Turun yliopisto
University of Turku

NASOPHARYNGEAL COLONIZATION BY PATHOGENIC BACTERIA: EFFECT OF POLYMORPHISMS IN INNATE IMMUNE GENES OF YOUNG CHILDREN

Juho Vuononvirta

TURUN YLIOPISTON JULKAISUJA – ANNALES UNIVERSITATIS TURKUENSIS
Sarja - ser. D osa - tom. 1168 | Medica - Odontologica | Turku 2015

University of Turku

Faculty of Medicine
Department of Paediatrics
Turku University Hospital
University of Turku Doctoral Program of Clinical Investigation (TKT/CLIPD)
Department of Infectious Disease Surveillance and Control
National Institute for Health and Welfare, Turku

Supervised by

Adj. Professor Qiushui He, MD, PhD
The Department of Infectious Disease Surveillance and Control
National Institute for Health and Welfare
Turku, Finland

Professor Jussi Mertsola, MD, PhD
Department of Pediatrics
Turku University Hospital
University of Turku
Turku, Finland

Reviewed by

Professor Mikael Skurnik, PhD
Department of Bacteriology and Immunology
Haartman Institute, University of Helsinki
Helsinki, Finland

Adj. Professor Mikko Seppänen, MD, PhD
Immunodeficiency Unit
Division of Infectious Disease,
Inflammation Center
Helsinki University
Helsinki University Hospital
Helsinki, Finland

Opponent

Adj. Professor Risto Vuento, MD, PhD
Microbiology, Fimlab Laboratories PLC
The Hospital District of Pirkanmaa
Tampere, Finland

The originality of this thesis has been checked in accordance with the University of Turku quality assurance system using the Turnitin OriginalityCheck service.

ISBN 978-951-29-6075-0 (PRINT)
ISBN 978-951-29-6076-7 (PDF)
ISSN 0355-9483
Painosalama Oy - Turku, Finland 2015

I don't think you'll ever have a perfect world because we humans are prone to error, and so we're always in search of an upgrade.

-Henry Rollins-

ABSTRACT

JUHO VUONONVIRTA

Nasopharyngeal colonization by pathogenic bacteria: effect of polymorphisms in innate immune genes of young children

University of Turku, Faculty of Medicine, Department of Paediatrics; Turku University Hospital; University of Turku Doctoral Program of Clinical Investigation (CLIPD); Department of Infectious Disease Surveillance and Control, National Institute for Health and Welfare, Turku

Annales Universitatis Turkuensis, Medica – Odontologica, Painosalama Oy, Turku, Finland 2015

Nasopharyngeal bacteria can asymptomatically colonize the nasopharynx of infants and young children but are also associated with the development of respiratory infections and diseases. Such nasopharyngeal bacteria include *Streptococcus pneumoniae*, *Moraxella catarrhalis*, *Haemophilus influenzae* and *Staphylococcus aureus*. The host defense against invading pathogens is largely relies germline-encoded pattern recognition receptors (PRR), which are expressed on the cells of innate immunity, and different cytokines. These include toll-like receptors (TLR), mannose-binding lectin (MBL) and different cytokines such as IL-17A. Single nucleotide polymorphisms (SNP) in these receptors and cytokines have been reported. The aim of this study was to investigate genetic polymorphisms in the genes for TLR2, 3 and 4, MBL as well as for IL-17A and their associations with nasopharyngeal pathogenic bacterial colonization during a two-year follow-up.

The study revealed that polymorphisms in *TLRs*, *MBL*2 and *IL17A* are associated with the nasopharyngeal bacterial colonization in young children. Healthy young (2.6 months of age) children with variant types of *MBL2*, *TLR2* R753Q or *TLR4* D299G had an increased risk to be colonized by *S. pneumonia*, *S. aureus* or *M. catarrhalis*, respectively. Moreover, variant types of *MBL2* in healthy children with might facilitate human rhinovirus (HRV)-induced *S. pneumoniae* colonization at 2.6 months of age. The polymorphism of *TLR4* D299G was shown to be associated with *M. catarrhalis* colonization throughout the whole two-year follow-up (2.6, 13 and 24 months of age) and also with the bacterial load of this pathogen. Also, the polymorphism of *IL17A* G152A was shown to be associated with increased risk to be colonized by *S. pneumoniae* at 13 and 24 months of age. Furthermore, the results suggest that *IL17A* G152A has an effect on production of serum IL-17A already at young age.

In conclusion, the results of this study indicate that polymorphisms in the key PRRs and *IL17A* seem to play an important role to colonization of *S. pneumoniae*, *M. catarrhalis*, and *S. aureus* in healthy young Finnish children. The nasopharyngeal colonization by these pathogenic bacteria may further promote the development of respiratory infections and may be related to development of asthma and allergy in the later life of children. These findings offer a possible explanation why some children have more respiratory infections than other children and provide a rational basis for future studies in this field.

Keywords: Bacterial colonization, *S. pneumoniae*, *M.catarrhalis*, *S. aureus*, SNP, TLR, MBL, IL-17A, young children, respiratory infections

TIIVISTELMÄ

JUHO VUONONVIRTA

Luontaista immuniteettiä ohjaavien geenien vaihtelevuuden vaikutus tauteja aiheuttavien bakteereiden asustamiseen nuorten lasten nenänieluissa

Turun yliopisto, Lääketieteellinen tiedekunta, Lastentautioppi; Turun yliopistollinen keskussairaala, Turun yliopiston kliininen tohtoriohjelma (TKT); Tartuntatautiseurannan ja – torjunnan osasto, Terveyden ja hyvinvoinnin laitos, Turku

Annales Universitatis Turkuensis, Medica – Odontologica, Painosalama Oy, Turku, Suomi 2015

Nenänielu on erittäin monimuotoinen ekosysteemi, joka sisältää useita erilaisia bakteereja. Monet näistä bakteereista asustavat oireita aiheuttamatta nuorten lasten nenänielussa, mutta ne voivat usein aiheuttaa myös hengitystieinfektioita ja sairauksia. Yleisimmät taudinaiheuttajabakteerit nenänielussa lapsilla ovat *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Moraxella catarrhalis* ja *Staphylococcus aureus*. Luontaisen immuniteetin reseptorit, jotka ilmentyvät luontaisen immuniteetin solujen pinnalla, kontrolloivat elimistön puolustusta bakteereja vastaan. Tällaisia molekyylejä ovat muun muassa Toll-proteiinin kaltaiset reseptorit (TLR), mannoosia sitova lektiini (MBL) ja erilaiset sytokiinit, kuten IL-17A. Useita erilaisia polymorfismeja eli mutaatioita on tunnistettu geeneissä, jotka koodittavat luontaisen immuniteetin reseptoreita ja sytokiinejä. Tämän väitöskirjatyön tarkoituksena oli tutkia luontaista immuniteettiä ohjaavien geenien vaihtelevuuden vaikutusta tauteja aiheuttavien bakteereiden asustamiseen terveiden suomalaisten lasten nenänieluissa lasten kahden ensimmäisen elinvuoden aikana.

Tehty tutkimus osoitti, että *TLR*:ien, *MBL2*:n ja *IL17A*:n geneettiset polymorfismit vaikuttavat nuorten lasten nenänieluissa asustaviin bakteereihin. Terveillä 2.6 kuukauden ikäisillä lapsilla, joilla todettiin polymorfismeja *MBL2*:ssä, sekä polymorfismit *TLR2* R753Q ja *TLR4* D299G, oli suurempi riski kantaa *S. pneumonia*, *S. aureus* ja *M. catarrhalis* bakteereita nenänielussa, tässä järjestyksessä. Sen lisäksi tutkimuksessa havaittiin, että 2.6 kk ikäisillä lapsilla polymorfismit *MBL2*:ssa saattavat johtaa rinoviruksen indusoimaan *S. pneumoniae* nenänieluasustamiseen. Noin kahden vuoden seurannan aikana (2.6kk-24kk) huomattiin *TLR4* D299G polymorfismin olevan mahdollisesti yhteydessä nenänielun kolonisoitumiseen *M. catarrhalis* bakteereilla ja mahdollisesti myös *M. catarrhalis* bakteerien kantajuuteen nenänielussa. Lisäksi tutkimus osoitti, että 13 ja 24 kuukauden ikäisillä lapsilla *IL17A* G152A polymorfismi oli yhteydessä nenänielun kolonisoitumiseen *S. pneumoniae* bakteereilla ja että *IL-17A* G152A polymorfismilla oli vaikutus IL-17A sytokiinin seerumipitoisuuteen.

Johtopäätöksenä voidaan todeta tutkimuksessa saatujen tulosten viittaavan siihen, että luontaisen immuniteetin reseptoreiden ja sytokiinien polymorfismeilla on mahdollisesti yhteyttä terveiden nuorten suomalaisten lasten nenänielujen kolonisoitumiseen *S. pneumoniae*, *M. catarrhalis* ja *S. aureus* bakteereilla. Tästä voi lasten myöhemmässä elämässä olla seurauksena hengitystieinfektioiden lisääntyminen ja toisaalta se voi myös nostaa astman tai allergian kehittymisen riskiä. Tutkimuksessa havaitut polymorfismien ja nenänieluasustamisen mahdolliset yhteydet voivat antaa selityksen sille, minkä takia jotkut lapset saattavat sairastua hengitystieinfektioihin useammin kuin toiset lapset.

Avainsanat: nenänielukolonisaatio, *S. pneumoniae*, *M. catarrhalis*, *S. aureus,* polymorfismi, TLR, MBL, IL-17A, nuoret lapset, hengitystieinfektiot

CONTENTS

ABBREVATIONS

AOM	acute otitis media
ARI	acute respiratory infection
CAP	community acquired pneumonia
CD	cluster of differentiation
COPD	chronic obstructive pulmonary disease
CRD	carbohydrate-recognition domains
DC	dendritic cell
DNA	deoxyribonucleic acid
ds	double stranded
Hib	Type b *Haemophilus influenzae*
HIV	human immunodeficiency virus
HRV	human rhinovirus
Hsp	heat shock protein
IFN	interferon
Ig	immunoglobulin
IL	interleukin
IL-1R	interleukin-1 receptor
IRAK	IL-1 receptor-associated kinase
IRF	interferon regulatory factor
LAM	lipoarabinomannan
LBP	LPS-binding protein
LPS	lipopolysaccharide
LRI	lower respiratory tract infection
LRR	leucine-rich-repeat
LTA	lipoteichoic acid
MAC	membrane attack complex
MAL	MyD88-adaptor-like
MALP2	macrophage-activating lipopeptide 2
MAP	mitogen activated protein kinase
MASP	MBL-associated serine proteases
MBL	mannose-binding lectin
MHC	major histocompatibility complex
MMTV	mouse mammary tumor virus

mRNA	messenger RNA
MRSA	methicillin resistant *S. aureus*
MyD88	myeloid differentiation factor 88
NF	nuclear factor
NFAT	nuclear factor activated T cell
NS	nasopharyngeal samples
NTHi	nontypeable *Haemophilus influenzae*
ODN	oligodeoxynucleotide
OM	otitis media
PAM3CSK4	synthetic triacylated lipoprotein
PAMP	pathogen associated molecular pattern
PCR	polymerase chain reaction
PCV	pneumococcal conjugate vaccine
PGN	peptidoglycan
PKR	protein kinase receptor
Poly:IC	polyinosinic-polycytidylic acid
PRR	pattern recognition receptor
RAPD	randomly amplified polymorphic DNA
RNA	ribonucleic acid
RSV	respiratory syncytial virus
SARM	sterile α- and armadillo-motif-containing protein
SNP	single nucleotide polymorphism
ss	single stranded
STEPS	Steps to Healthy Development and Wellbeing of Children
Th	T helper
THL	National Institute for Health and Welfare
TIR	toll/interleukin-1 receptor
TLR	toll-like receptor
TNF	tumor necrosis factor
TRAF	TNF receptor–associated factor
TRAM	TRIF-related adaptor molecule
TRIF	TIR-domain-containing adaptor protein inducing IFN-β
URI	upper respiratory tract infection

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications, which are referred in the text by their corresponding Roman numerals. Previously unpublished data are also included.

I Vuononvirta Juho, Toivonen Laura, Gröndahl-Yli-Hannuksela Kirsi, Barkoff Alex-Mikael, Lindholm Laura, Mertsola Jussi, Peltola Ville, He Qiushui. Nasopharyngeal bacterial colonization and gene polymorphisms of mannose-binding lectin and toll-like receptors 2 and 4 in infants. PLoS One. 2011;6(10):e26198.

II Karppinen Sinikka*, Vuononvirta Juho*, He Qiushui, Waris Matti, Peltola Ville. 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 Pediatr Infect Dis Soc. 2013;2 (3): 240-247.

III Vuononvirta Juho, Peltola Ville, Mertsola Jussi, He Qiushui. Risk of repeated *Moraxella catarrhalis* colonization is increased in children with toll-like receptor 4 Asp299Gly Polymorphism. Pediatr Infect Dis J. 2013;32(11):1185-1188.

IV Vuononvirta Juho, Peltola Ville, Ilonen Jorma, Mertsola Jussi, He Qiushui. The gene polymorphism of *IL17* G152A is associated with increased colonization of *Streptococcus pneumoniae* in young finnish children. Pediatr Infect Dis J. (accepted).

* Equally contributed

1. INTRODUCTION

Respiratory infections are common during childhood. Acute respiratory infections are the most frequent cause of absence from school or day care. Viruses and bacteria are common causative agents of these infections. In addition to these two classes of pathogens, fungi can cause respiratory infections, but rather rarely and endemically. Bacterial respiratory infections are mainly caused by extracellular encapsulated bacteria such as *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Moraxella catarrhalis* and in some cases *Staphylococcus aureus*. *S. pneumoniae* and *M. catarrhalis* colonize as many as 54% and 72% of children, respectively, by 1 year of age (García-Rodríguez & Fresnadillo Martínez, 2002). *S. aureus* colonizes up to 35% of young children and is associated with a wide range of diseases. Infants who have been colonized by these pathogens at young age (>3 months) have a greater risk of developing respiratory infections in the next 6 to 9 months during their early life (Givon-Lavi *et al*. 2002; Bisgaard *et al*. 2007).

Toll-like receptors (TLRs) are important proteins of the innate immunity by recognizing several pathogen associated molecular patterns (PAMPs) from different organisms. Single nucleotide polymorphisms (SNPs) within *TLR*s can result in human susceptibility to infectious diseases (Schröder *et al.* 2005). Mannose-binding lectin (MBL) is also an important protein of innate immune system and it activates complement via lectin pathway. Polymorphisms in *TLR2*, *TLR3*, *TLR4* and *MBL2* are found to be associated with a higher risk of infectious diseases caused by gram-positive bacteria and to a reduced synthesis of pro-inflammatory cytokines (TLR2); reduced synthesis of pro-inflammatory cytokines and susceptibility to viral infections (TLR3); decreased response to lipopolysaccharide (LPS), lower plasma concentrations of several pro-inflammatory markers and increased risk of infectious diseases caused by Gram-negative bacteria (TLR4) and low serum MBL concentration that is related to an increased risk of different infectious diseases (Van Rijn *et al*. 2004; Schröder *et al*. 2005; Woehrle *et al*. 2008; Dhiman *et al*. 2008; Rantala *et al*. 2008).

Recent studies in mice have suggested that IL-17s play an important role in naturally acquired immunity to extracellular pathogens including *S. pneumonia*. However, little is known about role of IL-17s in protection against pathogenic bacterial colonization in humans. One SNP in *IL17A* has been shown to be associated with childhood asthma and to link bacterial colonization and the onset of asthma (Chen *et al*. 2010).

The study population for this thesis project was recruited from Finnish children, who are taking part in the ongoing study called Steps to Children's Healthy Development and Wellbeing (STEPS)-study. The STEPS-study is a prospective and observational cohort study in which 1827 children are followed-up from before birth. The outcome of this thesis is to provide a comprehensive view on the bacterial etiology and gene polymorphisms in the innate immunity. This project can also provide rational basis for further information and improvement to study respiratory infections in children.

2. REVIEW OF THE LITERATURE

2.1 Respiratory infections

Acute respiratory infections (ARIs) are one of the leading causes of mortality among young children, especially in developing countries but also in developed countries. It is estimated that in 1990's among children who were less than five years of age, three to five million deaths were caused by ARIs, and 75% of these caused by pneumonia (Smith *et al*. 2000). Since then, the number of deaths caused by ARIs in young children has remained rather similar, approximately 4.25 million deaths each year, and 930000 (22%) of them were due to pneumonia (World Lung Foundation, 2010; WHO, 2014). This decrease in mortality rates has been achieved with vaccines and increased immunization coverage and better understading of the hygiene. Despite the decrease of mortality rates throughout the world in the past decades, ARIs remain a major problem in many countries and is even increasing in some countries due to factors such as improper use of antibiotics, under utilization of rapid diagnostic methods, suboptimal breastfeeding and malnutrion often caused by political unrest and poverty (Chen *et al*. 2013; Nair *et al*. 2013; Selvaraj *et al*. 2014).

2.1.1 Different types of respiratory infections

UPPER RESPIRATORY TRACT INFECTION (URI)

Most upper respiratory tract infections (URIs) are of viral etiology and are often complicated and followed by secondary bacterial infections. Common URIs includes rhinosinusitis, acute otitis media (AOM), pharyngitis and laryngitis. Most prevalent causative agents of these URIs are human rhinovirus (HRV) with its 100 different serotypes and respiratory syncytial virus followed by bacteria such as *S. pneumoniae*, *M. catarrhalis*, *H. influenzae* and, in some cases, *S. aureus.* All these organisms show seasonal variation in incidence. Although they are listed as potential pathogens, they still can colonize the nasopharynx of a young child asymptomatically (Jourdain *et al*. 2011; Rodrigues *et al*. 2013 Hamilos *et al*. 2014). Mortality rates due to upper respiratory tract infections are not high in developed countries. In 2010, URIs caused approximately 3000 child deaths in United States and are are leading cause for a child to miss a day care or school (Lozano *et al*. 2012).

LOWER RESPIRATORY TRACT INFECTION (LRI)

Infections of the lower respiratory tract are often severe and can be fatal. The most common LRIs include bronchitis, bronchiolitis and pneumonia. Influenza can affect both the upper and lower respiratory tracts. Bacteria are dominant causative agents of LRIs in children. Approximately 60% of hospitalized pneumonia cases are caused by pathogens such as *S. pneumoniae* (the major causative agent), *H. influenzae*, *S. aureus* and *Klebsiella pneumoniae*. There are also atypical organisms such as *Mycoplasma pneumoniae*, which causes approximately 10% of hospitalized pneumonia cases (Michelow *et al*. 2004). Although bacteria are dominant agents behind pneumonia, nearly 40% of pneumonia cases in children are of viral etiology. Common viral pathogens include influenza A virus, respiratory syncytial virus (RSV) and human metapneumovirus (Nair *et al*. 2010; Krilov, 2011). It has been estimated that pneumonia accounts for approximately 20% of all paediatric deaths world wide. In the United States alone, pneumonia is the sixth leading cause of death and there has been little change in mortality due to LRIs (Carroll *et al*. 2002; Mizgerd *et al*. 2006).

