoniae*, *M. catarrhalis* or *S. aureus*, the infants with wild type TLR3 and TLR4 were less commonly colonized with *S. aureus* and more commonly colonized with *M. catarrhalis* during rhinovirus infection compared to children without rhinovirus infection. Rhinovirus infection has previously been related to colonization with *M. catarrhalis* (Kloepfer*, et al*. 2014). However, a negative association between *M. catarrhalis* and *S. aureus* has been described suggesting a competitive interaction between these bacteria (Regev-Yochay*, et al*. 2004, Kwambana*, et al*. 2011). Our results are in accordance with these earlier observations.

In addition to the host immune system also age, number of siblings, use of antibiotics, vaccinations and viral infections have effects on the rate of bacterial colonization. In our study, infants at the age of 2 months were most commonly colonized with *S. aureus* followed by *M. catarrhalis* and *S. pneumoniae* (28%, 25% and 14%, respectively). This is in line with previous studies. The rate of *S. aureus* colonization has been described to be most common in infants at the age of 2 months, and it decreases by the age of 1 year,

while the colonization rates of *S. pneumoniae* increase during the first years (Garcia-Rodriguez and Fresnadillo Martinez. 2002). In addition to age, the number of siblings and presence of respiratory symptoms were associated with pneumococcal and *M. catarrhalis* colonization, while infants colonized with *S. aureus* were more often asymptomatic. Because colonization with *S. pneumoniae* and *M. catarrhalis* is more common in older children, they efficiently transmit these bacteria to younger siblings within families as described in previous studies (Mosser, *et al*. 2014, Knox, *et al*. 2015). In our study, the prevalence of rhinovirus infection was 8% at the age of 2 months. Symptoms of rhinovirus infection are usually mild also in infants, however, most rhinovirus findings are associated respiratory tract infections when specifically asked (Toivonen, *et al*. 2016). In our study 43% of the rhinovirus positive samples were collected during symptoms of infection and 13% from children who were asymptomatic at the time of the visit, which is in line with previous studies (Kusel, *et al*. 2006, Jartti, *et al*. 2008).

The impact of viral-bacterial synergism is important, because rhinovirus infections cause a great burden on small children (Toivonen, *et al*. 2016). Based on other studies from the STEPS study population, children had a mean of 5.9 rhinovirus infections annually during the first two years of life. Notably, rhinovirus was associated with almost one-half of antibiotic treatments at this age. In our study, it was noteworthy that the rate of rhinovirus infection was not different among infants with polymorphisms of MBL, TLR3 or TLR4. The MBL polymorphism was detected in one-third of this study population, and its importance seems to be not in susceptibility to rhinovirus infection or bacterial colonization alone, but in affecting the development of bacterial colonization during viral infection. Here, we did not study bacterial infections, but colonization is the first step in the development of infection. Bacterial complications cause a great burden to individuals but also an economic burden. By finding individuals who are more susceptible to bacterial complications, prevention could be targeted to these children.

6.2 Viral interference (Study II)

In this study, we showed an antagonistic association between RSV and rhinovirus, the two most common viruses that cause respiratory infections in small children. Rhinovirus was detected less often concurrently with RSV than in control children. However, in co-infections, symptoms lasted longer than in single infections, suggesting that both viruses cause symptoms in these children. The strength in our study was that we were able to compare the rate of rhinovirus infections in control children and in children with RSV during the same season within the same birth cohort.

The prevalence of rhinovirus (14%) in the control group represents the rate of rhinovirus infections in small children in their usual state of health. Most of the children were asymptomatic during sampling but some had incidental, mostly mild symptoms. In our

earlier analysis of the cohort data, the prevalence of rhinovirus regardless of symtoms was 14% at 2 months of age, 18% at 13 months of age and 24% at 24 months of age. (Toivonen, *et al*. 2016). A higher prevalence of rhinovirus infections has been reported during symptomatic RTIs and even 70% of RTIs can be caused by rhinovirus infections in young children (van der Zalm, *et al*. 2009, Toivonen, *et al*. 2016b). It has been proposed that rhinovirus finding in asymptomatic children could be explained by preceding rhinovirus infection. The shedding of rhinovirus infection after RTI has been reported to be 11 days (mean) and even longer in immunocompromised children (Peltola, *et al*. 2013). Symptoms might also be so mild that parents do not report them or the sample has been collected during incubation of infection.