2.2 Microbiology

2.2.1 Colonization of the nasopharynx

Nasopharynx contains various microorganisms, making it a complex ecosystem. In children, the nasopharyngeal flora becomes established within the first 12 months of their life including both commensal bacteria and potential pathogens (García-Rodríguez *et al*. 2002, Figure 1). This mixture of bacterial flora is in constant interaction between each other. Immune response of the host takes also part in this interaction and by doing this, it has an effect on the composition of the colonizing bacterial flora. Furthermore, there are external factors such as vaccines and antimicrobial treatment that can also affect bacterial flora of the nasopharynx. Commonly pathogens are persistently colonizing nasopharynx for months. Different strains of certain pathogen may colonize nasopharynx at the same time (Faden *et al.* 1995; Faden *et al*. 1997; Revai *et al*. 2006). Furthermore, Harrison *et al* studied nasopharyngeal bacterial flora in 72 infants from England and Wales since birth until 18 months of age, and observed that colonization of the nasopharynx by pathogenic bacteria in children younger than 3 months increases their risk to develop upper respiratory tract infections in the next 6 to 9 months of their early life (Harrison *et al.* 1999)**.**

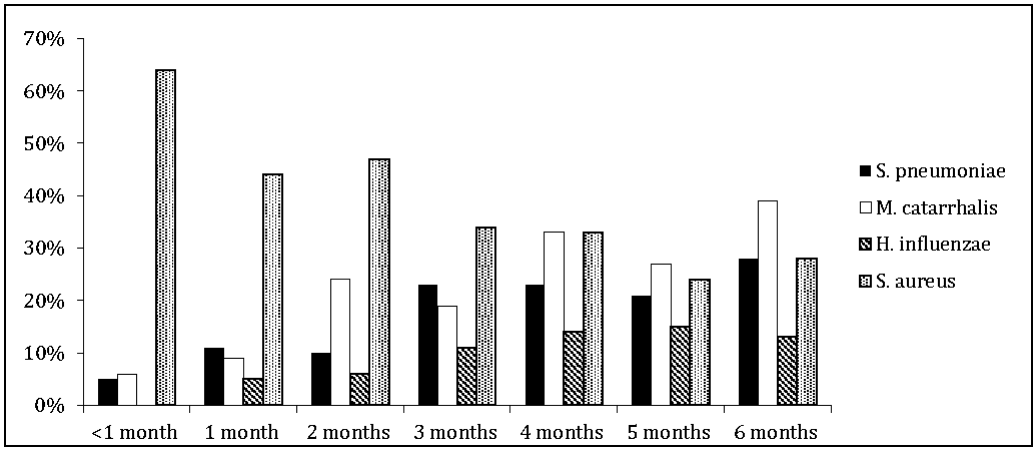


Figure 1. The carriage rate of four important pathogenic bacteria in children during the first six months of life. Figure modified from Harrison *et al*. 1999

There are variations in reported rates of bacterial carriage between studies and geographical sites. These differences have been related to genetic backgrounds and socio-economic conditions such as housing, access to health care, family size, number of siblings, day-care centers, overcrowded living conditions and poor hygiene (García-Rodríguez *et* al. 2002).

2.2.2 Characteristics of respiratory viruses

There are several viruses that colonize the respiratory tract and cause acute respiratory infections in children. One of the most common viral agents that cause URIs and common colds in young children are HRV belonging to the genus *Enterovirus* of the family *Picornaviridae*. HRV are isolated from all different age groups, but there have been studies showing greater incidence in HRV colonization and infection among children rather than adolescents and adults (Blomqvist *et al*. 2002). Although HRV is known to be associated in respiratory tract illnesses in children, it has not been thought to play a role in respiratory tract infections in infants. During the past decade, studies by using sensitive techiques such as Real Time-PCR have shown that HRV is associated with a significant burden of disease in infants and young children (Miller *et al*. 2011; Midulla *et al*. 2010). In a recent study carried out in Finland, it was oberved that from 77 healthy children (no symptoms) who were at 2 or 13 months of age, 5% were HRV positive and from 33 children with respiratory symtoms at 2 or 13 months of age, 45% were HRV positive (Toivonen *et al*. 2015). Bronchiolitis caused by HRV infection during infancy has been shown to cause wheezing, which in turn has been shown to be associated with development of asthma in later childhood (Gern *et al*. 2002; Jartti *et al*. 2004; Miller *et al*. 2007).

Human respiratory syncytial virus (RSV) is another common respiratory pathogen in infants and young children. It belongs to genus *Pneumovirus* in the family *Paramyxoviridae.* These include other respiratory viruses such as those causing measles. Most infants experience RSV infection during their first RSV season and approximately 20% of infants experience RSV associated wheezing during the first 12 months of their life. It is shown that about 3% of these infants require hospitalization (Glezen *et al*. 1986; Welliver *et al*. 2003). Toivonen *et al* observed that of Finnish children aged between 2 to 13 months and with respiratory symptoms, 3% were positive for RSV and that of children between the same ages but without respiratory symptoms, 3% as well were positive for RSV (Toivonen *et al*. 2015). In Finland, the latest large RSV epidemic season was 2012 when 2345 laboratory confirmed cases were reported and approximately 90% of these cases were from children aged 0-4 years (National Institute for Health and Welfare, Finland, Annual Infectious Diseases Surveillance Report 2013, www.thl.fi visited 14th January 2015). During the epidemic seasons, almost 90% of acute wheezing disease may be ascribed to RSV. Other acute wheezing disease episodes during the epidemic are attributed to other viruses such as HRV, human metapneumovirus and less frequently enterovirus, coronavirus or bocavirus (Marguet *et al*. 2009). RSV is one of the major causative agent in bronchiolitis among young children, a disease that has been shown to be associated with development of recurrent wheezing and asthma during childhood (Pullan *et al*. 1982; Sigurs *et al*. 2000). However, although RSV is the most common cause of bronchiolitis worldwide, this clinical condition may also be caused by parainfluenza viruses, influenza viruses and adenovirus.

2.2.3 Charasteristics of respiratory bacteria

S. pneumoniae

S. pneumoniae is a Gram-positive encapsulated diplococcus and it produces many virulence factors, the most important being its polysaccharide capsule which protects the bacterial cell from phagocytosis and hampers the complement activation. The polysaccharide capsule determines the serotype of *S. pneumoniae*; so far 93 different serotypes have been described. Other important virulence factors include pneumolysin, a pore-forming toxin which is able to lyse eukaryotic cells, and pneumococcal surface protein A that can protect the bacteria from host immune defence mechanisms (Hausdorff *et al*. 2005; Mitchell & Mitchell, 2010; Croucher *et al*. 2013).

S. pneumoniae is a common bacterium colonizing the nasopharynx of humans. Its carriage rate is higher in children than in adults and up to 90% of healthy children are able to carry *S. pneumoniae* in their nasopharynx asymptomatically (Bogaert *et al*. 2004; Rupa *et al*. 2014). Over 50% of children are colonized by *S. pneumoniae* at least once

during the first 12 months of life and its prevalence increases during the first 24 months of life and then starts to decrease at 3-5 years of age (Syrjänen *et al*. 2001; Murphy *et al*. 2009; Jourdain *et al*. 2011). However, apart of being a part of normal nasal microbiota, *S. pneumoniae* is also a major pathogen among children. It is a common cause of community-acquired pneumonia (CAP) (Table 1), AOM, rhinosinusitis and meningitis (Jacobs, 2004; Lynch & Zhanel, 2010). It accounts for more than half of all community acquired bacterial pneumonias and one third of acute bacterial rhinosinusitis (Anon *et al*. 2004; Johansson *et al*. 2010). Its also the most common bacterial finding in AOM since nearly 50% cases are positive for *S. pneumoniae* (Ruohola *et al*. 2006).

Each year, infections caused by *S. pneumoniae* contribute to a significant morbidity and mortality world wide each year leading to high burden and cost to health care systems. In developing countries, especially in children under 5 years of age, mortality rates for pneumococcal infections can be up to 50%, whereas in developed countries mortality rates from pneumococcal infections can be as low as 1-4% (O'Brien *et al* 2009; Rodgers *et al*. 2011; Gessner *et al*. 2010). In Finland, it is estimated that the annual incidence rate for *S. pneumoniae* community acquired pneumonia is 6.4/100 000 in children (Heiskanen-Kosma *et al*. 2003). In addition, in 2013 there were 724 invasive pneumococcal cases reported of which 4.6% were from children under 5 years of age (National Institute for Health and Welfare (Finland), Annual Infectious Diseases Surveillance Report 2013, www.thl.fi visited 14th January 2015). Furthermore, it is estimated that 500 000 cases of AOM occur annually, of which up to 60% have, major causative factor is *S. pneumoniae* (Niemelä *et al*. 1999; Kilpi *et al*. 2001). The incidence rates of these pneumococcal infections have remained quite stable within the past decade.

There are two types of vaccines available to prevent pneumococcal infection: a capsular polysaccharide and a conjugate vaccine (Mirsaeidi *et al*. 2014). Pneumococcal vaccines have had positive effect on decreasing the disease severity in community acquired pneumoniae and effectively reducing the incidence of invasive pneumococcal disease in children (Poehling *et al*. 2004; Pletz *et al*. 2008).

Table 1. Pathogens isolated from patients with community-acquired pneumonia

Common causes	Less common causes	Uncommon causes
S. pneumoniae	*Pseudomonas aeruginosa*	*Mycobacterium tuberculosis*
H. influenzae	*Pneumocystis jirovecii*	*M. pneumoniae*
S. aureus	*M. catarrhalis*	*Chlamydophila pneumoniae*
Influenza virus		

Modified from Musher & Thorner, 2014

M. catarrhalis

M. catarrhalis, previously known as *Branhamella catarrhalis*, is a human restricted, uncapsulated, Gram-negative bacterium. For a long period of time, *M. catarrhalis* was thought be just a normal commensal of the upper respiratory tract. During the past decades, this bacterium has started be recognized as a pathogen of the upper and lower respiratory tracts (de Vries *et al*. 2009). *M. catarrhalis* is the third most common pathogen causing AOM during childhood; it being responsible for approximately 15%-20% of AOM cases (Verduin *et al*. 2002). Moreover, approximately 20% of sinusitis cases in young children are caused by *M. catarrhalis* (Wald, 1998). It is also the second most common causative agent in chronic obstructive pulmonary disease (COPD) in adults, and estimated to be responsible for 15% of acute exacerbations in COPD (de Vries *et al.* 2009; Aebi, 2011). The carriage rate of *M. catarrhalis* is rather high in healthy children; 8% at one month of age and increasing up to 80% at two years of age. Its carriage rate in healthy adults is considerablely lower, around 3% to 5% (Faden *et al.*1997; Murphy *et al.* 2005; Bisgaard *et al*. 2007).

M. catarrhalis has specific receptors that allow it to bind to host epithelia and extracellular matrix. Due to these receptors, it is able to colonize the mucosal surface of the middle ear of young children (Tan *et al*. 2006; Hall-Stoodley *et al*. 2006; Hallström *et al*. 2011). *M. catarrhalis* is able to invade the host epithelial cells and thus evade the host's immune surveillance. It also interacts and competes with other commensal flora and is able to survive and multiply under challenging conditions by forming microcolonies and biofilm (de Vries *et al*. 2009). Currently, there are no vaccines available to prevent *M. catarrhalis* infections; there have been some vaccine studies have showed promising results in animal models (Hassan, 2013).

H. influenzae

H. influenzae is a gram-negative bacterium and a part of normal human respiratory tract microbiota. It is also a major pathogen to cause several respiratory conditions in children. The nasopharynegal colonization of *H. influenzae* starts in early childhood (King, 2012). It is estimated that up to 20% of children will be colonized by *H. influenzae* during the first 12 months of their life while by the age of 5 years approximately 50% of children are colonized by it (Howard *et al*. 1988).

H. influenzae is divided into typeable and nontypeable *H. influenzae* (NTHi), based on the precence or absence of a polysaccharide capsule. The typeable *H. influenzae* strains have a polysaccharide capsule and they include six serotypes (a to f). Serotype b *H. influenzae* (Hib) is the most notable and can cause severe invasive diseases

such as meningitis, epiglottitis and pneumonia (King, 2012). However, incidence of invasive diseases has decreased dramatically since the introduction of Hib vaccine in 1989 (Wenger *et al*. 1993; Madore *et al*. 1996). In contrast to typeable *H. influenzae*, NTHi rarely causes infections outside the respiratory tract and is responsible for majority of respiratory tract infections and diseases together with *S. pneumoniae* and *M. catarrhalis* (Klein, 1997). Nontypeable *H. influenzae* are responsible for one-third of AOM episodes in children and it is the most common cause of recurrent AOM (Murphy *et al*. 2009). The 10-valent pneumococcal conjugate vaccine (PCV) includes a protein D antigen, which is expressed by some NTHi and could therefore indirectly reduce the incidence of NTHi. Results of one studies suggest that this vaccine may prevent episodes of AOM more effectively than the previous 7-valent PCV (Prymula *et al*. 2006).

S. aureus

S. aureus is a gram-positive commensal bacterium that frequently colonizes various epithelial surfaces such as the human respiratory tract, especially the nasopharynx. Despite being a commensal bacterium *S. aureus* also causes many infectious syndromes. It is the most common cause of surgical wound infections and the second most common cause of bloodstream infections (Lowy *et al*. 1998). *S. aureus* bacteremia can be complicated by a.o.t. endocarditis, metastatic infections or the sepsis syndrome. Nasopharyngeal carriage rate of *S. aureus* is high in young children, the rate being close to 50% during the first eight weeks of life and then decreasing to 20% by the age of six months. Moreover, more than 70% neonates are at least once positive for nasal culture (Peacock *et al*. 2003). Children are also considered to be persistent carriers of *S. aureus* more frequently than adults and there is a transition from persistent carriage to non-persistent state during adolescence (Wertheim *et al*. 2005).

S. aureus expresses many potential virulence factors such as surface proteins that promote the colonization of host tissues and membrane damaging toxins that can lyse eukaryotic cell membranes and damage host tissues (Lowy *et al*. 1998). *S. aureus* has become increasingly antibiotic resistant over the decades, a good exmaple of this being methicillin-resistant *S. aureus* (MRSA) which is strongly associated with skin and soft tissue infections (Blumental *et al*. 2013). Currently there is no vaccine against *S. aureus*. Clinical human trials aiming to develop efficient vaccines have unfortunately failed (Fowler *et al*. 2014).

2.3 Immunology

2.3.1 Innate and adaptive immunity

The human immune system comprises two integrated and cooperative sub-systems, innate and adaptive immunity. The innate immunity is the first line of host defense against the invading pathogens and it consists of phagocytes such as macrophages and dendritic cells (DCs) that trigger immune system as well as humoral factors and surface barriers (Kumar *et* al.2011). The cells of innate immunity express a set of germline encoded pattern recognition receptors (PRRs). These recognize many different pathogen-associated molecular patterns (PAMPs). The PAMPs, such as lipopolysaccharide (LPS), peptidoglycan (PGN), lipoteichoic acid (LTA), lipoarabinomannan (LAM), lipopeptides, yeast wall mannans, bacterial DNA and flagellin are capable of stimulating innate immunity responses. This recognition of PAMPs by PRRs results in phagocytosis and/or activation of pro-inflammatory pathways (Maródi, 2006; Strunk *et al*. 2011). Adaptive immunity is involved in the "late" phase of the infection by eliminating invading pathogens as well as generating the immunological memory (Akira *et al*. 2006; Iwasaki & Medzhitov, 2010). In contrast to innate immunity, adaptive immunity is mediated by two types of antigen receptors, T-cell receptors and B-cell receptors that are expressed on T and B lymphocytes. These receptors recognize specific antigen epitopes that are presented the cells of adaptive immunity by the cells of innate immunity or, in the case of thymus independent antibody responses, antigens are directly recognized by the B cell receptor (Iwasaki & Medzhitov, 2010). The recognition of antigen epitopes is a very effective mechanism to protect the host but is activated more slowly compared to innate immunity. In addition to T and B lymphocytes there are important innate lymphoid cells (ILCs). These ILCs produce many Th cell-associated cytokines but they do not express cell-surface markes that are associated with other immune cell lineages. Moreover, these ILCs subsets do not express T cell receptor and therefore do not respond in an antigen-specific manner (Walker *et al*. 2013). However, when the immune system of the host encouters the same infective antigenic agent, the activation of the adaptive immunity is rapid as a result of the immunological memory created after the first encouter with the same antigenic agent (Iwasaki & Medzhitov, 2010).

2.3.2 Pattern recognition receptors (PRRs)

Host defense is largely relies on germline encoded PRRs which are expressed on the cells of innate immunity. These PRRs recognize PAMPs from different organisms. Recent evidence suggests that PRRs can also recognize endogenous molecules from damaged cells (Takeuchi *et al*. 2010). PRRs are generally divided into three distinct groups based on their localization and functional properties. The first group consists of

soluble PRRs such as MBL, the second group consists of endocytic receptors such as the macrophage scavenger receptor and the third group are signaling receptors consisting of membrane-bound and cytoplasmic receptors such as TLRs. Soluble receptors are involved in activating the complement cascades and opsonizing invading pathogens, whereas the signaling receptors are responsible of activation of inflammatory signaling after pathogen exposure (Takeuchi *et al*. 2010; Schlapbach *et al*. 2010; Deban *et al*. 2011). The endocytic PRRs play an important role in uptake and clearance of components, such as modified host molecules, apoptotic cells and microorganisms. However, the "signaling pathways" of endocytic PRRs are not well understood since they function without any intracellular signal (Mukhopadhyay *et al*. 2004; Canton *et al*. 2013).

2.4 Toll-like receptors (TLRs)

The story of TLRs started in 1980s, when antimicrobial peptides were found to be key mechanisms of innate host defense in insects. A decade later it was found that the fruit fly *Drosophila melanogaster* had a protein called Toll and it had an intracellular Toll/interleukin-1 receptor (TIR) domain in common with mammalian interleukin-1 receptor (IL-1R) (Casanova *et al*. 2011). This quickly led to a finding of the mammalian TLR family. The first mammalian TLR was found and described in 1997 (Medzhitov *et al*. 1997; Rock *et al*. 1998). The human TLRs act as receptors for pathogens and there are 10 active human TLRs described thus far (Lee *et al*. 2014). TLRs are characterized by the varying numbers of leucine-rich-repeat (LRR) motifs in their extracellular domains and the cytoplasmic TIR domain. Each LRR domain consists 19-25 LRR motifs, each of which is 24-29 amino acids in length (Akira *et al*. 2006; Botos *et al*. 2011). TLRs can be divided into five subfamilies based on their genomic structure and amino acid sequence and these five subfamilies are TLR2, TLR3, TLR4, TLR5 and TLR9. The TLR2 subfamily is composed of TLR1, TLR2, TLR6 and TLR10 whereas the TLR9 subfamily is composed of TLR7, TLR8 and TLR9. TLR 1 and TLR6 can form heterodimers with TLR2 (Kawai & Akira, 2010). Various immune cells such as dendritic cells, macrophages, B cells, T cells and even non-immune cells express TLRs extra- or intracellularly. TLRs 1, 2, 4, 5 and 6 are expressed on the cell surface whereas TLRs 3, 7, 8, and are expressed in intracellular compartments like endosomes (Moresco *et al*. 2011). TLR10 is the only TLR in which ligand and function have not been defined. However, a recent study has shown that TLR10 is able to dampen TLR2 responses, thereby suppressing immune responses through production of IL-1Ra (Oosting *et al*. 2014).

TLR4 was the first TLR to be indentified as a signal transducer for LPS which is a major cell wall component of gram-negative bacteria (Poltorak *et al*. 1998). Thereafter,

TLRs have been shown to recognize and transmit signals for wide range of microbial components (Table 2). In addition to microbial ligands, TLRs also recognize some endogenous ligands such as heat shock proteins (Hsp) and some synthetic ligands such as polyinosinic-polycytidylic acid (Poly-IC). The interaction between TLRs and PAMPs indicate the presence of foreign antigen and may activate specific signaling pathways leading to inflammatory and immune responses (Yu *et al*. 2010). The recognition of PAMPs by TLR2, TLR3 and TLR4 as well as the activation of specific signaling pathways of TLRs will be discussed in more detail.

Table 2. Some examples of microbial, endogenous and synthetic ligands for TLRs.

Toll-like receptor	Microbial, endogenous and synthetic ligand
TLR1/2 heterodimer	Triacyl lipopeptides (bacterial), PAM3CSK4 (synthetic)
TLR2	Lipoproteins and Peptidoglycans (bacterial), MALP2 and PAM3CSK4 (synthetic), zymosan (fungi)
TLR3	dsRNA (viral), Poly-IC (synthetic)
TLR4	LPS (bacterial), HSPs (endogenous)
TLR5	Flagellin (bacterial)
TLR6/2 heterodimer	Lipoproteins and PGN (bacterial), MALP2 (synthetic), Zymosan (fungi)
TLR7	ssRNA (viral), Imidazoquinoline (synthetic)
TLR8	ssRNA (viral), Imidazoquinoline (synthetic)
TLR9	CpG-DNA (bacteria), CpG-ODN (synthetic)
TLR10	Not yet identified

2.4.1 Recognition of PAMPs by TLR2, TLR3 and TLR4

TLR2 is a transmembrane signaling receptor that can recognize various type of PAMPs and it was shown to recognise the widest spectrum of different ligands among all TLRs as described in Table 3. Earlier *in vitro* studies on *TLR2* knockout mice and by the deletion of *TLR2* to macrophages has resulted in loss of lipoprotein and peptidoglycan recognition and responsiveness (Medzhitov&Janeway 2000). TLR2 has an unique ability to form heterodimers with TLR1 and TLR6 and this is thought to enable TLR2 to recognize such a large number of ligands (Kawai & Akira, 2010). Studies employing diacylated and triacylated lipoproteins have shown that they require TLR2/6 heterodimers for cell activation, while for TLR2/1 heterodimers are required for triacylated lipoprotein-induced activation (Lee *et al*. 2012). Moreover, Hoebe *et al*. showed that TLR2/6 heterodimers require CD36 to be able to sense diacylated lipoproteins but for TLR2/1 heterodimers this is not needed (Hoebe *et al*. 2005).

TLR3 is an intracellular signaling receptor that recognizes viral double-stranded RNA (dsRNA). DsRNA is known to bind only intracellular targets such as dsRNA-dependent

protein kinase (PKR). The existense of TLR3 was discovered when PKR-deficient mice where it was found to respond to Poly-IC which is a synthetic version of viral dsRNA (Alexopoulou *et al*. 2001). Furthermore, TLR3 has been shown to recognise endogenous messenger RNA (mRNA) and sequence-independent small interfering RNAs (Karikó *et al*. 2004; Karikó *et al*. 2004). TLR 3 has evolved principally to recognize viral dsRNA for example during viral replication (Zhang *et al*. 2013)

TLR4 is a trans-membrane signaling receptor and principally expressed in macrophages and dendritic cells. Studies have shown that several different PAMPs are capable of stimulating TLR4 (Takeuchi *et al*. 1999; Medzhitov&Janeway, 2000). These PAMPs include LPS from Gram-negative bacteria, the fusion protein F from RSV and the envelope protein from mouse mammary tumor virus (MMTV). In addition, endogenous molecules such as heat-shock proteins can also interact directly or indirectly with TLR4 (Erridge, 2010). LPS, an important bacterial cell wall component for gram-negative bacteria, is the best characterized and described ligand for TLR4 (Sender & Stamme, 2014). Functional TLR4 requires MD-2 molecule to form a complexed with the extracellular domain of TLR4 to enable effective LPS recognition (Park & Lee, 2013). Lipopolysaccharide binds to serum LPS-binding protein (LBP) which rapidly then catalyzes the transfer of LPS to membrane bound CD14. CD14 presents LPS to the TLR4/MD-2 complex leading to activation of TLR4 signaling pathway (Peri *et al*. 2010).

2.4.2 Toll-like receptor signaling

The specific signaling pathways that are activated by different TLRs after they have recognised PAMPs in the host, are important for innate immunity. TLR signaling involves five adaptor proteins which link with downstream protein kinases eventually leading to the activation of transcription factors such as members of nuclear factor-kappaB (NF-kappaB) and interferon (IFN)-regulatory factor (IRF) families. TIR domain is the key signaling domain in the TLR system. It is located in the cytosolic face of each TLR and also in the adaptor proteins (Jenkins & Mansell, 2010). The TIR-domain containing adaptor proteins are myeloid differentiation factor 88 (MyD88), MyD88-adaptor-like (MAL or TIRAP), TIR-domain-containing adaptor protein inducing IFN-β (TRIF or TICAM1), TRIF-related adaptor molecule (TRAM or TICAM2) and sterile α- and armadillo-motif-containing protein (SARM) (Jenkins & Mansell, 2010).

MyD88 is a key adaptor protein that is shared by all TLRs except TLR3 and by this way TLR signaling is divided into two pathways, the MyD88-dependent pathway and the MyD88-independent pathway (Figure 2). TLR4 can activate either MyD88-dependent or MyD88-independent pathway, of these the latter pathway is unique to TLR3 (Yamamoto

& Takeda, 2010; Frans *et al*. 2014). In MyD88-dependent pathway MyD88 associates with TIRAP and recruits IL-1 receptor-associated kinases (IRAK1-4) to TLRs. After IRAK (1/2/4) recruiment, they are activated by phosporylation and associate with TNF receptor–associated factor-6 (TRAF-6) eventually leading to activation of NF-kβ and MAP-kinase pathways as well as expression of pro-inflammatory cytokines (Yamamoto & Takeda, 2010; Frans *et al*. 2014). The MyD88-independent pathway is activated by TLR3 and TLR4 through TRIF leading to activation of IRF-3 and NF-kβ. TLR3 can directly associate with TRIF, whereas TLR4 needs an additional adaptor protein called TRAM to be able to associate with TRIF. IRF-3 is phosporylated and translocated to the nucleus where it induces INF-β gene and several other targets. Moreover, the association of TLR3 and 4 with TRIF can also lead to NF-kβ activation through TRAF-6 which leads to gene expression of IFN-β and inflammatory cytokines (Kawasaki & Kawai, 2014). Human monogenic immunodefiencies in these signaling pathways are known to be caused by mutations in *MYD88*, *IRAK4*, *TLR3*, *UNC93B1*, *TRIF*, *TRAF3* and *TBK1*, and cause severe susceptibility to certain bacteria and viruses (Alsina *et al*. 2014)

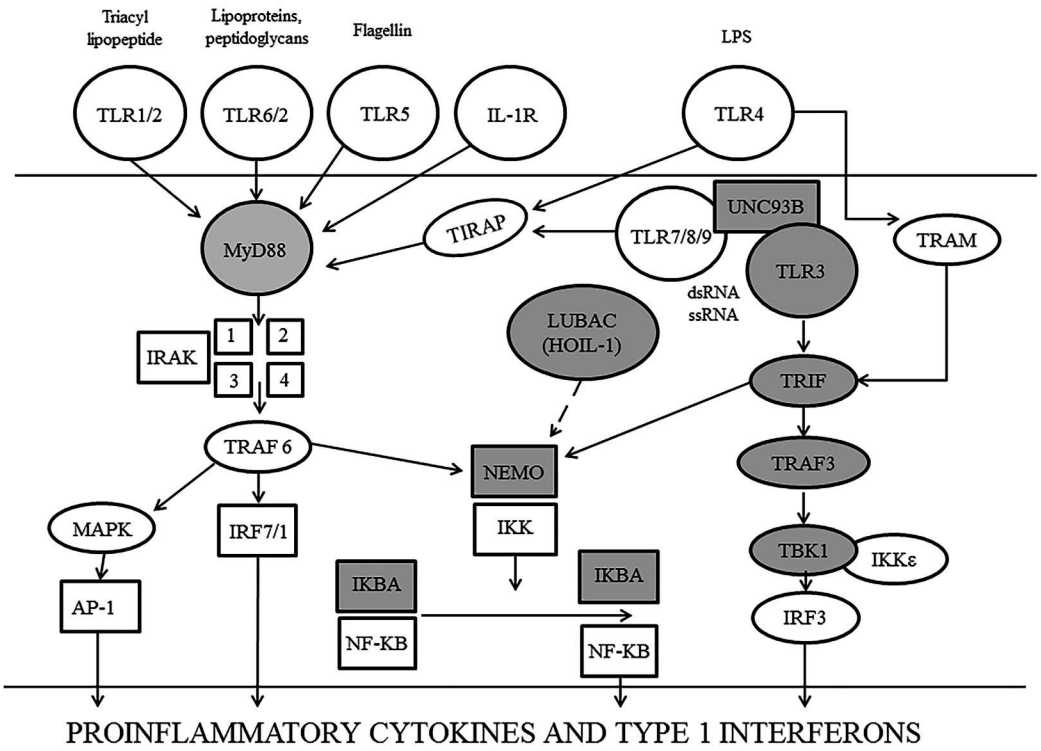


Figure 2. TLR-mediated immune responses: MyD88-dependent and MyD88-independent pathways. Figure modified from Frans *et* al.2014.

2.5 Mannose-binding lectin

MBL initiates complement lectin pathway (Daha, 2010). MBL belongs to the collectin family of proteins and has an oligomeric structure, each monomere containins a C-type carbohydrate recognition domain that is joined to a collagenous region. MBL shares similarities to C1q which is the antibody-recognizing moiety of the first component of the classical pathway of complement activation. MBL is mainly synthesized by the liver and it circulates as a serum protein. However, MBL has been identified also from other sites such as middle ear fluid and nasopharyngeal secretions (Garred *et al.* 1993). In blood circulation, MBL associates with four proteins that are structurally related to it. These proteins are MBL-associated serine proteases (MASP)-1, 2, 3 and Map19 of which MBL-MASP-2 complex seems to be the most efficient activator of the lectin pathway of the complement system (Beltrame *et al*. 2015). In addition to activation of complement system, MBL has also been shown to promote the complement-independent opsonophagocytosis, modulate inflammation and to promote apoptosis (Daha, 2010).

2.5.1 MBL binding to microorganisms

MBL has the ability to bind sugar groups such as *N*-acetylglucosamine, mannose, *N*-acetylmannosamine and fucose, which are displayed on the surfaces of wide range of microorganims through multiple carbohydrate-recognition domains (CRD). These microorganisms include gram-positive and gram-negative bacteria, viruses, fungi, and protozoa (Table 3). MBL's ability to bind to these specific sugar groups of different microorganisms makes it possible to differentiate self from non-self. MBL can also bind to nucleic acids, phospholipids and non-glycosylated proteins (Palaniyar *et al*. 2004; Dommett *et al*. 2006).

Table 3. Some microorganisms that bind to MBL. Table modified from Dommett *et al*. 2006.

Gram +/- bacteria	Viruses	Fungi	Protozoa
S. pneumoniae	HIV*	*Aspergillus fumigatus*	*Cryptosporidium parvum*
H. influenzae	Herpes simplex 2	*Candida albicans*	*Plasmodium falciparum*
S. aureus	Influenza A	*Cryptococcus neoformans*	*Trypanosoma cruzi*
Escherichia. coli	SARS-CoV*		
Klebsiella aerogenes			

*SARs-CoV, severe acute respiratory syndrome–coronavirus; *HIV, human immunodeficiency virus

As described earlier, in circulation MBL is bound with MASP proteins. The activation of MBL-MASP complex leads to activation of the lectin pathway. MASP-2 cleaves C4 to produce C4b and ultimately C4b2a, which is the C3 convertase. The following production of C3b, which is a key opsonic molecule, alleviates the activity of C4b2a3b (C5-convertase). The following cleavage of C5 produces anaphylatoxin C5a and finally C5b fragment activates membrane-attack complex formation that eventually causes the lysis of invading pathogen (Figure 3, Beltrame *et al*. 2015).

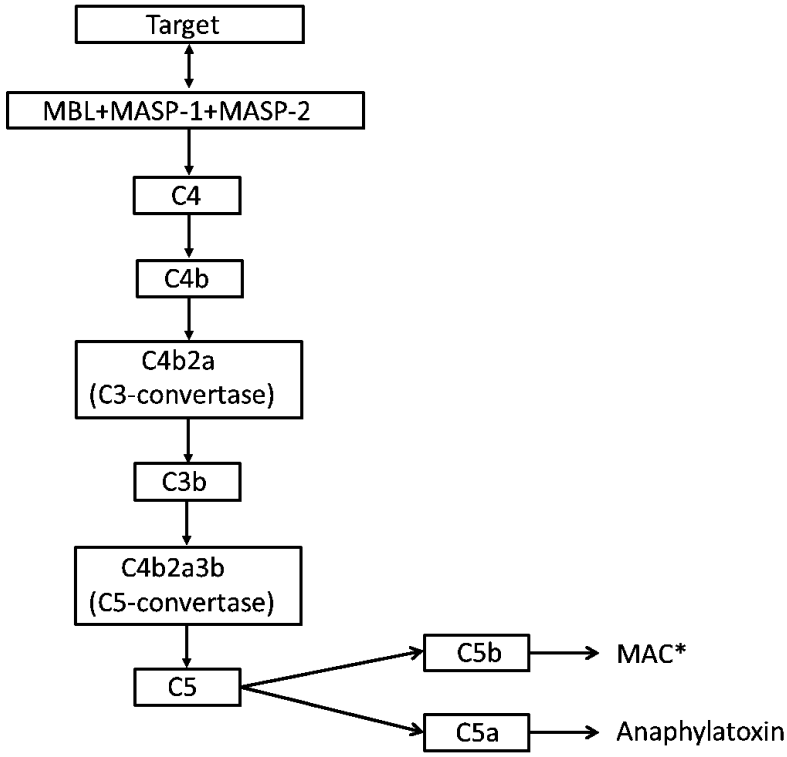


Figure 3. Complement activation (lectin) pathway. Based on information from Beltrame *et* al. 2015.

2.6 T helper cells and Th17 cytokines

Cytokines are responsible for most of the biological effects in the immune system, such as cell-mediated immunity and allergic responses. They can be functionally divided to pro- and anti-inflammatory cytokines. T lymphocytes are the major cytokine producing cells and express CD3. There are two main subsets of T lymphocytes, distinguished by the presence of cell surface molecules known as CD4 and CD8. T lymphocytes expressing

CD4, called T helper (Th) cells, are proficient cytokine producers. They can be divided to several subsets, including Th1, Th2, Th9, Th17, Th22 and regulatory T cells (Tregs) (Figure 4) (Raphael *et al*. 2014). CD4 T lymphocyte differentiation is initiated when dendritic cells recognize invading pathogens through their TLRs (Iwasaki & Medzhitov, 2010). DCs express all the reported TLRs, major histocompatibility complex (MHC) and the co-stimulatory molecules CD80 and CD86 (Schreibelt *et al*. 2010). DCs migrate to lymph nodes to activate antigen-specific naïve T cells where the cytokine environment surrounding the naïve CD4 cells determines their differentiation: production of IL-12 drives naïve T cells to Th1 cells and they produce INF-γ, IL-2 and tumor necrosis factor (TNF)-β, whereas IL-4 drives naïve T cells into Th2 cells that produce IL-4, IL-5, IL-6, IL-10 and IL-13 (Figure 4, Coomes *et al*. 2013). A new paradigm in naïve T cells biology indicates that T cells are much more flexible than what was previously thought. T cells have shown plasticity with the ability to change between helper phenotypes and even between helper and regulatory functions (Coomes *et al*. 2013)

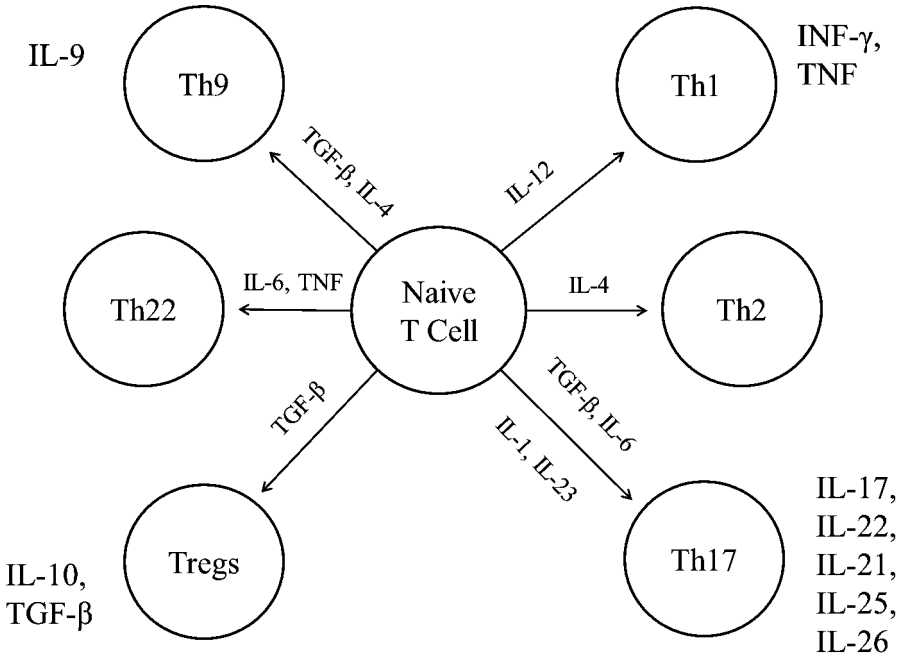


Figure 4. Th0 cell differentiation. Modified from Raphael *et* al. 2014.

The identification of IL-17 family of cytokines together with IL23- mediated expansion of IL-17 producing T cells led to the discovery of newer subset of Th cells defined as Th17 cells. Th17 cells produce IL-17A, -B, -C, IL-17E (IL-25), IL-17F, IL-21 and IL-22 (Song *et al*. 2014). The majority of research related to Th17 cells has been focused on IL-

17A, commonly known as IL-17. IL-17 is involved in host defense against infection and development of inflammation by inducing production of pro-inflammatory cytokines, chemokines and antimicrobial peptides. Moreover, IL-17 is a key cytokine for recruiting neutrophils to the site of inflammation and infection (Reynolds, 2010). IL-17 has been shown to play an important role in protecting the host from extracellular pathogens such as *S. pneumoniae* and *S. aureus* in epithelial and mucosal tissues (Ishigame *et al*. 2009; Wright *et al*. 2013). Recent studies have linked IL-17 to the development of allergic and autoimmune diseases such as asthma, multiple sclerosis and rheumatoid arthritis (Iwakura *et al*. 2011; Chien *et al*. 2013).

2.7 Single nucleotide polymorphisms in the genes of innate immunity

Humans and other multicellular organisms have evolved immunological defense mechanisms, categorized as innate and adaptive immunity, under the pressure from infectious microorganisms. The initial response to infection is provided by the innate immune system enabling the host to discriminate among pathogens and to trigger subsequent adaptive immune responses through antigen-specific mechanisms. Many single nucleotide polymorphisms (SNPs) have been reported in the genes of innate immunity and SNPs in these genes have been associated to various bacterial/viral infections, autoimmune diseases and cancers. Innate immunity genes where potentially important SNPs have been identified include genes such as those for the TLR family, MBL (*MBL2*), caspase recruitment domain-containing protein 15 (*CARD15*/*NOD2*) and macrophage migration inhibitory factor (*MIF*) (Forrest *et al*. 2006). Below, polymorphisms in *TLR2*, *-3* and *-4*, *MBL2* and *IL17A* will be described in more detail.

2.7.1 SNPs in *TLR*s

TLRs can be classified based on their known agonists. Intracellular TLRs recognise nucleic acid-based agonists whereas cell-surface TLRs recognise other products such as glycolipids, lipopeptides and flagellin. Intracellular TLRs (TLR3, -7, -8 and -9) are mostly involved in recognition of different viruses. These TLRs have been evolving under a strong purifying selection and it is thought that neither nonsense nor missense mutations that are predicted to be damaging are endured in these four TLRs (Ramensky *et* al. 2002). This selection demostrates that intracellular TLRs have an essential biological role in host survival and polymorphisms in these genes could be expected lead to severe disorders. This hypothesis has been validated by clinical genetic studies on *TLR3* (Zhang *et al*. 2007; Gorbea *et al*. 2010; Guo *et al*. 2011). For cell-surface TLRs (TLR1, 2, 4, 5, 6 and 10) which recognise PAMPs other than nucleic acids, the purifying selection

described for intracellular TLRs is not as strong as with intracellular TLRs. It has been shown that up to 23% in general population have damaging missense polymorphisms at least in one of the cell-surface TLRs. However, some of the SNPs in cell-surface TLRs are unlikely to play critical role in host survival (Barreiro *et al*.2009). Furthermore, some polymorphisms in cell-surface TLRs can, under particular circumstances, be advantageous for protective immunity and polymorphisms in these TLRs are not only endured but may also be subject to positive selection in certain populations. In other words, impaired TLR-mediated responses may in some cases be beneficial for the survival of the host (Casanova *et al*. 2011).