The rate of rhinovirus infection was significantly lower during RSV infections. The negative association between RSV and rhinovirus has been reported also in previous studies (Greer, *et al*. 2009, Martin, *et al*. 2013). The reason for a lower rate of rhinovirus infection during RSV infection might be mediated through an antiviral interferon response. Activation of an intracellular signaling cascade after recognition of viral structures or products by PRRs results in production of interferons, cytokines and chemokines. These products can limit the viral replication and spread of infection (Lester and Li. 2014, Takeuchi and Akira. 2009). The host immune response may lead to development of an antiviral state that inhibits not only the current infection, but also a new infection by another virus. Because we did not collect repeated samples, we are not able to say which virus infected the child first. Although it seems that RSV inhibited rhinovirus infections in our study, it is also possible that rhinovirus infection caused an antiviral state and reduced the possibility of RSV infection.

The copy numbers of these two viruses cannot be directly compared due to differences in replication kinetics among different viruses. Interestingly, the viral load was significantly higher in single rhinovirus infections compared to co-infections with RSV. Previous studies have reported that the viral load of rhinovirus is highest during the first days of infection and decreases thereafter (Peltola, *et al*. 2008). This would support a view that a high copy-number of rhinovirus with strong immune response at the beginning of the infection could inhibit RSV infection, whereas low copy numbers later during the infection could not. Those children with RSV infection had already bypassed acute rhinovirus infection and had lower viral loads.

RSV causes both upper and lower respiratory tract infections, and it is often associated with AOM. We showed that symptoms in RSV-rhinovirus co-infections lasted longer than in single RSV infections. There was no difference in the rate of wheezing, AOM or pneumonia episodes between children with single or co-infection, but our study was not powered to detect differences in these variables. It has been shown previously that children with bronchiolitis and positive for RSV and rhinovirus need longer treatment in the hospital, and their symptoms last longer compared to children with bronchiolitis

caused by a single virus (Mansbach, *et al*. 2012, Martin, *et al*. 2012). This might be explained by stronger immune response in co-infections, but also by overlapping symptomatic periods of two different viral infections.

The effects of other viruses and bacteria on rhinovirus and RSV infections were not analyzed in this study. As we know that co-infections with other viruses are also common and the microbiological environment in the nasopharynx is diverse, there may be also other factors in the microbiome and host defense system that influence the synergism between these two microbes. However, this study demonstrates the possible inhibiting interactions between viruses that affect the presence of different pathogens in the nasopharynx during respiratory tract infections.

6.3 Effects of rhinovirus infection on pneumococcal acquisition and transmission in families (Study III)

Viruses are commonly detected concurrently with bacteria during respiratory tract infections in children, and synergism between rhinovirus and *S. pneumoniae* has been shown in ecological and *in vitro* studies (Heikkinen, *et al*. 1999, Honkinen, *et al*. 2012). Also, we demonstrated the link between viral infection and bacterial colonization in infants with polymorphisms in the *MBL* gene. However, the impact of rhinovirus infection on bacterial transmission has been assessed only in a few studies (Gwaltney, *et al*. 1975). In our study, concurrent rhinovirus infection increased both the acquisition and transmission of *S. pneumoniae* in children. The rate of pneumococcal acquisition was 4 times higher and the transmission rate was 15 times higher in children with concurrent rhinovirus infection compared to those children who did not have rhinovirus infection. In adults, the impact of rhinovirus infection on pneumococcal colonization could not be analyzed because of a small number of pneumococcal acquisition rates.

Pneumococcal colonization is common in small children, and up to 87% have been colonized with pneumococcus during the first two years of life at least once (Syrjanen *et al*. 2001). Risk factors for pneumococcal colonization are a young age, crowding and viral infections but also host immune system and vaccination have an effect on pneumococcal colonization. In our study, 46% of children and 10% of adults were colonized with pneumococcus during the three weeks follow-up, and it was more common in families with three or more children and in children who attended daycare which is in line with previous studies.