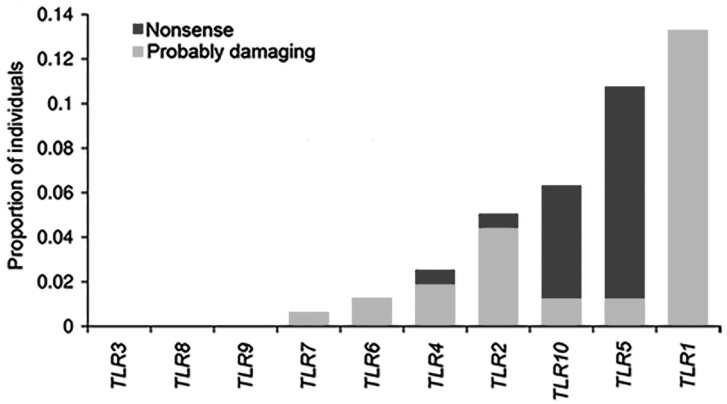


Figure 5. Proportion of individuals in the general population (158 healthy individuals from Sub-Saharan Africa, Europe and East Asia) carrying a probably damaging or a nonsense mutation for each individual *TLR* (Barreiro *et al*. 2009).

TLR2 recognises a wide spectrum of ligands through its unique ability to form heterodimers with TLR1 and TLR6. Several SNPs in *TLR2* have been reported in functional and genetic association studies. A polymorphism R753Q (arginine changes to glutamine in amino acid position 753 as a result of nucleotide substitution G>A in mRNA position 2258; rs5743708 is a reference SNP cluster report code for *TLR2* R753Q, Table 4) which is located in the TIR-domain is the best characterized in functional studies and has been shown to be associated with altered signaling (Schröder *et al*. 2005). It has been suggested that the compromised signaling by *TLR2* R753Q is not due to lower expression of polymorphic receptor but instead due to deficient tyrosine phospholyration. This defect compromises TLR2-TLR6 assembly to form heterodimer and impairs the recruitment of MyD88 (Xiong *et al*. 2012). The SNP R753Q has been shown to be associated with different diseases and infections. It is associated with pneumococcal meningitis, staphylococcal infections, *S .aureus* colonization and with increased risk for

tuberculosis in adult patients (Lorenz *et al*. 2000; Tellería-Orriols *et al*. 2014). However, *TLR2* R753Q polymorphism has a protective effect against late stage Lyme disease in Europeans (Schröder *et al*. 2005). The frequency of *TLR2* R753Q differs among different case cohorts and control cohorts. A study carried out by Ma *et al*. showed variation between three ethnic groups, African American, European American and Hispanic. In African American control group (n=194), none of the subjects (0%) had heterozygote *TLR2* R753Q (guanine changes to adenine [G/A] in mRNA position 2258 creating two different alleles of a gene) and, in the same ethnic group, from 339 cases only two subjects (0.6%) had *TLR2* R753Q. In European American controls, five subjects (4.5%) out of 110 had *TLR2* R753Q and in cases nine subjects (5%) out of 180 had *TLR2* R753Q. In Hispanic controls, four subjects (4%) out of 110 had *TLR2* R753Q and in cases only one subject (0.3%) out of 374 had *TLR2* R753Q (Ma *et al*. 2007). Similar frequencies were observed in another study that was carried out using a New Zealand Caucasian cohort. From 182 Crohn's disease patients and from 188 ethnically matched controls, 3% and 2%, respectively, had *TLR2* R753Q (Hong *et al*. 2007, Table 5). In none of these studies, homozygote *TLR2* R753Q (guanine changes to adenine in mRNA position 2258 to create two identical alleles of a gene [A/A] that are present in both homologous chromosomes) was detected in the study subjects. However, frequency of *TLR2* R753Q and its associations with infections and nasopharyngeal bacterial colonization in Finnish population have not been reported.

TLR3 is an important intracellular signaling receptor responsible for recognition of viral dsRNA. Several SNPs in *TLR3* has been recently studied. The SNP L412F (leucine changes to phenylalanine in amino acid position 412 as a result of nucleotide substitution C>T in mRNA position 1234; rs3775291, Table 4) is one of the most well-described polymorphisms of *TLR3*. It affects a residue localized near the concave surface of the TLR3 ectodomain. It has been hypothesized that the *TLR3* L412F polymorphism will alter hydrophobic interactions and glycosylation of neighboring residues which are critical for TLR3 function. Moreover, *TLR3* L412F has an effect on a residue that is highly conserved from human to fish implying that a polymorphism in this site is likely to be obstrusive for the structure and function of TLR3 (Choe *et al*. 2005; Bell *et al*. 2006; Ranjith-Kumar *et al*. 2007). For instance, *TLR3* L412F has been shown to be associated with increased susceptibility to chronic mucocutaneous candidiasis, with decreased secretion of INF-γ in response to stimulation with Poly:IC *in vitro* in HEK293 cells, with risk for recurrent herpes simplex virus infection and with bronchiolitis leading to hospitalization and postbronchiolitis wheezing in young infants (Nahum *et al*. 2011; Yang *et al*. 2012; Nuolivirta *et al*. 2012). The frequency of *TLR3* L412F in different ethnic groups is rather high when compared for example to SNPs reported in *TLR2*

and *TLR4* and it also differs between ethnic groups. For *TLR3* L412F, in a study that consisted of 135 healthy ethnic Lithuanians, 29% were heterozygotes (cytosine changes to thymine [C/T] in mRNA position 1234 creating two different alleles of a gene) and 19% homozygotes (cytosine changes to thymine in mRNA position 1234 to create two identical alleles of a gene [T/T] that are present in both homologous chromosomes) (Kindberg *et al*. 2011). In healthy 131 Hispanic controls, 40% were heterozygotes and 12% were homozygous for *TLR3* L412F (Sironi *et al*. 2012). In 274 healthy Asian controls, 53% were heterozygous and 5% were homozygotes for *TLR3* L412F (Palikhe *et al*. 2011, Table 5). The frequency of *TLR3* L412F and its possible association in Finnish infants with bronchiolitis and postbronchiolitis wheezing have been studied (Nuolivirta *et al*. 2012). However, frequencies for *TLR3* L412F polymorphism, possible disease associations and associations to nasopharyngeal bacterial or viral colonization have not been reported in Finnish population.

TLR4 is the major receptor for innate immune response to LPS or endotoxin which are the key components of the Gram-negative bacteria. The *TLR4* gene has two important SNPs, D299G and T399I, that are in linkage disequilibrium. From these two SNPs, *TLR4* D299G has been intensively studied in functional and genetic association studies (Trejo de la *et al*. 2014). The *TLR4* D299G (aspartic acid changes to glycine in amino acid position 299 as a result of nucleotide substitution A>G in mRNA position 1194; rs4986790, Table 4) polymorphism lies within the extracellular domain of the receptor but the mechanism behind the dysregulated *TLR4* D299G signaling is unclear. It has been shown with crystallography that LPS binds to TLR4/MD2, where *TLR4* gene contains D299G polymorphism, to form receptor dimers in the same manner as the *TLR4* normal type but local conformational differences in the formed receptor dimers are observed between the normal type and the variant type (Ohto *et al*. 2012). *TLR4* D299G has been shown to be associated with increased susceptibility to infections caused by gram-negative bacteria with a hyporesponsiveness to LPS, resulting in improper activation of TLR4 signaling pathways and insufficient production of proinflammatory cytokines and interferons (Agnese *et al*. 2002; van der Graaf *et al*. 2005; Miller *et al*. 2005). Furthermore, *TLR4* D299G has been associated with increased susceptibility to infections caused by RSV and increased risk to Gram-negative septic shock (Tal *et al*. 2004; Lorenz *et al*. 2002). The frequency of *TLR4* D299G differs marginally between different ethnic groups. In 1482 ethnic German volunteers, 10% were heterozygote for *TLR4* D299G (adenine changes to guanine [A/G] in mRNA position 1194 to create two different alleles of a gene), only 0.2% were homozygote *TLR4* D299G (adenine changes to guanine in mRNA position 1194 to create two identical alleles of a gene [G/G] that are present in both homologous chromosomes) (Weyrich *et al*. 2010). In 140 healthy

African control subjects, 13% were heterozygotes, while 1.4% were homozygoous for *TLR4* D299G (Omrane *et al*. 2014). In a study consisting 439 American caucasian controls, 6% heterozygotes and only 0.2% homozygous for *TLR4* D299G (Zareparsi *et al*. 2005). Furthermore, in a study carried out in China, 13% subjects out of 287 controls were heterozygote for *TLR4* D299G (Yang *et al*. 2012). However, in one study it has been observed that the frequency of *TLR4* D299G was not that strong in African populations and that it was almost totally absent from Asian populations (Ferwerda *et al*. 2007, Table 5). The *TLR4* D299G frequency has been reported in Finnish population. *TLR4* D299G was shown to be associated with increased carotid artery compliance in young Finnish adults. The study consisted 2201 study subjects and 17% of them were heterozygote for *TLR4* D299G and 1% of them had the rare homozygote of *TLR4* D299G (Hernesniemi *et al*. 2008). Another study by Löfgren J *et al*. suggested that *TLR4* D299G could be associated with increased risk of severe RSV infection in Finnish infants. The study included 356 controls and 16.5% of them were for *TLR4* D299G and 2% were homozygotes (Löfgren *et al*. 2010). However, the possible association between *TLR4* D299G and nasopharyngeal bacterial colonization in Finnish children is not known.

Table 4. Information of nonsynonomous SNPs in *TLR2, TLR3* and *TLR4* gene.

Gene	SNP id (rs-code)	Nucleotide substitution	Amino acid change
TLR2	rs5743708	2258G>A	753 Arginine>Glutamine
TLR3	rs3775291	1234C>T	412 Leucine> Phenylalanine
TLR4	rs4986790	1194A>G	299 Aspartic acid>Glycine

Table 5. The frequency of heterozygote and homozygote variants of *TLR2* R753Q, *TLR3* L412F and *TLR4* D299G in different ethnic groups.

Ethnicity	*TLR2* R753Q		*TLR3* L412F		*TLR4* D299G	
	G/A	A/A	C/T	T/T	A/G	G/G
African	1%	0%	0%	0%	13%	1%
Caucasian	5%	0%	29%	19%	6-17%	1%
Asian	0%	0%	53%	5%	13%	0%
Hispanic	4%	0%	40%	12%	3%	0%

2.7.2 SNPs in *MBL2*

MBL serves in host defence by recognizing a broad range of microorganisms and activating the lectin-pathway of the complement system. MBL is important during the period of time when maternal-derived antibodies start to disappear and the child's own immune system is still being matured and before a primary immune response is

generated following microbial challenges (Garred *et al*. 2006). Polymorphisms located in the promoter region and exon 1 of *MBL2* gene have been described. In the promoter region, two promoter variants, H allele and L allele at position -550 (H/L) are in linkage disequilibrium with two other promoter variants X allele and Y allele at position −221 (X/Y). Other promoter variants, P allele and Q allele occur at position −66 (P/Q). In the presence of normal allele A, the *MBL2* haplotypes HYP (HYPA), LYP (LYPA) and LXP (LXPA) have been shown to be associated with high, intermediate and low MBL concentrations, respectively (Madsen *et al*. 1995; Steffensen *et al*. 2000). Three different genetic polymorphisms located in exon 1 of human *MBL2* have been shown to lead to dramatic reduction of the serum MBL concentration by distrupting the collagenous structure of the MBL protein. The designation of these *MBL2* variant alleles is B, C and D. The *MBL2*-B variant (glycine changes to aspartic acid in amino acid position 54 as a result of a nucleotide substitution G>A in mRNA position 161; rs1800450, Table 6) occurs in codon 54, the *MBL2*-C variant (glycine changes to glutamic acid in amino acid position 57 as a result of a nucleotide substitution G>A in mRNA position 170; rs1800451, Table 6) occurs in codon 57 and the *MBL2*-D variant (arginine changes to cysteine in amino acid position 52 as a result of a nucleotide substitution C>T in mRNA position 154; rs5030737, Table 6) occurs in codon 52 (Larsen *et al*. 2004; Verdu *et al*. 2006). As mentioned before, all these three polymorphisms have a dominant effect on the level of MBL blood concentration. In the heterozygous situation, the level of functional MBL in the serum might be decreased as much as 90%. Homozygosity leads to a total deficiency of functional MBL in the blood. These polymorphisms make the MBL protein unstable, easily degraded to lower oligomeric forms and the mutated proteins probably have a shorter half-life in the circulation (Heitzeneder *et al*. 2012). The presence of these *MBL2* polymorphisms have been found to be associated with increased risk of several infection types such as acute respiratory tract infection during childhood and in particular during the vulnerable time period of infancy from 6-18 months when the immune system is still maturing, with increased risk of meningococcal disease, with increased risk to invasive pneumococcal infection, with mycobacterial infection and with HIV susceptibility and disease progress (Koch *et al*. 2001; Eisen & Minchinton, 2003; Larsen *et al*. 2004). *MBL2* variant frequencies differ among different ethnic populations. The B-allele is almost absent in Sub-Saharan Africans or occurs with a low frequency, 0-6%, whereas in Caucasians (European) and Asians the B-allele is rather common exceeding frequencies between 12-14%. However, in South American Indians the frequency of the B-allele may exceed 50%. The C-allele is rather common (12-30%) in Sub-Saharan Africans whereas it is rare (≤3%) in Caucasians and absent in Asians and South American Indians. The frequency for D-allele is low (5-8%) and occurs mostly in Caucasian and Northern African populations (Garred *et al*. 2006; Ermini *et al*. 2012, Table 7). The frequencies of

these three *MBL2* gene polymorphisms and disease associations have been described in Finnish population. In a study carried out by Rantala *et al*. they showed that *MBL2* gene polymorphisms affecting the MBL serum concentration were associated with respiratory tract infcetions among young Finnish men. Of the 362 healthy Finnish men as a control group in this this study, the frequency for normal allele-A (A/A) was 65%, for B-allele (A/O; in literature heterozygote variants of *MBL2* [A/B, A/C and A/D] are referred as A/O) was 21%, for C-allele (A/O) 2% and for D-allele (A/O) 7%. The homozygous variants (in literature homozygote variants of *MBL2* [B/C, B/D, B/B, C/C, C/D and D/D] are referred as O/O) frequency was 5% (Rantala *et al*. 2008). In another Finnish study, Seppänen *et al*. showed that *MBL2* gene polymorphisms were associated with recurrent herpes simplex virus 2 infection. Their study included 147 healthy Finnish controls and the frequency for B-allele was 16%, for C-allele 1% and for D-allele 5%. The homozygous O/O frequency was 3% (Seppänen *et al*. 2009). However, the information about possible association between *MBL2* gene polymorphisms and nasopharyngeal bacterial colonization with respiratory pathogens in Finnish children is unknown.

Table 6. Information of nonsynonomous SNP located in exon 1 of *MBL2*.

Gene variant	SNP id (rs-code)	Nucleotide substitution	Amoni acid change
MBL2-B	rs1800450	161G>A	54 Glycine>Aspartic acid
MBL2-C	rs1800451	170G>A	57 Glycine>Glutamic acid
MBL2-D	rs5030737	154C>T	52 Arginine>Cysteine

Table 7. The frequency of different *MBL2* allele variants in different ethnic groups

Ethnicity	***MBL2*-B**	***MBL*-C**	***MBL2*-D**
African	0-6%	12-30%	5%
Caucasian	12-30%	3%	7%
Asian	15-20%	0%	0%
South American Indians	50%	0%	0%

2.7.3 SNP in Th17 cytokine IL-17A

The Th17 cytokine IL-17A is involved in host defense against infection and development of inflammation by inducing production of pro-inflammatory cytokines, chemokines and antimicrobial peptides as well as recruiting neutrophils to the site of inflammation and infection. IL-17A has received considerable attention since its discovery. One of the SNPs in *IL17A* gene, *IL17A* G152A (nucleotide substitution G>A in mRNA position 152, rs2275913), is interesting since it is located within the binding motif for the nuclear factor

activated T cells (NFAT), which is a critical regulator of the IL-17 promoter. Because of this, it is perceivable that *IL17A* G152A has an effect on the transcriptional regulation of IL-17A (Espinoza *et al*. 2011). The presence of *IL17A* G152A has been shown to be associated with pulmonary tuberculosis, with childhood asthma, and with bacterial colonizations of *S. pneumoniae* or *M. catarrhalis* in patients having bronchiolitis (Chen *et* al. 2010; Ocejo-Vinyals *et al*. 2013). Moreover, *IL17A* G152A has been found to be associated with cancer (Omrane *et al*. 2014). Omrane *et al*. demonstrated that *IL17A* G152A was associated with colorectal cancer (Omrane *et al*. 2014). In their study, of 139 healthy Sub-Saharan African controls 27% were heterozygote G/A and 4% homozygote A/A (Omrane *et al*. 2014). Another study carried out by Narbutt J *et al*. showed that A/A variant of *IL17A* G152A predisposed to increased severity of atopic dermatitis and to its coexistence with asthma. In this study, they had 153 healthy controls with Caucasian background and the frequency for heterozygote G/A was 48% and for homozygote A/A it was 7% (Narbutt *et al*. 2014). A study by Chen J *et al*. showed that *IL17A* G152A was associated with childhood asthma and bacterial colonization of the hypopharynx in patients with bronchiolitis. In this study, they had 205 healthy Asian controls and the frequency for the heterozygote G/A was 51% and for the homozygote A/A it was 16% (Chen *et al*. 2010, Table 8). Based on these recent studies, it can be seen that the frequency for *IL17A* G152A differs among the different ethnic populations. The frequency and possible disease and colonization associations for *IL17A* G152A have not been reported in Finland before.

Table 8. The frequency of *IL17A* G152A in different ethnic groups.

Ethnicity	***IL17A* G152A**	
	G/A	A/A
African	27%	4%
Caucasian	48%	7%
Asian	51%	16%

3. AIMS OF THE STUDY

The main object of this study was to investigate nasopharyngeal pathogenic bacterial colonization and genetic polymorphisms in the genes of the innate immunity that could be potential risk factors for respiratory infections in infants and young children. We investigated nasopharyngeal bacteria such as *S. pneumoniae*, *M. catarrhalis*, *H. influenza*, *S. aureus* and their association with SNPs in different *TLRs*, *MBL2* and Th17 cytokine gene *IL17A*.

We studied the frequencies of common respiratory pathogens in healthy infants and young children during two-year follow-up to explore the possible changes in bacterial colonization rates. We also investigated the frequencies of SNPs in the genes encoding the different TLRs, MBL and IL-17A in the same study cohort, mostly previously unstudied in this research field before.

More specific aims of this study were to investigate:

1. The association between nasopharyngeal bacterial colonization and polymorphisms of the *MBL2* and *TLR*s in Finnish healthy infants.

2. The possible effects of HRV infection on nasopharyngeal bacterial colonization in Finnish infants with wild or variant types of MBL and TLRs.

3. The possible association between *TLR4* D299G polymorphisms and *M. catarrhalis* colonization in healthy children during a two-year period of follow-up

4. The possible differences in IL-17A cytokine production among healthy infants and infants with *IL17A* G152A polymorphism and its association with nasopharyngeal pneumococcal colonization.

4. MATERIALS AND METHODS

4.1 Study design and study subjects (I, II, III, IV)

Finnish children taking part in the ongoing STEPS-study were recruited. The STEPS-study was designed as a prospective, observational cohort study which consisted of all mothers who had live deliveries in the Hospital District of Southwest Finland from January 2008 to April 2010 and their children (n = 9811 mothers, n = 9936 children). Of these, 1797 mothers and their 1827 children were recruited to an intensive follow-up group during the first trimester of pregnancy or soon after delivery (Lagström *et al*. 2013).

Healthy infants or young children visited the study clinic at the ages of <3 months, 12 months and 24 months on Monday or Tuesday during the period between August 2008 and June 2012. Three-month visit was preferably scheduled before universal vaccinations at 3 months, resulting in a mean age of 0.22 years (standard deviation, 0.08 years). Nasopharyngeal (NP) samples were collected from 489 children at 2.6 months of age, 202 children at 13 months of age and 176 children at 24 months of age (Figure 6). There were 161 children whose NP samples were available at all three different time points. NP samples were collected by using flocked NP swabs (Copan, Brescia, Italy). Duplicated NP swabs were taken from nostrils of each study subject, one of which was used for bacterial culture and the other for viral culture and antigen detection. The swab was immersed in 1 mL of 0.9% NaCl, and homogenized by vortexing and the sample was transported within 3 hours to laboratory for bacterial culture. Blood samples were collected from a total of 412 children for DNA isolation at three months of age.

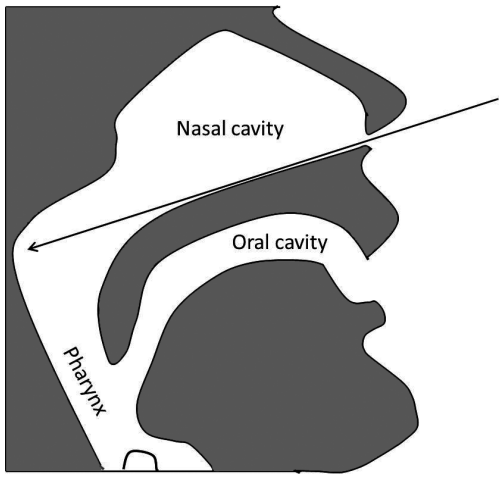


Figure 6. NP swab should be taken precisely from back of the nasopharynx (arrow).

The study protocol was approved by the Ethics Committee of the Hospital District of the South-Western Finland, Turku, Finland. All parents of participating children gave their written informed consent.

4.2 Bacteriology (I, II, III, IV)

For bacterial culturing, a semi quantitative culturing method was used where 10 µl-loopful of bacterial suspension was plated and spread over one-quarter of the plate and then streaked onto remaining three quadrants by using the same 10 µl loop. For the semi-quantitative culture, the number of colonies on each of 4 quadrants of the plates were counted and categorized as growth 1 to 5: growth 1, <25 colonies in quadrant 1; growth 2, ≥25 colonies in quadrant 1 and <25 colonies in quadrant 2; growth 3, ≥25 colonies in quadrant 2 and <25 colonies in quadrant 3; growth 4, ≥25 colonies in quadrant 3 and <25 colonies in quadrant 4; and growth 5, ≥25 colonies in quadrant 4 (Figure 7).

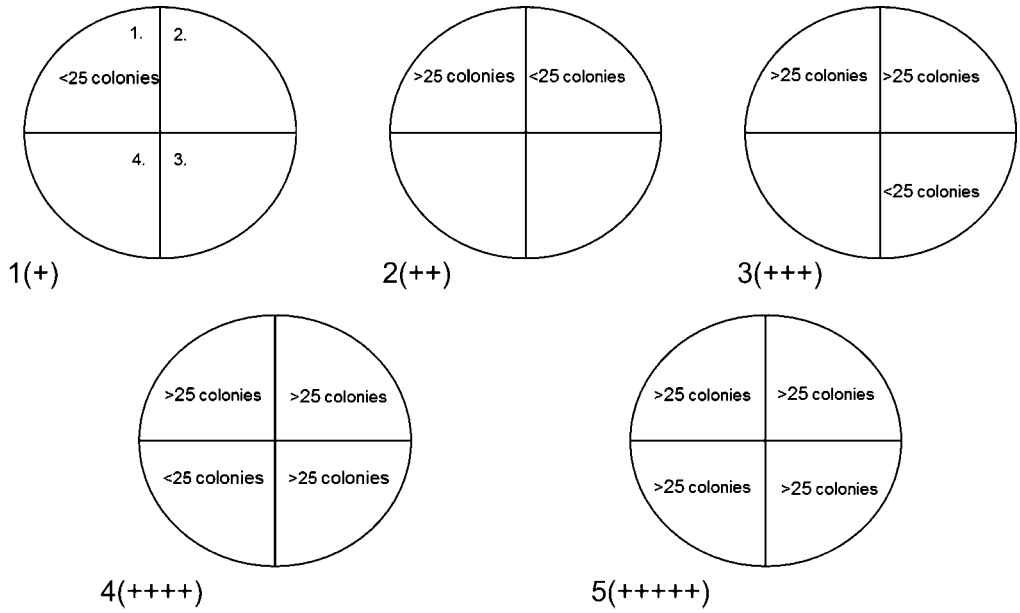


Figure 7. A semi quantitative culturing method and the number of colonies on each of 4 quadrants to be counted.

Four different culture plates were used: a blood agar plate containing 5% sheep blood, a heated blood agar (chocolate agar) plate, a *H. influenzae* selective plate (a heated blood agar plate containing 300 mg/l bacitracin) and a *S. pneumoniae* selective plate (sheep blood agar plate containing 5 mg/l colistin and 2.5 mg/l oxolinic acid). Plates

were incubated in 5% CO_2 at 35°C for 48 hours. The plates were inspected every day. Suspected colonies of each species were identified as follows: *S. pneumoniae* isolates by using the optochin disk susceptibility test (Oxoid, Basingstoke, England), *H. influenzae* isolates by the X, V and X+V factor test (Oxoid), *M. catarrhalis* isolates by the oxidase and tributyrin test (Rosco Diagnostica, Taastrup, Denmark) and *S. aureus* isolates by the coagulase, catalase and latex agglutination test (Staphaurex, Remel Inc., Lenexa, KS, USA). Other genera or species of bacteria were identified based on the morphological appearance and by standard biochemical methods such as catalase positivity test, oxidase test and Gram-staining.

All different bacteria isolates from three different time points were stored at -70 °C in glycerol containing medium for future studies.

4.3 Virology (II)

The nasopharyngeal swab was eluted with 1.0 or 0.5 ml of phosphate buffered saline, of which 550 µl or 200 µl was used for the extraction by Nuclisense easyMag (BioMerieux, Boxtel, The Netherlands) or MagnaPure 96 system (Roche Applied Science) automated extractor, with an elution volume of 55 µl or 50 µl, respectively. Rhinoviruses were detected by reverse transcription and a real-time PCR assays for rhino-, entero-, and respiratory syncytial virus as described earlier (Peltola *et al*. 2008) with the modification that proprietary dual-label probes specific for rhino- and enteroviruses were used for their differentiation. In this present project, we focused on the rhinoviruses.

4.4 DNA isolation (I, II, III, IV)

DNA was extracted from 200 µl of whole blood by QIAGEN QIAamp DNA Blood Mini Kit 250 (Qiagen, Hilden, Germany) according to the manufacturer's protocol.

4.5 SNP detection (I, II, III, IV)

The genotyping of *MBL2* gene in codons 52 (allele D, rs5030737), 54 (allele B, rs1800450) and 57 (allele C, rs1800451), *TLR2* R753Q (rs5743708), *TLR3* L412F (rs3775291) and *TLR4* D299G (rs4986790) was performed by pyrosequencing (PSQ™96MA Pyrosequencer, Biotage, Uppsala, Sweden), using a PSQ™96 Pyro Gold Q96 reagent kit according to the manufacturer's protocol. The PCR and sequencing primers used for the genotyping have been described earlier (Roos *et al*. 2006; Woehrle *et al*. 2008; Ahmad-Nejad *et al*. 2009). All the primers were HPLC purified and synthesized by Sigma-Aldrich, Finland. Presence of the PCR products was verified on a stained agarose gel.

The genotyping of *IL17A G152A* (rs2275913) was performed from extracted DNA by Sequenom massARRAY iPlex Gold® system (Sequenom Inc, CA, USA) in University of Eastern Finland, Kuopio, Finland.

4.6 Randomly amplified polymorphic DNA analysis (III)

To study whether colonization by *M. catarrhalis* represents repeated acquisition of different strains or persistent colonization by the same strain, a simple method "randomly amplified polymorphic DNA analysis (RAPD)" was used to see differences between *M. catarrhalis* strains that were isolated at 3, 13 and 24-month time point within same individual. The method for RAPD used for this study has been published 1999 (Vu-Thien, 1999). We used the following primer: 5´-GCAATTAACCCTCACTAAAG-3´ (SIGMA-ALDRICH, Finland). The PCR program consisted 40 cycles 95°C for 1 minute, 49°C for 1 minute and 72°C for 1 minute. The first denaturation at 95°C lasted for 3 minutes and the extension at 72°C lasted for 5 minutes. After the PCR, the PCR products were visualized by gel electrophoresis in a 1.5% agarose gel and stained by ethidium bromide.

4.7 Cytokine measurements with Luminex (IV)

Bio-Plex Pro Human Cytokine Group IL-17A Assay Kit (Bio-Rad Laboratories, Finland) was used to measure cytokine IL-17A production from selected samples. These assay kits were allowed to quantitatively measure the IL-17A concentration from as little a volume as 15µL of serum within 4 hours. A total of 93 sera collected from 93 subjects aged 13 months were tested by Luminex (Luminex 100/200TM System, Luminex B.V., The Netherlands). The standard curve ran from 1.0 to 20000pg/mL. Cytokine IL-17A measurements were performed according to manufacturer's protocol.

4.8 Statistical analyses (I, II, III, IV)

For the original publications I, III and IV, the differences between groups were analyzed and evaluated with GraphPad Prism 4 (San Diego, CA), using the Chi-square test or Fisher's exact test. For the original publication IV, Kruskal-Wallis test was applied to analyze the differences in IL-17A production between subjects with different genotypes of *IL17A* G152A. Odds ratios, relative risk and 95% confidential intervals were also calculated in original publications I, III and IV. In the original publication II the statistical analyses were carried out in two steps. First, the effect of rhinovirus on bacterial colonization was examined by cross-tabulation and tested with Chi-Square test,

Fisher's exact test, and unadjusted binary logistic regression analysis. In second, the gene polymorphisms of MBL, TLR3 and TLR4 and socio-demographic background variables were included to adjusted logistic regression analysis to adjust the effect of rhinovirus on bacterial colonization in order to exclude the possible confusing factors. Two tailed *P*-value <0.05 was considered as significant in all analyses. Statistical analyses were carried out using SPSS version 16 and SAS version 9.1.

5. RESULTS

5.1 Bacterial colonization at 2.6 months of age (I)

A total of 489 NP samples were collected from study subjects at 2.6 months of age. From these 489 samples, 290 (59%) were culture positive for at least one of the four bacterial species: 55 (11%) for *S. pneumoniae*, 114 (23%) for *M. catarrhalis*, 122 (25%) for *S. aureus* and 5 (1%) for *H. influenzae* (Figure 8, Figure 1 in the original publication I). Twenty-four (5%) children were found to be culture negative. This, however, does not mean that these children did not have any bacteria in their nasopharynx since our semi-quantitative culturing method was optimized for the bacterial species listed in Figure 8. Since the bacterial prevalence for *H. influenzae* was only 1%, it is noteworthy to mention that a larger volume (50 µL) of the original bacterial suspension, together with 10 µL, was spread on the plates with the semi-quantitative culture method. However, this addition did not increase prevalence of *H. influenzae* observed from the plates. From 290 children who were culture positive for one of the four pathogens, 128 (26%) were culture positive for two different pathogens at the same time. Of the 55 children who were positive for *S. pneumoniae*, 19 (35%) were also positive for *M. catarrhalis* and 8 (15%) for *S. aureus*. There were only four children who were positive for *S. pneumoniae*, *M. catarrhalis* and *S. aureus* at the same time. When the possible seasonal variation in the colonization rates of these pathogens was studied, no significant difference was observed (Figure 9).

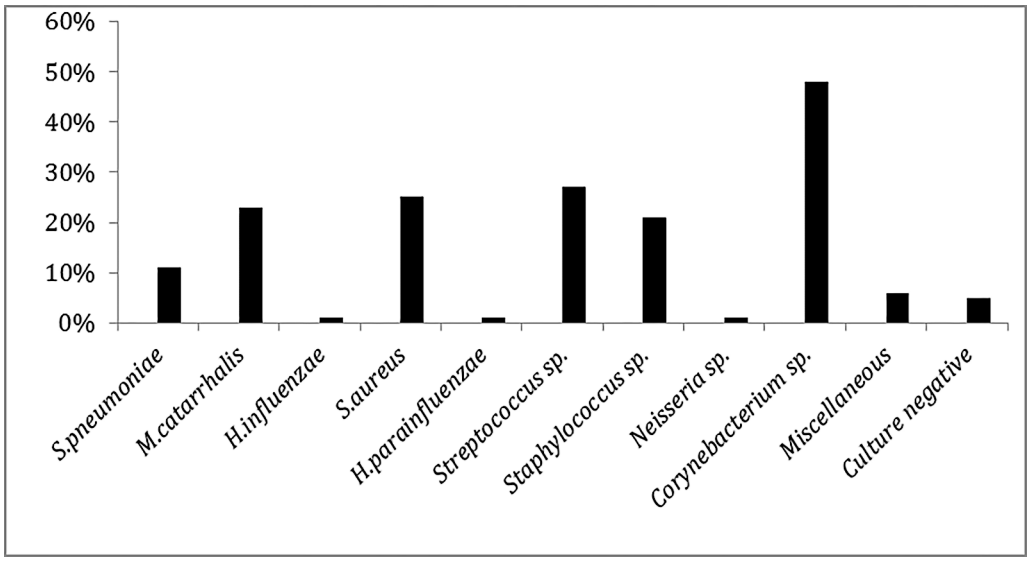


Figure 8. Different bacterial colonization rates from 489 healthy Finnish children at 2.6 months of age.

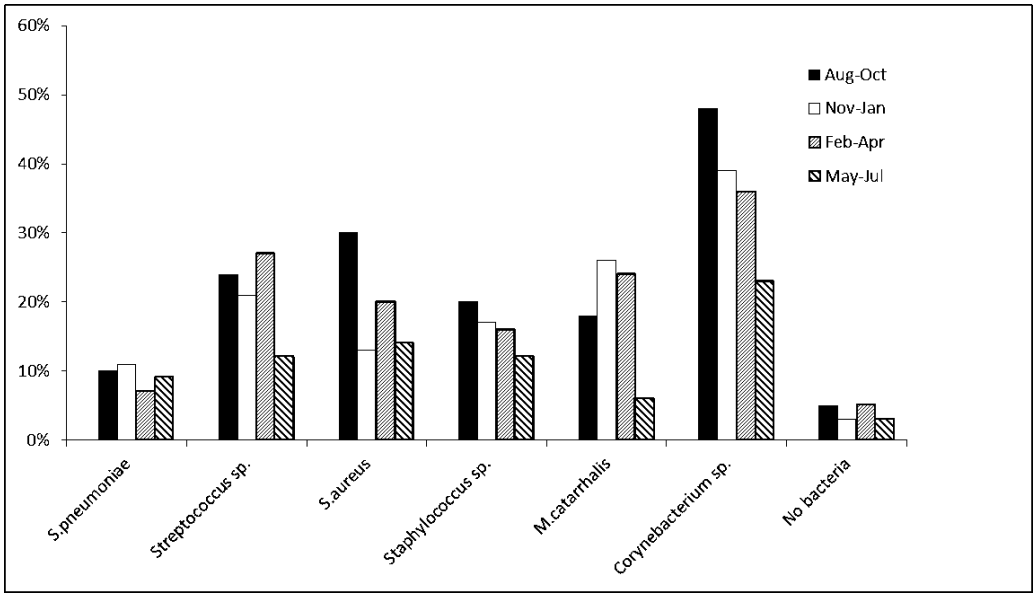


Figure 9. Seasonal variation among different nasopharyngeal bacteria isolated from 2.6 months old infants (August 2008 – July 2009, N=136).

5.1.1 The frequency of *TLR2* R753Q, *TLR4* D299G and SNPs in *MBL2* together with bacterial colonization at 2.6 months of age (I)

Altogether, there were 412 extracted DNA samples available from the study cohort. For *TLR2* R753Q, 392 (95%) of the children were C/C homozygote normal allele and 20 (5%) were C/T heterozygotes (Table 9). No subjects with homozygote for T/T were found in this study cohort. For *TLR4* D299G, 340 (83%) children were A/A homozygote normal allele, 69 (17%) were A/G heterozygotes and three were G/G homozygotes (Table 9). Genotypes with mutant alleles were combined for statistical analyses. For the *MBL2* polymorphisms, 279 (68%) children were A/A normal allele, 124 (30%) were A/O heterozygotes and 9 (2%) were O/O homozygotes. Of the 124 children who had A/O, 86 were A/B, 37 were A/D and one child was A/C. Of the nine children who had O/O homozygote, five were B/B, two were C/B, one was B/D and one was D/D (Table 9).

Table 9. The frequency of *TLR2* R753Q, *TLR4* D299G and SNPs in *MBL2* 412 children.

TLR2 R753Q		*TLR4* D299G		*MBL2* variants	
C/T (%)	T/T (%)	A/G (%)	G/G (%)	A/O (%)	O/O (%)
20 (5)	0 (0)	69 (17)	3 (1)	124 (30)	9 (2)

Of the 124 children with *MBL2* A/O, 24 (19%) were colonized by *S. pneumoniae*, one (11%) child out 9 children with *MBL2* O/O was *S. pneumoniae* positive and 27 (10%) children out of 279 with *MBL2* A/A were positive for *S. pneumoniae*. Carrying one or two mutant alleles (A/O or O/O, respectively) were combined together for statistical analyses and referred to below as "A/O". Interestingly, it was observed that children with *MBL2* A/O (A/O or O/O) had a higher risk (OR 2.16) to be colonized by *S. pneumoniae* at 2.6 months of age compared to the children with *MBL2* A/A (P=0.011). Moreover, these same children with *MBL2* A/O had a higher risk (OR 1.75) to be colonized by *S. aureus* and *Staphylococcus sp*. at 2.6 months of age (P=0.024 and P=0.016, Table 1 in the original publication I). Of the 20 children with *TLR2* heterozygote C/T, 9 (45%) children were positive for *S. aureus,* while only 86 (23%) of children with C/C normal allele were nasopharyngeal carriers of *S. aureus*. Also, *TLR2* heterozyote C/T carriers had a higher risk (OR 2.91) to be colonized by *S. aureus* at 2.6 months of age compared with C/C normal allele carriers (P=0.027, Table 2 in the original publication I). Of the 72 children with *TLR4* heterozygote A/G, 38 (40%) were positive for *M. catarrhalis* while 72 (21%) with A/A normal allele were *M. catarrhalis* positive. Children with *TLR4* D299G A/G variant type had a higher risk (OR 2.37) to be colonized by *M. catarrhalis* at 2.6 months of age compared to the children with A/A normal allele (P=0.002, Table 3 in the original publication I).

Further associations between any of the SNPs studied and colonization with other bacteria were not found in study I.

5.2 Respiratory symptoms and viral findings together with bacterial colonization at 2.6 months of age (II)

A total of 337 NP samples were available for both viral and bacterial detection from children at 2.6 months of age. Of the 337 children, 61 (18%) were positive for HRV, 46 (14%) were positive for *S. pneumoniae,* 86 (25%) were positive for *M. catarrhalis*, 96 (28%) were positive for *S. aureus* and only three (1%) were positive for *H. influenza* (Table 2 in the original publication II). When bacterial co-colonization was studied, 18 children were found to be positive for *S. pneumoniae* and *M. catarrhalis*. In addition, 4 children were found to be positive for co-colonization of *S. pneumoniae*, *M. catarrhalis* and *S. aureus* and 8 children were co-colonized by *S. pneumoniae* and *S. aureus*.