Small children with viral infection and higher bacterial colonization rates with bacteria are reservoirs for bacterial transmission between family members. It has been previously shown in ferrets that symptoms of donor and recipient during influenza infection increase the risk for bacterial transmission (Diavatopoulos, *et al*. 2010). In our study, the

symptoms of the donors could not be analyzed because of the small number of cases. Transmission between family members was associated with symptomatic rhinovirus infection in recipient but not with asymptomatic rhinovirus infection. This suggests that the host response to rhinovirus infection may predispose the individual to pneumococcal colonization.

It has been shown that bacterial density increases during viral infection, and increased density has been related to spread within the respiratory tract and in development of bacterial diseases like AOM and pneumonia (Syrjanen, *et al*. 2001, Diavatopoulos, *et al*. 2010, Wolter, *et al*. 2014). We used bacterial culture, serotyping and whole genome sequencing to analyze whether the viral infection increases the density of already existing bacteria in each individual or the transmission of bacteria between family members. In this study, only one serotype usually dominated in the families and the genetic constellation of the strains isolated from the same family was nearly identical. In some cases, genetically different pneumococci of the same serotype were detected in the beginning of the follow-up and nearly identical isolates towards to the end of the follow-up. During long-term colonization, there occurs mutations and slight divergence among different pneumococcal strains. Our findings from whole genome sequencing suggest transmission between family members instead of growth of existing, non-detectable pneumococci with low-level colonization before rhinovirus infection. This conclusion would have been difficult to achieve by using traditional serotyping methods only.

There were some methodological considerations in this study. Parents collected samples from a 2 cm depth of the nostrils at home and sent the samples to our study clinic. Even though it is not recommended to use nasopharyngeal specimens to detect pneumococcal colonization, similar levels of bacterial colonization have been reported by using nasal swabs or nasopharyngeal samples (van den Bergh, *et al*. 2012b, Rapola, *et al*. 1997). This sampling method made it possible to start sample collection immediately at the beginning of the symptoms and made the sampling more pleasant for the families than collecting nasopharyngeal samples.

Previously, it has been shown that pneumococcal carriage rates in self-collected nasal swabs are similar when compared with samples collected by health care professionals (Coughtrie, *et al*. 2014). After receiving the samples, we did the commercial antigen test for all samples. We have previously shown that sensitivity of this pneumococcal antigen test is higher than the sensitivity of culture of samples that have been collected at home and sent by mail and stored in RTI buffer (Terasjarvi *et al*. 2016). The rate of pneumococcal colonization in children (46%) in this study was similar to the rates of pneumococcal colonization in Finnish children of the same age (Syrjanen, *et al*. 2001). This suggests that the antigen test sensitivity is comparable with the sensitivity of culture of fresh samples.

Because of the high incidence of rhinovirus infections and their bacterial complications in small children, it is important to understand the development of bacterial colonization in the setting of viral infection. In our study, the rate of bacterial infections was low; we studied colonization, which is needed for development of pneumococcal disease. The factors that turn the bacterial colonization to disease are subjects for further studies.

6.4 Impact of pneumococcal conjugate vaccine on respiratory tract infections in children (Study IV)

Pneumococcal conjugate vaccines reduce the incidence of invasive pneumococcal diseases, pneumonia, otitis media and prescriptions of antibiotics (Eskola, *et al*. 2001, Palmu, *et al*. 2014, Griffin and Grijalva. 2013). A decrease in the rate of these diseases has been explained by the reduction of colonization by the vaccine type pneumococcus, in that way decreasing the development of pneumococcal diseases (Simell, *et al*. 2012, Saez-Llorens, *et al*. 2017). *S. pneumoniae* is just one pathogen causing AOM and pneumonia, and based on previous studies, it has been suggested that pneumococcal vaccines have an effect beyond strictly pneumococcal diseases (Sigurdsson, *et al*. 2015, Dagan, *et al*. 2001). In Iceland, it has been shown that pneumococcal conjugate vaccine reduced all-cause AOMs, and Dagan et al. described the impact of pneumococcal conjugate vaccine on the occurrence of RTIs regardless of their cause. In our study, the rate of all-cause RTIs was lower during the first two years of life, when children were vaccinated with the 10-valent pneumococcal conjugate vaccine before the age of 19 months compared to children vaccinated with a hepatitis vaccine.