From 337 children, 53 (16%) were reported to have mild respiratory symptoms such as nasal congestion, runny nose or cough on the day of sampling. Twenty-three (43%) of 53 children with respiratory symptoms were positive for HRV compared

to 38 (13%) of 284 children without respiratory symptoms (*P*=<0.001). Moreover, 27 (51%) children out of 53 with respiratory symptoms were positive for *M. catarrhalis* compared to 57 (20%) children out of 284 without respiratory symptoms (*P*=<0.001) (Table 2 in the original publication II). No significant associations were observed between other bacterial colonization rates and children with or without mild respiratory symptoms.

There was no significant association observed between HRV infection and *S. pneumoniae* colonization. Those 46 children out of 337 who were positive for *S. pneumoniae*, 11 (24%) were also positive for HRV at the same time compared to those 50 (17%) children out of 291 who were negative for *S. pneumoniae* but who had HRV infection (*P*=0.271). When looking at *M. catarrhalis* colonization it was observed that from those 84 children out of 337 who were *M. catarrhalis* positive, 22 (26%) were also positive for HRV at the time of sampling. When this was compared to those 39 (15%) children out of 253 who were *M. catarrhalis* negative but who had had HRV infection, an association was noticed between these two variables (*P*=0.026). No significant association was observed between HRV infection and *S. aureus* colonization. Those 96 children out of 337 who were positive for *S. aureus,* 11 (12%) were also positive for HRV at the time of sampling compared to those 50 (21%) children out of 241 who were *S. aureus* negative but who had HRV infection (*P*=0.046, Table 3 in the original publication II).

5.2.1 Nasopharyngeal bacterial colonization in relation to background variables (II)

Different background variables such as sex, breastfeeding at 3 months of age, two or more children in family, smoking and antibiotics use before 3 months of age were studied to see whether there was any association with NP bacterial colonization. It was observed that having one or more siblings in a family was associated with *S. pneumoniae* colonization (*P*=<0.001) and with *M. catarrhalis* colonization (*P*=0.001). No other associations between the different background variables and colonization with other NP bacteria were observed.

5.2.2 Nasopharyngeal bacterial colonization, HRV infection and gene polymorphisms in *MBL2, TLR3* L412F and *TLR4* D299G (II)

The associations between polymorphisms in *MBL2* (230 [68%] with A/A, 101 [30%] with A/O and six [2%] with O/O), *TLR3* L412F (128 [49%] with C/C, 134 [51%] with C/T and T/T) and *TLR4* D299G (277 [82%] with A/A, 60 [18%] with A/G) as well as HRV with bacterial colonization were analyzed. In children with *MBL2* wild type

alleles A/A, there was no significant association observed between the colonization of *S. pneumoniae* and HRV positivity or HRV negativity. However, when looking at children with variant type of *MBL2* (A/O and O/O combined) colonization rate of *S. pneumoniae* was significantly higher in children with HRV infection compared to the children who were HRV negative (8 of 20 [40%] with HRV, vs. 9 of 87 [10%] without HRV, *P*=0.003, Table 4 in the original publication II).

In children with *TLR4* D299G wild type alleles A/A, the colonization rate of *M. catarrhalis* was significantly higher in those children with HRV infection compared to children without HRV infection (18 of 51 [35%] with HRV vs. 44 of 226 [19%] without HRV, *P*=0.014). When looking at children with *TLR4* D299G heterozygosity (A/G), it was noticed that there was no significant difference in colonization rates of any of the three pathogens studied between children who had HRV infection and who were HRV negative. In children with wild type *TLR4* A/A, the colonization rates of *S. aureus* were significantly lower in children with HRV infection compared to those children without HRV infection (8 of 51 [16%] with HRV vs. 72 of 226 [32%] without HRV, *P*=0.021, Table 4 in the original publication II).

In children with *TLR3* L412F wild type alleles C/C, the colonization rates of *M. catarrhalis* were higher in children with HRV infection than in those children without HRV infection (9 of 23 [39%] with HRV vs. 21 of 105 [20%] without HRV, *P*=0.050). Moreover, in children with wild type *TLR3* L412F C/C, the colonization rates of *S. aureus* were significantly higher in children without HRV infection compared to children with HRV infection (48 of 105 [46%] without HRV vs. 5 of 23 [22%] with HRV, *P*=0.034). When looking at children with *TLR3* L412F C/T and T/T, it was observed that there was no significant difference in colonization rates of any of the three pathogens studied between children who had HRV infection and children who were HRV negative (Table 4 in the original publication II).

5.3 Nasopharyngeal bacterial colonization during the follow-up in 161 children (III)

Altogether there were 161 children who had their NP samples available at all three different time points. It was observed that the colonization rates of *S. pneumoniae*, *M. catarrhalis* and *H. influenzae* increased during the follow-up, whereas the nasopharyngeal carriage of *S. aureus* decreased with time (Figure 10). Of these 161 children 103 (64%) were culture positive for at least one of the four bacterial species at 2.6 months of age, 102 (63%) at 12 months of age and 119 at 24 months of age.

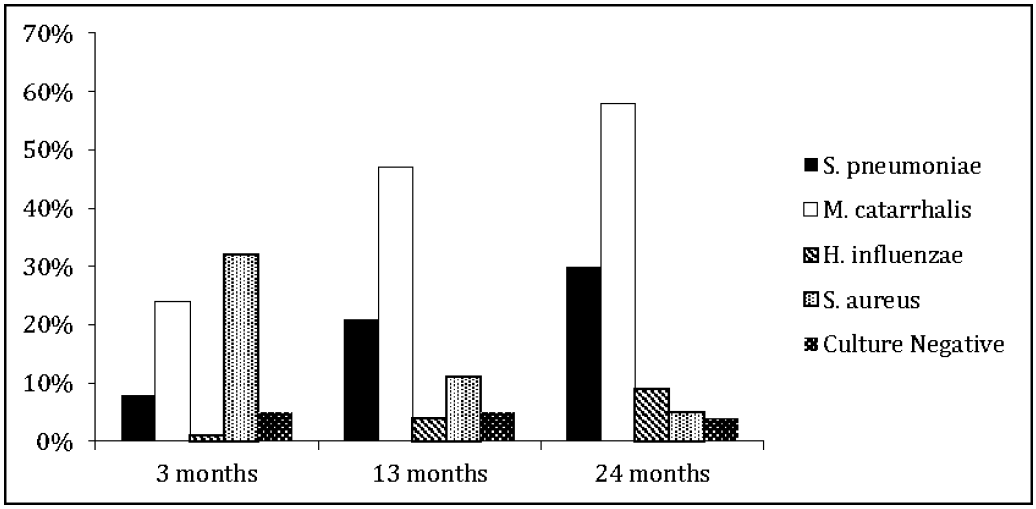


Figure 10. Nasopharyngeal bacterial colonization of four potentially pathogenic bacteria in 161 children at 2.6 months, 13 months and 24 months of age.

5.3.1 Nasopharyngeal bacterial colonization and *TLR4* D299G polymorphism in 161 children during the follow-up (III)

In this study cohort, 126 children (78%) had *TLR4* D299G wild type allele (A/A) while 35 (22%) had heterozygosity A/G and there were no G/G homozygotes in this cohort. From those 126 children with A/A, 23 (18%) were positive for *M. catarrhalis* at 2.6 months of age, 54 (43%) were positive at 13 months of age and 74 (59%) were positive at 24 months of age. From those 35 children with A/G, 15 (43%) were positive for *M. catarrhalis* colonization at 2.6 months of age, 21 (60%) were positive at 13 months of age and 20 (57%) were positive at 24 months of age.

It was observed that the *M. catarrhalis* nasopharyngeal carriage was significantly higher in those children with heterozygote A/G alleles compared with the children with wild type alleles A/A at 2.6 months of age (P=0.005). However, this phenomenon was not observed at 13 months and 24 months of age. When looking at *H. influenzae* colonization rates between different *TLR4* D299G genotypes in three different time points, it was noticed that children with heterozygous A/G had a higher nasopharyngeal carriage rate of *H. influenzae* at 24 months of age when compared to the children with wild type alleles A/A (20% vs. 6%, P=0.020). No significant differences were observed in colonization rates of *S. pneumoniae* and *S. aureus* between the different genotypes of *TLR4* D299G during the follow-up.

5.3.2 TLR4 D299G gene polymorphism and repeated nasopharyngeal colonization and load of *M. catarrhalis* during the follow-up (III)

There were 23 (18%) children out of 126 with wild type alleles A/A who were colonized by *M. catarrhalis* at 2.6 months of age. From these 23 children, 18 were also colonized by *M. catarrhalis* at 13 months of age and 11 children at 24 months of age. From children with heterozygous A/G, there were 15 (43%) out of 35 who were colonized by *M. catarrhalis* at 2.6 months of age. Again, the same 15 children were also colonized by *M. catarrhalis* at 13 months and 24 months of age. Repeated colonization rate for *M. catarrhalis* was significantly higher in children with the heterozygous A/G compared to the children with A/A (15 of 35 [43%] vs. 11 of 126 [9%], P=0.001, Figure 2 A and B in the original publication III).

As mentioned before, 15 children out of 35 with heterozygous A/G were colonized by *M. catarrhalis* in all three different time points. Altogether 45 bacterial cultures were performed from these 15 children by using the semi quantitative culture method. From these 45 bacterial cultures, 30 (67%) had bacterial growth category 3 to 5 for *M. catarrhalis*. From those 126 children with wild type A/A only 11 were colonized by *M. catarrhalis* in all three different time points. Altogether 33 bacterial cultures were performed from these 11 children only 10 (30%) bacterial cultures had the growth category 3 to 5 for *M. catarrhalis*. Children with the heterozygous A/G had significantly higher bacterial load of *M. catarrhalis* (P=0.003). RAPD testing showed that the children were colonized by different strains of *M. catarrhalis* during the two-year study period indicating that these children were not persistently colonized by *M. catarrhalis*.

5.4 Nasopharyngeal bacterial colonization and gene polymorphism of *IL17A* G152A during the two years follow-up (IV)

The colonization rates of *S. pneumoniae*, *M. catarrhalis* and *H. influenzae* increased from 2.6 months of age to 24 months of age whereas the colonization rates of *S. aureus* decreased during the same period of time (Figure 11).

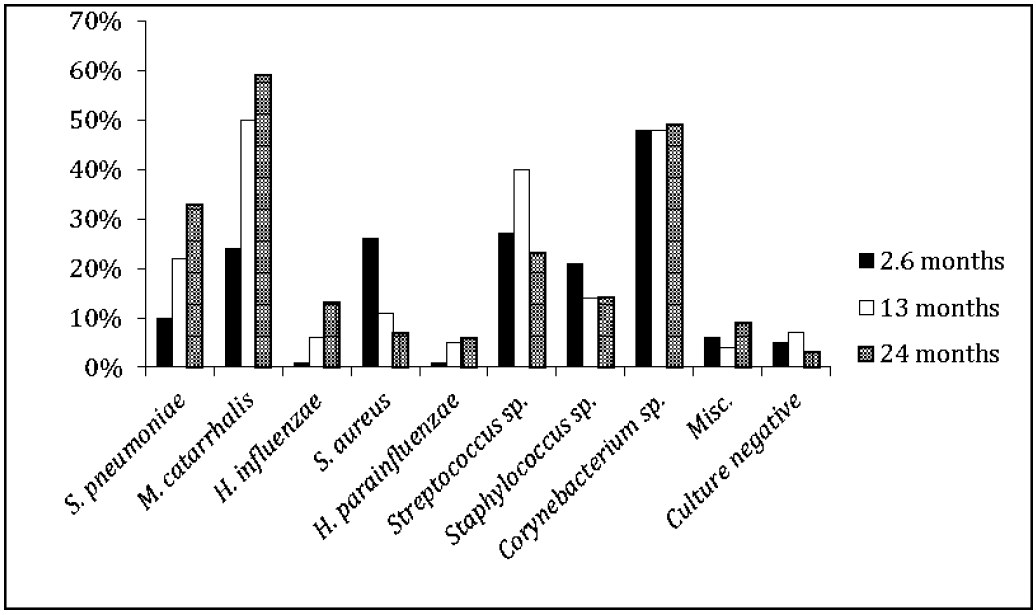


Figure 11. Nasopharyngeal bacterial colonization at 2.6 months, 13 months and 24 months of age (N=405 at 2.6 months, N=198 at 13 months and N=176 at 24 months).

At 2.6 months of age, there were NP samples available from 405 children that also had *IL17A* G152A data available. Of these 405 children, 146 (36%) had wild type allele G/G, 181 (45%) had heterozygous G/A and 78 (19%) had homozygous mutant A/A. The colonization rates of *S. pneumoniae* were found to be significantly higher in children with homozygous A/A in 13 months and 24 months of age when comparing to the children with the two other genotypes of *IL17A* G152A. This phenomenon was not observed at 2.6 months of age between the same genotypes (Table 10). No significant differences were observed between any other bacterial colonization such as *S. aureus* and the three different genotypes of *IL17A* G152A. When looking for differences in *S. pneumoniae* colonization rates between baby boys and girls, no associations were observed.

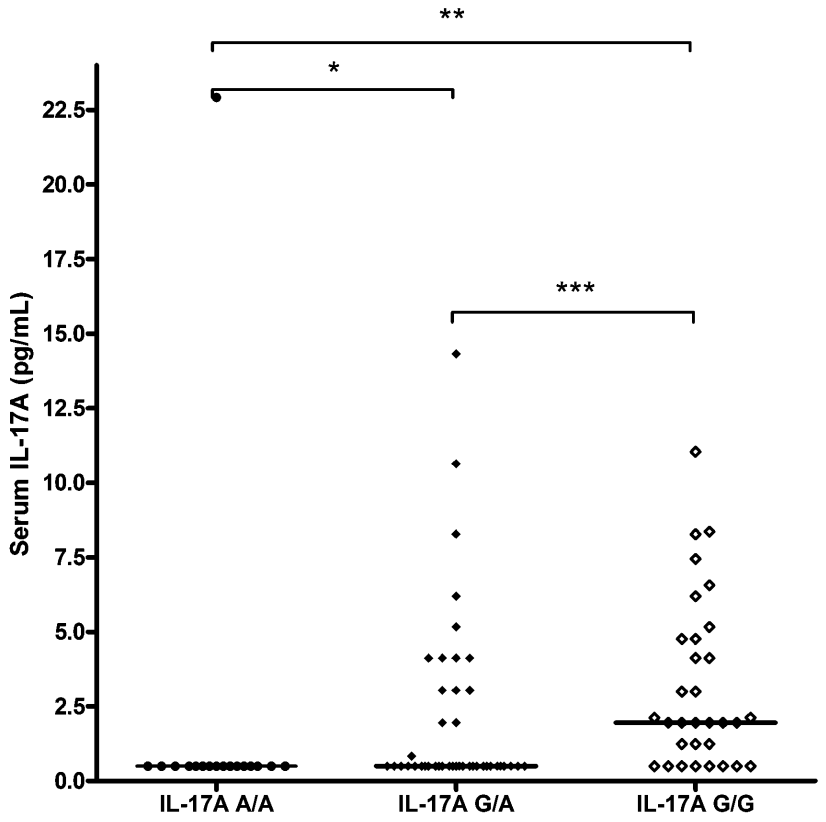
Table 10. *S. pneumoniae* colonization rates (%) in subjects with different *IL17A* genotypes at 2.6, 13 and 24 months of age.

Time point (months)	*N*	*S. pneumoniae* (%)		
		***IL17A* G/G**	***IL17A* G/A**	***IL17A* A/A**
2.6	405	17/146 (12)	17/181 (9)	8/78 (10)
***13**	198	12/70 (17)	16/90 (18)	15/38 (39)
****24**	176	14/52 (27)	25/87 (29)	19/37 (51)

* A/A vs. G/G: P= 0.02, RR=2.30, 95% CI=1.20 - 4.40.
** A/A vs. G/G: P= 0.03, RR=1.91, 95% CI=1.10 – 3.30

5.4.1 Gene polymorphism *IL17A* G152A and serum IL-17A concentration in study cohort (IV)

Altogether there were 93 serum samples available from the children at 13 months of age. Of these 93 children, 18 were A/A homozygotes and only one (6%) of these 18 children had detectable IL-17A in the serum compared to 15 (35%) heterozygotes G/A and 24 (75%) of 32 with homozygous wild type allele G/G. Levels of IL-17A were significantly lower in children with homozygote A/A and heterozygote G/A than in children with the normal allele IL-17A (Figure 12). In total, of the 93 serum samples at 13 months of age, 40 (43%) children had detectable serum IL-17A.



* A/A vs. G/A (*P* >0.050),** A/A vs. G/G (*P* =0.001), *** G/A vs. G/G (*P* <0.050)

Figure 12. IL-17A serum levels from 93 children with different *IL17A* G152A genotypes at 13 months of age.

6. DISCUSSION

6.1 Nasopharyngeal bacterial colonization in young children

The microbiota in the nasopharynx of young child is a complex ecosystem. It can include several non-pathogenic (commensals) and pathogenic bacteria such as *S. pneumoniae*, *M. catarrhalis*, *H. influenzae* and *S. aureus*. The nasopharynx of a young child becomes colonized rather rapidly after birth and serves as a major reservoir for transmission of these bacterial species (García-Rodríguez&Fresnadillo Martínez, 2002). There are changes in the bacterial microbiota since bacteria are aquired, eliminated and re-aquired constantly during the first months and years of life. Most of young children are colonized by potentially pathogenic bacteria during the first year of life and probably in most cases these bacteria are colonizing the nasopharynx without causing any respiratory symptoms. It has also been demonstrated that non-pathogenic bacteria can antagonize the colonization of pathogenic bacteria. *In vitro* studies carried out by Tano *et al.* have suggested that α-hemolytic streptococci may inhibit the colonization of *S. pneumoniae*, *M. catarrhalis* and *H. influenzae* (Tano *et al*. 1999; Tano *et al*. 2000). Pathogenic bacteria can colonize the nasopharynx for 4-5 months, in a persistent manner, before they are eliminated by the host immune system or by some other factor (Faden *et al*. 1995). However, pathogenic bacterial colonization is one of the key factors in development of respiratory infections in children and this is closely related to the outcome of respiratory infections and studies have demonstrated that an early bacterial colonization (< 3months of age) can increase the risk of having respiratory infections in the next 6-9 months of early life and even that the early colonization by pathogenic bacteria is associated with development of asthma in young children (Harrison *et al*. 1999; Bisgaard *et al*. 2007).

6.1.1 The prevalence of different respiratory bacteria in children (I, II, III and IV)

The colonization rates of common nasopharyngeal pathogenic bacteria can vary between different studies and different countries (García-Rodríguez&Fresnadillo Martínez, 2002; Xu *et al*. 2012). There are several factors such as age, ethinicity, socioeconomic living conditions, vaccine programs and differences in sampling and isolation techniques, that can have an effect on colonization rates. In this study we demonstrated the prevalency of common pathogenic respiratory bacteria in healthy Finnish children at 2.6 months, 13 months and 24 months of age. Based on cultures we found that 11% children were colonized by *S. pneumoniae* at 2.6 months of age and this corroborates to a study where it was demonstrated that approximately 12% of healthy children were colonized by *S.*

pneumoniae at 3 months of age (Syrjänen *et al*. 2001). In another study, 10% of infants already at one month of age were colonized by *S. pneumoniae* (von Linstow *et al*. 2013). Since colonization rates of over 20% for *S. pneumoniae* in 3 months old children have been found, this indicates differences among different populations (Harrison *et al*.1999). *S. pneumoniae* colonizes approximately 54% of children by one year of age and the mean age of first acquisition of *S. pneumoniae* is 6 months and within a range of 1-30 months of age. However, the colonization can happen as early as 8-10 days in particularly predisposed populations such as Australian aboriginal infants (Leach *et al*. 1994; Faden *et al*. 1997; García-Rodríguez&Fresnadillo Martínez, 2002). We also investigated the seasonal variation nasopharyngeal bacterial colonization in 2.6 months. The nasopharyngeal colonization rates for *S. pneumoniae* were stable throughout all seasons (Aug-Oct, Nov-Jan, Feb-Apr and May-Jul). No data about serotype variation during the four seasons were available since we did not perform any serotyping. It has been demonstrated that cold air exposure can stimulates increased secretion by nasal glands in adults, but wheter this increased secretion could enhance nasopharyngeal bacterial colonization, is not known (Hendley *et al*. 2005).

Another important respiratory pathogen, *M. catarrhalis*, was found to be present in 23% of children at 2.6 months of age. Our finding is in line with other studies where it has been demonstrated colonization rate of *M. catarrhalis* vary from 8% up to 30% in young children (Bisgaard *et al*. 2007; Verhaegh *et al*. 2010). However, there appears to be great geographical and socioeconomical variation in the colonization rates. Up to 100% of Australian aboriginal children were colonized by *M. catarrhalis* within the first 12 months of their life, suggested mainly to be caused by overcrowding, poor hygiene and high rates of bacterial carriage (Leach *et al*. 1994). Like some other pathogenic respiratory bacteria, *M. catarrhalis* is capable of colonizing the nasopharynx of young children without causing disease and symptoms, and because the very reason it was thought to be just a commensal pathogen for decades. Since *M. catarrhalis* was recognized as a real respiratory pathogen, the dynamics of the colonization has been carefully studied in young children. For instance, Faden *et al* demonstrated with restriction enzyme digestion that a child acquires and eliminates 3 to 4 different strains of *M. catarrhalis* during the first two years of life (Faden *et al*. 1994). We did study seasonal variation for *M. catarrhalis* colonization at 2.6 months of age. The colonization rates of *M. catarrhalis* seemed to be higher during the winter months, but the observed differencies turned out to be statistically insignificant. Our finding corroborates those from another study (Verhaegh *et al*. 2011). Both of these findings suggest that *M. catarrhalis* nasopharyngeal colonization occurs via regular cycles of colonization and

clearance with virtual increase during the winter months perhaps due to increased viral infections at that time of a year (Verhaegh *et al*. 2011).

H. influenzae is a normal commensal bacteria of the nasopharynx in healthy children. Although in many studies the colonization rate of *H. influenzae* is rather low, its colonization is established early in life and increases with age. In this present study the prevalence of *H. influenzae* colonization was only 1% at 2.6 months of age. The recorded prevalence is slightly lower compared with other studies with colonization prevalencies as high as 10% at three months of age (Harrison *et al*. 1999). However, a recent study exists, where the colonization rate of *H. influenzae* is rather similar and in line with the findings of this present study (Shiri *et al*. 2013). The recent drop in colonization rates may be caused by the cross protection against NTHi offered by the recently introduced conjugated pneumococcal vaccines and potential herd immunity incited. The colonization of *H. influenzae* is dynamic and the transmission among children is quite frequent, as shown by studying *H. influenzae* strains among children attending the same day care center (Farjo *et al*. 2004). The low prevalence that was recorded in this present study could also be due to the young age (2.6 months) of the study children as it is known that the colonization rates are generally low in very young children (Leach *et al*. 1994; Shiri *et al*. 2013). As described previously in the results section, a larger volume (50µL) of the original bacterial suspension was introduced on the culture plates in addition to normal volume (10µL). However, this adjustment to methodology did not increase *H. influenzae* detection rate at 2.6 months of age. The same volume (10µL) for bacterial culture was used throughout the whole study and it did not have an effect on the detection rates of other respiratory bacteria. Due to low number of children positive for *H. influenzae* nasopharyngeal colonization, it was impossible to study seasonal variation for this respiratory pathogen. However, it has been suggested that nasopharyngeal colonization by *H. influenzae* peaks during winter/spring months (Verhaegh *et al*. 2011).

The colonization prevalence of *S. aureus* among the study children was 25% at 2.6 months of age. The colonization rates of *S. aureus* become markedly high soon after birth and up to 30%-50% of children are colonized by this bacteria during the first eight weeks of their life, after which the colonization rates start to decrease around six months of age. However, it has been demonstrated that 70% infants are colonized by *S. aureus* (Harrison *et al*. 1999; Peacock *et al*. 2003). It has been described that up 10%-35% of healthy children carry one strain of *S. aureus* in their nasopharynx almost all the time and these children are regarded as persistent carriers. There is transmission from the persistent carriage status to intermittent and non-carrier status during adolecense (Kluytmans & Wertheim, 2005; Wertheim *et al*. 2005). Because strain typing was not

performed in this study, we cannot tell whether there were persistent carriers among the children positive for the same species at different time points of the follow-up. At young age (2.6 months), infants are in very close contact with the mother and it is possible that the rather high *S. aureus* nasopharyngeal colonization rates observed could be due to transmission between healthy, lactating mothers and their infants by breastfeeding (Kawada *et al*. 2003). There were no statistical differencies in *S. aureus* nasopharyngeal colonization rates when different seasons were studied. However, an apparent increase was observed during autumn and winter months. Our findings corroborate earlier findings (Harrison *et al*. 1999).

In the present study, the colonization rates of *S. pneumoniae*, *M. catarrhalis* and *H. influenzae* continued to increase during the follow-up between 2.6 to 24 months of age. For *S. pneumoniae*, the prevalence increased from 11% to 33%, for *M. catarrhalis* from 23% to 59% and for *H. influenzae* from 1% to 11%, perhaps due to increased contacts with other children for example in day-care centers (Figure 10). The reported constant increases in the colonization rates of these respiratory bacteria are typical during childhood (Gunnarsson *et al*. 1998; García-Rodríguez *et al*. 2002). On the contrary, the colonization rate of *S. aureus* decreased constantly between the 2.6 and 24 months of age from 26% to 8%. Our findings corroborate those of a previous study (Regev-Yochay *et al*. 2004). Though approximately 5% of children were found to be culture negative, the semi-quantitative culture method used in this present study has been optimized for detection of these four respiratory bacteria and other commensal or pathogenic nasopharyngeal bacteria have likely been missed. There are several issues that can have an effect on the early nasopharyngeal bacterial colonization in children, such as microbial (bacteria and viruses) interactions in the respiratory tract and genetic host factors, and next these will be discussed in more detail.

6.2 Bacterial interactions in the respiratory tract (I, III and IV)

There are two, positive and negative, types of associations between respiratory bacteria. A positive association occurs when two different bacteria are found together more often that it is expected to happen by chance. A negative association occurs when two different bacteria compete for growth within the same environment (Pettigrew *et al*. 2008).

It is known that there is a negative association between *S. pneumoniae* and *S. aureus* (Regev-Yochay *et al*. 2004; Bae *et al*. 2012). Both of these bacteria are common pathogens colonizing the respiratory tract of children, however, not at the same colonization rates and not necessarily colonizing at the same time and this indicates

that one organism interferes with colonization of the other organism. In the original publication I, the prevalence of *S. pneumoniae* was 9% and for *S. aureus* it was 26% whereas dual carriage of both of these respiratory bacteria was only 2%. The existing evidence of negative association between *S. pneumoniae* and *S. aureus* could partially explain the colonization rates reported in this current study for these two bacteria at 2.6 months of age. Furthermore, when looking at the colonization rates at 13 and 24 months of age, the colonization rates of *S. pneumoniae* increased constantly while those of *S. aureus* decreased, again suggesting a negative association. The biological mechanisms behind the negative association between these two bacteria are incompletely understood. *S. pneumoniae* is capable of killing *S. aureus in vitro* via the production of soluble hydrogen peroxide (Regev-Yochay *et al*. 2006). Furthermore, there is evidence that soluble hydrogen peroxide can have an impact on lysogenic prophages in *S. aureus* (Selva *et al*. 2009). Regev-Yochay *et al* found that the *S. aureus* colonization rate was lower (7%) in children aged 40 months or younger with *S. pneumoniae* colonization than without (13%) and that *S. pneumoniae* colonization was lower (28%) in children with *S. aureus* colonization than without (45%). The highest colonization rate for *S. aureus* was 30% and 9% for *S. pneumoniae* at 3 months old children (Regev-Yochay *et al*. 2004). The colonization rates in our study for both, *S. pneumoniae* and *S. aureus*, are in line with those reported above.

Negative association between *S. aureus* and *Corynebacterium sp*. has also been suggested. In a study carried out by Uehara *et al*, the colonization rate of *S. aureus* in the nasal cavity of healthy hospital staff with *Corynebacterium sp*., was significantly lower than in those without. Furthermore, those with extremely large bacterial loads of *Corynebacterium sp.* in their nasal cavity never acquired *S. aureus* during the three year study period (Uehara *et al*. 2000). These findings suggest competition between bacteria which may partly be explained by for example competition for a specific attachment molecule. In our study the prevalence for *Corynebacterium sp.* were around 50% in all three different timepoints and this rather high prevalence could partially explain the low positivity rate of *S. aureus* in children at 13 and 24 months of age.

Contrary to what has been observed with *S. aureus*, positive associations have been observed between colonizing *S. pneumoniae*, *M. catarrhalis* and *H. influenza* in the nasopharynx. Why this positive association occurs between them is not clearly understood. Jacoby *et al*. have demonstrated the positive association between *S. pneumoniae* and *H. influenzae* in aboriginal and non-aboriginal children in Australia (Jacoby *et al*. 2007). Furthermore, several other studies have showed the positive association between these two pathogens and even the carriage densities of these pathogens are

positively correlated (Dunne *et al*. 2013; Chien *et al*. 2013). In original publication I, the colonization prevalence for H. influenzae was only 1% and for S. pneumoniae 11% at 2.6 months of age. As evidence exist that there is a positive association between these two respiratory pathogens one would have expected to find more even colonization rates than 1% vs. 11%. The interaction between *S. pneumoniae* and *H. influenzae* is not always beneficial. A similar phenomenon like with *S. aureus* is possible when soluble hydrogen peroxide inhibits the growth of *H. influenzae in vitro* under aerobic conditions (Pericone *et al*. 2000). The ability of *S. pneumoniae* to produce hydrogen peroxide and neuraminidase could inhibit the growth of *H. influenzae*, and may partly explain the low colonization rate of the nasopharynx of the 2.6 month old children. Constant increases in the colonization rates were observed for both pathogens (Figure 10) during the follow-up at 13 (6% vs. 22%) and 24 (13% vs. 33%) months of age which seems to be typical for both pathogens. However, the continuously low prevalence of *H. influenzae* could be due to the factors mentioned above and the received conjugate pneumococcal vaccine offering immunity against NTHi expressing group D antigen. When looking at *M. catarrhalis* colonization in children, a positive association with *S. pneumoniae* has been observed in several studies (Pettigrew *et al*. 2008; Dunne *et al*. 2013). Again, the information about the mechanisms behind this positive association is very limited. One possible explanation could be the ability of the majority of *M. catarrhalis* isolates to produce β-lactamase which has a self-protective role for *M. catarrhalis* but at the same time may indirectly affect pathogenicity in mixed infections. Our study offered further evidence for the previously found positive association between *M. catarrhalis* and *S. pneumoniae.* In original publication I, the prevalence for *M. catarrhalis* was 23% and of these children 17% were positive for *S. pneumoniae* indicating the possibility for positive association for these two pathogens already at 2.6 months of age.

6.2.1 Viral and bacterial interactions in the respiratory tract (II)

Nontypeable *H. influenzae* and *S. pneumoniae* have been isolated more frequently from patients with viral respiratory infections when compared to subjects without viral infections, indicating a possible link between viral infection and bacterial colonization (White *et al*. 2003). Neuraminidase seems to play a role also in virus and bacteria interactions. It is known that influenza and parainfluenza virus produce neuraminidase that could promote *S. pneumoniae* colonization in the host (Peltola & McCullers, 2004). Moreover, it has been demonstrated in mice that influenza A virus can promote fatal septicemia which is followed by the infection by *S. pneumoniae* serotype 3 (Speshock *et al*. 2007). There is also debate going on about viral-bacterial synergy, which of them is colonizing and causing the infection first? The overall opinion seems to be that it is

the virus that is causing the infection which can further promote the infection possibly caused by colonizing bacteria.. The presence of influenzae A virus is usually essential for bacterial colonization of lower respiratory tract (Bakaletz, 1995). The use of influenza A virus and *S. pneumoniae* by Giebink *et* al. have shown an increase in AOM in which *S. pneumoniae* could be cultured in co-inoculated chichillas (67%) compared to those given either the virus or the bacteria alone, 4% and 21%, respectively (Giebink, 1989; Bakaletz, 1995). In original publication II, the interaction between HRV infection and bacterial colonization was studied at 2.6 months of age. There were no discernible associations between HRV infection and colonizations caused by *S. pneumoniae* or *S. aureus* alone. Interestingly, there was an association between HRV and bacterial colonization by both *S. pneumoniae* and *S. aureus* simultaneously. As already stated, there is competition in colonization by *S. pneumoniae* and *S. aureus*. It is rather difficult to speculate the reasons behind this phenomenon since evidence for synergy between HRV and *S. pneumoniae* exists (Ishizuka *et al*. 2003; Wang *et al*. 2009). Children in original publication II were 2.6 months old and perhaps the viral-bacterial synergy is not yet established in them or it is not detected as these children are rarely colonized by *S. pneumoniae*. The situation could be different with older children. In original publication II we also noted the association between HRV infection and *M. catarrhalis* colonization, since 26% of children who had HRV infection were also positive for *M. catarrhalis* colonization at the time of sampling (P=0.026). Our findings corroborate those from children during URI. In a recent study, 22% of children who had HRV infection were also positive for *M. catarrhalis* (Marom *et al*. 2014). In another study where bacterial detection together with HRV was evaluated on children with and without asthma, HRV infection was more likely to precede *M. catarrhalis* detection (Kloepfer *et al*. 2014). However, the mechanisms for the positive associations between these two pathogens are not fully understood. Unfortunately, any association between HRV infection and *H. influenzae* colonization was impossible to be evaluate due to the low positivity rate of *H. influenzae* in children at 2.6 months of age.

6.3 TLR2 R753Q and nasopharyngeal bacterial colonization (I)

In the original publication I, the association between *TLR2* R753Q and colonization by different respiratory bacteria at 2.6 months of age was investigated. We oberved that children who had heterozygote C/T had higher risk (OR 2.91) to be colonized by *S. aureus* when compared with those with normal allele C/C of *TLR2* at position 753 (P=0.027). There were no association between *TLR2* R753Q and other nasopharyngeal bacteria. It has been shown that *TLR2* R753Q is also associated with staphylococcal and other gram-positive bacteria. This may partly be explained by susceptibility to colonization

(Lorenz *et al*. 2000). Moreover, Iwaki *et al* have demonstrated that the extracellular domain of *TLR2* binds directly to PGN, a major cell wall component of *S. aureus* (Iwaki *et al*. 2002). Structural changes in *TLR2* caused by R753Q polymorphism may affect the ability of TLR2 to recognize, and activate corresponding signaling pathways prior to *S. aureus* colonization. However, it should be kept in mind that the frequency of this polymorphism in Caucasian population is generally below <5%. Whether there are critical structural differences between recognition sites of PGN and the studied *TLR2* polymorphic site in *S. aureus* and *S. pneumoniae* remains to be studied. We found no association between *TLR2* R753Q and *S. pneumoniae* colonization, This may have been caused by the low numbers of events, since the number of children who were positive for *S. pneumoniae* at this age was low as was the frequency (5%) of mutated *TLR2* at position 753. Indeed, only 4 subjects with mutated *TLR2* were positive for *S. pneumoniae*. The reported frequency for *TLR2* R753Q in Finnish population is similar to those found in other Caucasian populations (Lorenz *et al*. 2000; Ma *et al*. 2007).

6.4 TLR3 Leu412Phe and nasopharyngeal bacterial-viral colonization (II)

In the original publication II, the effects of *TLR3* L412F on bacterial colonization and HRV infection at 2.6 months of age were studied. No significant associations were found. We did however observe that *S. aureus* colonization decreased in those children with *TLR3* normal allele C/C who also had HRV infection (P=0.034). *TLR3* L412F is associated with risk for recurrent herpes simplex virus infection and with bronchiolitis leading to hospitalization and postbronchiolitis wheezing in young infants (Zhang *et al*. 2007; Yang *et al*. 2012; Nuolivirta *et al*. 2012). At 2.6 months of age, children with the normal allele and variant genotypes had almost similar prevalence for HRV infection (18% vs. 21%). It remains to be seen whether *TLR3* L412F has a role in early childhood of these children regarding bacterial-viral colonization.

6.5 TLR4 D299G and nasopharyngeal bacterial colonization (I, II and III)

TLR4 D299G has been in the scope of interest in many studies. In original publications I-III D299G polymorphism was assessed together with bacterial and viral colonization. In the original publication I, children with *TLR4* D299G heterozygosity A/G had higher risk (OR 2.37) to be colonized by *M. catarrhalis* than those with normal allele A/A, indicating that TLR4 plays an important role in protecting against *M. catarrhalis* and perhaps other gram-negative bacteria. In the original publication II, no associations were observed in children with *TLR4* D299G heterozygosity A/G or with wild type A/A between bacterial colonizations and HRV infection. In the original publication II,

children with normal allele A/A were more often colonized by *M. catarrhalis* when HRV infection was present compared to children with the same genotype but without HRV infection. This observation is most likely due to interaction between *M. catarrhalis* and HRV rather than the possibility that *TLR4* normal allele A/A would endanger the host for mixed colonization of these two pathogens. In the original publication III, where 161 children were followed from 2.6 to 24 months of age, children with *TLR4* D299G heterozygote A/G were found to have significantly higher *M. catarrhalis* colonization rates when compared to those with wild type allele A/A at 2.6 months of age. The phenomenon noticed at 2.6 months of age was no longer present at the ages of 13 and 24 months. However, it was noticed that those children with heterozygous A/G still had higher risk (OR 4.90) to have repeated colonizations of *M. catarrhalis* throughout the follow-up when compared to children with wild type A/A (P=0.001) and children with heterozygous A/G also had higher bacterial loads of *M. catarrhalis* implying that heterozygous A/G renders children still more susceptible to *M. catarrhalis.* We did not study the effect of antibiotics received between the different time points thus our findings do not reflect the natural history of patients having an access to antibiotics. The colonization rate of *H. influenzae* was also significantly higher in children with *TLR4* D299G heterozygosity at 24 months of age. No difference between the genotypes and bacterial colonization by gram-positive bacteria *S. pneumoniae* and *S. aureus* were observed. These findings seem plausible since TLR4 senses antigens on gram-negative bacteria. Our findings may support the suggestion that *TLR4* D299G polymorphism may be associated with hyporesponsiveness to LPS, a important bacterial cell wall component of gram-negative bacteria, leading to an improper activation of TLR4 specific signaling pathways and insufficient production of pro-inflammatory cytokines and interferons (van der Graaf; *et al*. 2005; Miller *et al*. 2005). This impaired response could be the factor that influences the host in the clearance of *M. catarrhalis* and other gram-negative bacteria. Furthermore, this information could support the findings reported in the original publication III that children are more susceptible to gram-negative bacterial colonizations as well as to increased bacterial loads already in early childhood. Even though the children with heterozygous A/G were at higher risk to be repeatedly colonized, based on RAPD analysis performed in original publication III, these children seemed to be colonized by different *M. catarrhalis* strains during the follow-up. This indicates that the children are not persistently colonized by the same strain and the colonizing strain is cleared at some point but is rapidly replaced by another strain. The findings from the original publication III support the role for *TLR4* D299G in predisposing young children to infections caused by gram-negative bacteria.