In this study, most of the infection episodes were mild viral-type infections. Still, the effect of pneumococcal vaccine was significant on all cause RTIs with VE of 12%. The effect was more obvious in episodes associated with AOMs, which is in line with previous studies (Sigurdsson, *et al*. 2018). As the viral-bacterial interaction affects the attachment of bacteria on the epithelium, it also modulates immune responses and has effects on the symptoms of infections. Bacterial colonization during viral infection may increase the symptoms of viral infection and symptoms have been reported to last longer (Cebey-Lopez, *et al*. 2016).

It can be hypothesized that the clearance of bacteria after the pneumococcal vaccination could reduce viral-bacterial interactions and have an effect on the development of symptomatic viral infections. Effects of bacterial colonization on the symptoms might be more evident in milder forms of RTIs. In our study, the effect of pneumococcal vaccine was more evident in episodes without collected nasal swab samples. Based on previous analysis (Toivonen, *et al*. 2016), these episodes were milder, and the duration of symptoms was shorter than in episodes with nasal swab samples. Those episodes that were tested for viruses did not differ regarding to viral findings between vaccinated and

control children showing that the pneumococcal vaccine does not have a direct effect on the occurrence of tested viruses.

In our study, we were able to compare the rate of RTIs during the same seasons between the groups, so the effects of seasonal variation in the rate of viral infections was excluded. Diagnosis of AOMs were mostly made by our study clinic physicians, diminishing the possibility of differences in diagnostic criteria among the groups. The mechanisms behind the vaccine effects were not analyzed in this study. As we did not collect bacterial samples during the episodes, we do not know the effects of vaccination on pneumococcal colonization. However, it has been shown in previous studies that the pneumococcal conjugate vaccines reduce the colonization by vaccine serotypes but not as much on overall colonization, as replacement by other serotypes occurs (Weinberger*, et al*. 2011).

There were slight differences between the PHiD-CV10 and control vaccine groups regarding the living environment, number of siblings and parents' educational level. In Finland, geographical differences between urban and rural areas are small and barely have an effect on the incidence of infections or bacterial colonization. The possible effects of background differences between the study groups were taken into account by adjusting the estimates of vaccine effectiveness for cluster effects and the presence of siblings.

Because viral infections cause a great burden on small children and are often complicated with bacterial disease, the knowledge regarding the effects of pneumococcal vaccine on all-cause RTIs is important. Even though on an individual level the mean difference of one infection per year between PHiD-CV10 vaccinated and control children is small, this effect of pneumococcal conjugate vaccine on the rate of RTIs could be important at the population level.

6.5 Future prospectives

We found that genetic differences in the host innate immune system, virus infections and pneumococcal colonization, have effects on the complex viral-bacterial interaction in small children. Also, the use of a conjugate vaccine may modify the viral-bacterial interaction. Children suffer the most from RTIs during their first years of life. It is important to understand the factors that have effects on the development of these diseases, so that preventive strategies could be developed. By understanding the differences among individuals regarding the susceptibility to infections, we could be able to target possible interventions to the individuals who would benefit most.

These studies suggest that prevention strategies of common respiratory viral infections should be developed not only to prevent viral infections but also to prevent bacterial transmission, colonization and infections. Viral infections could be prevented with

smaller groups in daycare centers; however, this can be costly for the society. Currently influenza A and B viruses are the only respiratory viruses that can be treated with medications. Influenza virus infections can be treated with specific neuraminidase inhibitors. The use of neuraminidase inhibitors has led to reduced rates of AOM in small children and also to lower rates of hospitalizations and antibiotic descriptions (McNicholl and McNicholl. 2001, Kaiser*, et al*. 2003, Wang*, et al*. 2012). As neuraminidase activity facilitates pneumococcal adherence, the use of neuraminidase inhibitors inhibits also *S. pneumoniae* activity in both in vitro and *in vivo* studies (Trappetti*, et al*. 2009, McCullers. 2011).

Palivizumab, a monoclonal antibody, has been used for passive immunization against RSV in high-risk infants (e.g., children born prematurely, those with chronic lung disease or congenital heart disease), who have an increased risk for severe RSV infections (Cockerill*, et al*. 2018, Del Vecchio*, et al*. 2018). The use of palivizumab has led to reduction in all-cause mortality and hospitalization among preterm infants at high risk (Checchia*, et al*. 2011). Palivizumab must be given once each month during the RSV season, which, together with its high cost, makes it unfeasible for large-scale use. Better passive or active immunization methods and antiviral drugs against RSV would be needed to effectively prevent and treat RSV infection and to prevent its bacterial complications.

New antiviral medications and vaccines are under investigation. Rhinovirus is the most common virus causing respiratory tract infections, and it predisposes children to bacterial colonization and bacterial diseases. Different medications have been studied for prevention and treatment for rhinovirus. Mousnier et al. recently published data about a molecule that could prevent rhinovirus proliferation in infected cells (Mousnier*, et al*. 2018). The molecule, IMP-1088, a picomolar dual inhibitor of the human *N*-myristoyltransferases, could prevent the fatty-acid attachment of infected cells to viral proteins and in that way, prevent the rhinovirus replication without cytotoxic effects. A vaccine against rhinovirus would be highly important, but the development of a rhinovirus vaccine has been challenging because of a large number of rhinovirus types (McLean. 2014).

A pneumococcal vaccine was introduced into the Finnish National vaccination program in September 2010, and it has led to reduction of both mild and invasive pneumococcal diseases. In this study, we showed that a pneumococcal vaccine may reduce respiratory infections irrespective of the causative agent. More research is needed to understand the mechanisms behind this phenomenon.

Co-infections are common and the environment and inflammatory responses in the nasopharynx are affected by both viruses and bacteria. The pathogens have an important effect on each other before and during respiratory tract infections affecting also the severity and duration of symptoms. Thus, it would be important to focus studies rather on viral-bacterial co-infections than only on single infections in the future.

7 CONCLUSIONS

The aim of this study was to evaluate (I) the polymorphisms in innate immune genes with regard to microbial interactions in children, (II) negative interactions and (III) synergism between microbes causing RTIs and (IV) the effectiveness of 10-valent pneumococcal conjugate vaccine on all-cause RTIs in children.

In Study I we showed that bacterial colonization is common already in infants at the age of 2 months. Early pneumococcal colonization was associated with rhinovirus infection in children with variant-type MBL but not in those with wild type MBL. Genetic variations may explain susceptibility to viral-bacterial interactions in part of children and predispose them to bacterial complications of viral infections.

In Study II, we found a negative interaction between rhinovirus and RSV infection. Rhinovirus infection was significantly less common in children with RSV infection compared to the control children without RSV, matched for the age and time of sample collection. However, the presence of both viruses increased the duration of symptoms during RTI, which suggests that both viruses are clinically significant during co-infections in children.

In Study III, we found an association between rhinovirus infection and pneumococcal acquisition and transmission in families with children. Rhinovirus-infected children were more prone to acquire pneumococcus into their upper respiratory tract and transmit pneumococcus between family members. These results highlight the importance of rhinovirus infection in the first step of development of pneumococcal infections, namely, acquisition of a new pneumococcal serotype, and the importance of young children as a major transmitter of both rhinovirus and pneumococcus.

In Study IV, by vaccinating children with a pneumococcal conjugate vaccine, the rate of all RTI episodes could be decreased. This finding indicates possible effects of pneumococcal vaccine also on viral infections. The effect of the 10-valent pneumococcal vaccine was seen not only in AOMs but also in RTIs that are mainly caused by viruses. By affecting the bacterial colonization in the nasopharynx, the vaccine may have effect on development of symptomatic RTIs. This suggests that pneumococci are not always only passively colonizing the respiratory tract but may play a role in the development of symptoms even without a clearly identified pneumococcal infection.

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