The frequency for *TLR4* D299G was 18% in the present study. Similar frequencies were also reported in other studies carried out in Finland (Hernesniemi *et al*. 2008; Löfgren *et al*. 2010). Another common SNP of *TLR4*, T399I, was not tested in this study. However, it is known that the two SNPs are generally in linkage disequilibrium in Caucasian population (Schröder *et al*. 2005). The SNP T399I has been shown to be associated with symptomatic RSV disease in high-risk infants and support a dual role for *TLR4* SNPs (Awomoyi *et al*. 2007). Furthermore, *TLR4* T399I has been shown to influence susceptibility and severity of pulmonary tuberculosis (Najmi *et al*. 2010).

6.6 Polymorphisms in MBL and nasopharyngeal bacterial-viral colonization (I and II)

The role of MBL in innate immune system is most pronounced during the time when maternal-derived antibodies start to disappear and the immune system of a child is still under maturation before primary immune responses can be launched following pathogen challenges (Garred *et al*. 2006). In the original publications I and II, possible associations between *MBL2* polymorphisms and bacterial colonzations as well as HRV infection were studied. In the original publication I, it was observed that children with variant type of MBL (A/O and O/O combined) had a higher risk (OR 2.31) to be colonized by *S. pneumoniae* than children with the wild type MBL (A/A). Moreover, it was observed that these children were also more often colonized by *S. aureus* and other staphylococci. In the original publication II, it was observed that HRV infection was associated with *S. pneumoniae* colonization in children with variant types of *MBL2* and that these children had an increased risk (OR 5.78) to be colonized by this bacterium during the HRV infection. This information supports the results from the original publication I indicating that children with variant types of *MBL2* have an impaired clearance of the important respiratory pathogens *S. pneumoniae* and *S. aureus*. Impaired clearance and increased rates of colonization may partly explain why MBL deficiencies are associated with infectious complications at early life. Whether it is the lower concentrations of serum MBL or the disrupted structure of the molecule or both that make children more prone to certain bacterial colonizations, remains to be shown. In the original publication II, HRV infection was associated with *S. pneumoniae* colonization in children with variant types of MBL. Again, MBL deficiency has been associated with community-aquired pneumonia in patients with viral co-infections (Endeman *et al*. 2008). There was a positive association between HRV infection and *S. pneumoniae* colonization. Furthermore, children with variant types of *MBL2* were more often colonized by *S. pneumoniae* when HRV infection was present. It has been shown that HRV infection increases the expression of platelet-activating factor receptor in the respiratory tract

epithelium; this acts as a receptor for *S. pneumoniae* as well (Wang *et al*. 2009; Ishizuka *et al*. 2003). Children with variant types of *MBL2* may display somewhat compromised immune response to HRV infection, which while affecting the epithelium also makes it more favorable for *S. pneumoniae* to bind to it.

The frequency (32%) of variant types of *MBL2* in our study closely corroborates those found in earlier studies (Rantala *et al*. 2008; Seppänen *et al*. 2009).

6.7 IL17A G152A and nasopharyngeal bacterial colonization (IV)

IL-17A appears to be the major Th17 cytokine and is involved in host defense against infection and development of inflammation. In original publication IV association between *IL17A* G152A and bacterial colonization was studied during the follow-up period, and the effect of *IL17A* G152A on blood concentrations of this protein in young children was examined at 13 months of age. Children with *IL17A* G152A homozygote A/A had increased risk (OR 2.30) of being colonized by *S. pneumoniae* when compared to children with normal allele G/G at 13 and 24 months of age (table 4). This phenomenon was not observed at 2.6 months of age. No association between *IL17A* G152A and other studied colonizing bacteria were observed during any of the time points. It has been demonstrated that immunization of mice with *S. pneumoniae* endowes CD4+ T cell-dependent protection against its colonization and IL-17A production promotes the clearance of *S. pneumoniae* by neutrophilic infiltration (Lu *et al*. 2008). Results suggest that IL-17A has an important role in protection against *S. pneumoniae* at least in mice. Moreover, Lu *et al* demonstrated that IL-17A can accelerate pneumococcal killing by human neutrophils in both the absence and presence of antibodies and complement system, proposing a major role for IL-17A in pneumococcal immunity in humans. Based on this demonstration, individuals with Th17 cell deficiencies would be expected to be more prone to *S. pneumoniae* colonization and infections as well as diseases such as rare primary immune deficiency the classical hyper-IgE syndrome (HIES, caused by dominant negative mutations of *STAT3*) which is characterized by elevated serum IgE and recurrent bacterial infections of the lung (McDonald, 2012). Our results obtained in the original publication IV further suggest that *IL17A* G152A may influence the levels of IL-17A and cause impaired clearance of *S. pneumoniae* in children with homozygote A/A. Chen *et al* reported a similar finding and observed higher *S. pneumoniae* colonization rates in bronchiolitis patients with homozygote A/A of *IL17A* G152A (Chen *et al*. 2010). However, their study cohort was comprised of patients with bronchiolitis whereas our study recruited healthy young children. The lack of association between *S. pneumoniae* colonization and the homozygote A/A at 2.6 months of age could be due to the low

frequency of *S. pneumoniae* at this age. Also, serum IL-17A is generally low in young children and thus IL-17A may not play a critical role in epithelial and mucosal tissues yet at this age. The negative association reported with other bacterial species in all three age groups could indicate that the clearance of *S. pneumoniae* is more IL-17A-dependent as suggested by different studies in mice and humans (Wright *et al*. 2013; Wang *et al*. 2014; Kim *et al*. 2013). It is also known that IL-17A plays an important role against *S. aureus* colonization, the cause to our failure to find any association could be the low colonization rate of *S. aureus* at 13 and 24 months of age. However, Th17 cell deficiencies have been reported to be associated with recurrent *S. aureus* colonization for example in HIES (McDonald, 2012)

To study effects of different genotypes of the *IL17A* G152A polymorphism, we analyzed serum IL-17A concentrations of these subjects. Serum samples were selected from children at 13 months of age. This was done because there was only limited amount of sera available from different age groups; at 13 months of age sera was most readily available. Children with homozygote A/A had undetectable serum concentrations for IL-17A and children with heterozygote G/A had lower IL-17A concentrations than children with normal allele G/G (Figure 11). It is noteworthy to mention that generally the levels of circulating IL-17A were low in all tested subjects. However, those 43% of the children who had detectable concentrations, the concentrations were still within the detection limit of the test kit. The reported concentrations are in line with other studies indicating the levels of IL-17A may be low in young children and even in adults (Chien *et al*. 2013; Pavlovic *et al*. 2014). The findings in the original publication IV indicate that *IL17A* G152A genotype has an effect on the concentration of this particular cytokine already at young age and since IL-17A is a potent activator of neutrophils responsible for early pathogen clearance, children with homozygote A/A may display impaired eradication of pathogens (Wang *et al*. 2014).

The frequencies for *IL17A* G152A were 19% for homozygote A/A and 45% for heterozygote G/A. This is the first study to report the frequency of *IL17A* G152A in Finns. Similar frequencies have been observed in studies carried out in Norwegian and Spanish populations (Ocejo-Vinyals *et al*. 2013; Nordang *et al*. 2009).

6.8 Other external factors affecting nasopahryngeal bacterial colonization in young children (II)

Other known factors contributing to the bacterial colonization outside microbial interactions and host genetic factors include mostly socio-economical factors like the

number of siblings, day-care attendance, smoking of the parent, breastfeeding, housing, access to health care, vaccinations, antibiotics and poor healthcare (García-Rodríguez & Fresnadillo Martínez, 2002). The proportional role of the social factors compared to individual variation (genetic factors) in resistance or susceptibility to bacterial colonization is incompletely understood, although available evidence suggest that both acquisition and carriage of bacteria in young children is influenced by their socio-economic status (Leach *et al*. 1994). There are differences in bacterial carriage even in countries with similar environments and socio-economical conditions (De Lencastre *et al*. 1999; Principi *et al*. 1999). In the original publication II, *S. pneumoniae* and *M. catarrhalis* colonizations were found to be associated with having one or more siblings in a family. This indicates that elder siblings from the family, who are attending daycare or school might help transmit both bacteria and viruses to younger siblings especially at 2.6 months of age when these children are yet to receive their first vaccinations.

6.9 Limitations of the study

There are limitations in this study. The SNPs selected for this study from *TLRs*, *MBL2* and *IL17A* were based on their previously reported importance for bacterial colonization and respiratory infections. However, we did not perform any studies to characterize the functional consequences of these selected SNPs, but focused on studying associations of any of the SNPs with nasopharyngeal bacterial colonization. However, functional studies for TLRs have been carried out in mice and *in vitro* as described earlier in the text. It is important to see which functional mechanisms are behind the phenomenon that certain SNPs in *TLR*s, *MBL2* and *IL17A* increase the risk for certain bacterial colonization. There are several SNPs reported in each of *TLR*s, however, we only studied one SNP each from *TLR2*, *-3* and *-4*. Using high throughput SNP analysis would have enabled us to study more SNPs in these genes of the innate immunity. In addition, we did not perform haplotype analysis for the three studied *TLR*s. The sample size for NP samples was 489 at 2.6 months of age but the sample size decreased considerably to 202 at 13 months of age and to 176 at 24 months of age, making the sample size maintained throughout the whole study period relatively small.

7. SUMMARY AND CONCLUSIONS

Nasopharynx is a complex ecosystem and contains various bacteria and viruses. These micobes can asymptomatically colonize infants and young children but some are also associated with various respiratory infections. Common pathogenic bacteria include *S. pneumoniae*, *H. influenzae*, *M. catarrhalis* and *S. aureus*. Innate immunity molecules control the first line of defense against pathogenic bacteria. These molecules include TLRs, MBL and different cytokines. Gene polymorphisms in these key molecules have been reported. However, the overall effect of genetic variations in TLRs, MBL and different cytokines on bacterial colonization needs further study. The aims of this thesis project were (I) to investigate the association between nasopharyngeal bacterial colonization and gene polymorphisms of *MBL2*, *TLR2* and *-4* in Finnish healthy children at 2.6 months of age, (II) to study the possible effects of HRV infection on nasopharyngeal bacterial colonization in these children with respect to genetic variations in *MBL2*, *TLR3* and *-4*, (III) to investigate the association between *TLR4* D299G polymorphisms and *M. catarrhalis* colonization in healthy children during a two-year follow-up, and (IV) to investigate the possible differences in IL-17A cytokine production among healthy infants and infants with *IL17A* G152A polymorphism and its association with nasopharyngeal bacterial colonization.

I) The results from the study demonstrate that healthy young children with variant types of *MBL2*, *TLR2* or *TLR4* have an increased risk to be colonized by *S. pneumoniae*, *S. aureus* or *M. catarrhalis*, respectively. This supports the role of these genetic variants to make children perhaps more susceptible to nasopharyngeal carriage of respiratory pathogens which may render them more susceptible to respiratory infcetions..

II) It is unclear why some children are prone to acquire bacterial infections after viral infections. In this study it was demonstrated that variant types of *MBL2* in healthy children may facilitate HRV-induced *S. pneumoniae* colonization. This finding indicates that genetic variations may play specific roles on the viral–bacterial interactions and in the pathogenesis of certain respiratory infections.

III) In the first study children with *TLR4* D299G were more often colonized by *M. catarrhalis* at 2.6 months of age. The results from this third study showed that children with *TLR4* D299G had an increased risk of repeated *M. catarrhalis* colonization by different strains during the first two years of life. Furthermore, this polymorphism also had an effect on the bacterial load of *M. catarrhalis* colonization. Both of these findings

suggest that *TLR4* D299G has a role in the susceptibility to gram-negative bacterial infections.

IV) The results from the fourth study showed that children with *IL17A* G152A were at increased risk of being colonized by *S. pneumoniae* at 13 months and 24 months of age. Moreover, the results suggest that *IL17A* G152A has an effect on the levels of serum IL-17A. Both of these findings suggest that this polymorphism has a role in the susceptibility to *S. penumoanie* carriage and possibly in development of respiratory infections.

For the first time, this study assessed the frequenies of *TLR2* R753Q and *TLR3* L412F as well as *IL17A* G152A in Finnish population. In conclusion, genetic variations in important pattern recognition receptors and cytokines of the innate immunity seem to render healthy young children prone to bacterial colonization by respiratory pathogens. This further promote the development of respiratory infections and asthma in their later life. However, though these associations between genetic variations and bacterial colonization only serve as susceptibility factors to respiratory infections. Not necessarily all children with variant types of *TLR*s or *MBL2* rendered clinically more prone to infections than children with normal alleles. Though these polymorphisms may have an impact on the early life of a child, but whether these affect adolecents, remains to be seen. In addition to genetic factors socio-economical factors affect the susceptibility to colonization and may play as important a role as genetic factors, in the early chidlhood. The main findings of this thesis project suggest that genetic polymorphisms in the genes of innate immunity play an important role in the host-pathogen interaction. The studied children will be followed until 5 years of age. The consequent clinical follow-up data will help us to assess to which extent these genetic polymorphisms affect the course of infections in the early life of a child. This in turn will provide rational basis for further information and intervention for children who may develop respiratory infections.

ACKNOWLEDGEMENTS

This study was carried out at the Pertussis Reference Laboratory, Department of Infectious Disease Surveillance and Control, National Institute for Health and Welfare, Turku, Finland and at Department of Pediatrics, University of Turku, Turku, Finland as well as the Doctoral Program of Clinical Investigation (TKT/CLIPD), University of Turku, Turku, Finland.

I wish to offer my sincerest gratitude to my supervisor adj. professor Qiushui He, who gave me the chance to be a part of his research team in the former National Health Institute (KTL) now known as the National Institute for Health and Welfare (THL). You have supported me throughout my thesis with patience and knowledge. I have learned a lot from you.

I would like to express my profound gratitude and thanks to my second supervisor professor Jussi Mertsola for his guidance and support during my thesis.

Professors Erika Isolauri and Jussi Mertsola from the Department of Pediatrics are appreciated for giving me the opportunity to study as a graduate student. I would like to also than professors Pentti Huovinen and Jaana Vuopio from the Department of Medical Microbiology and Immunology for collaboration in basic and clinical research of microbiology and immunology.

I would like to thank all my co-authors: Laura Toivonen, Laura Lindholm, Kirsi Gröndahl-Yli-Hannuksela, Alex-Mikael Barkoff, Qiushui He, Jussi Mertsola, Ville Peltola, Matti Waris, Sinikka Karppinen and Jorma Ilonen for their invaluable contribution to laboratory experiments, manuscript preparations and comments. Your help has truly been appriciated. All the study nurses and families participating to this study are also greatly appriciated.

Thesis committee members professor Ville Peltola and adj. professor Matti Waris are warmly appricitated for their expertise and valuable guidance throughout my thesis studies.

The reviewers professor Mikael Skurnik and adj. professor Mikko Seppänen are thanked for their constructive comments and critisisms as well as for showing flexibility during the reviewing process due to my tight time schedule.

I would like to express my warmest gratitude to my past and present fellow co-workers at the Pertussis laboratory: Päivi Haaranen, Elisa Laakso, Leni Mannermaa, Teemu Kallonen, Annika Elomaa, Alex-Mikael Barkoff, Kirsi Gröndahl-Yli-Hannuksela and Johanna Teräsjärvi. You have made my thesis journey possible with your knowledge, encouragement, praises and push towards the end. Thank you for never being too busy, although I knew you were, to have a chat about everything possible, even science. Above all else, Alex-Mikael Barkoff, thank you for your friendship and honesty as well as the nights out from the lab: those are hard to remember but also hard to forget. Thanks also to all junior researchers at the National Institute for Health and Welfare.

I would like to thank the rest of the personnel of National Institute for Health and Welfare, Turku for the amazing working atmospehere and relaxing conversations and chats. I am thankfull for your help during my thesis, especially at its inception.

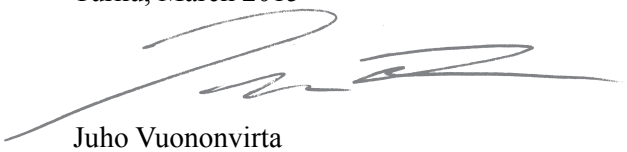
From when my journey into Academia started many years ago in England: Joona, Mike, Alex, Rob, James and Matt, my life would not be the same without you guys, and not necessarily in a good way ☺. The boys from Oulu and Turku Connections, you know who you are. Everything has always been better after a drink and a chat with you.

I owe my very deepest gratitude to all my family, Isä, Äiti, Reeta and Raisa for never doubting and always encouraging. I owe you so much.

Finally, my love Ida, eternal thanks for your love and support as well as for putting up with me in our everyday life, you kept me sane with your wonderful humour during this journey. I would not be in this point without you.

This work was financially supported by the Academy of Finland, the Special Governmental Fund for University Hospitals (EVO), the Doctoral Program of Clinical Investigation (TKT/CLIPD), University of Turku, THL annual budget, Orion-Farmos Foundation, Paulo Foundation, Maud Kuistila Memorial Foundation and Otto A. Malmi Foundation.

Turku, March 2015



Juho Vuononvirta

REFERENCES

Aebi, C. (2011). Moraxella catarrhalis – pathogen or commensal? *Adv Exp Med Biol*, 697:107-116.

Agnese, D. Calvano, J. Hahm, S. Coyle, S. Corbett, S. Calvano, S. & Lowry, S. (2002). Human toll-like receptor 4 mutations but not CD14 polymorphisms are associated with an increased risk of gram-negative infections. *J Infect Dis*, 186(10):1522-1525.

Ahmad-Nejad, P. (2009). Pyrosequencing of Toll-Like Receptor Polymorphisms of Functional Relevance; DNA and RNA Profiling in Human Blood. *Methods in Molecular Biology*, Volume 496, Part I: 73–87.

Alexopoulou, L. Holt, A. Medzhitov, R. & Flavell, R. (2001). Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. *Nature*, 413(6857):732-738.

Alsina, L. Israelsson, E. Altman, M. Dang, K. Ghandil, P. Israel, L. . . . Chaussabel, D. (2014). A narrow repertoire of transcriptional modules responsive to pyogenic bacteria is impaired in patients carrying loss-of-function mutations in MYD88 or IRAK4. *Nat Immunol*, 15(12):1134-1142.

Anderson, G. & Coyle, A. (1994). TH2 and 'TH2-like' cells in allergy and asthma: pharmacological perspectives. *Trends Pharmacol Sci*, 15(9):324-332.

Anon, J. Jacobs, M. Poole, M. Ambrose, P. Benninger, M. Hadley, J. . . . Partnership, S. A. (2004). Antimicrobial treatment guidelines for acute bacterial rhinosinusitis. *Otolaryngol Head Neck Surg*, 130(1 Suppl):1-45.

Arbour, N. Lorenz, E. Schutte, B. Zabner, J. Kline, J. Jones, M. . . . Schwartz, D. (2000). TLR4 mutations are associated with endotoxin hyporesponsiveness in humans. *Nat Genet*, 25(2):187-191.

Awomoyi, A. Rallabhandi, P. Pollin, T. Lorenz, E. Sztein, M. Boukhvalova, M. . . . Vogel, S. (2007). Association of TLR4 polymorphisms with symptomatic respiratory syncytial virus infection in high-risk infants and young children. *J Immunol*, 179(5):3171-3177.

Bae, S. Yu, J. Lee, K. Lee, S. Park, B. & Kang, Y. (2012). Nasal colonization by four potential respiratory bacteria in healthy children attending kindergarten or elementary school in Seoul, Korea. *J Med Microbiol*, 61(Pt 5):678-685.

Bakaletz, L. (1995). Viral potentiation of bacterial superinfection of the respiratory tract. *Trends Microbiol*, 3(3):110-114.

Barreiro, L. Ben-Ali, M. Quach, H. Laval, G. Patin, E. Pickrell, J. . . . Quintana-Murci, L. (2009). Evolutionary dynamics of human Toll-like receptors and their different contributions to host defense. *PLoS Genet*, 5(7):e1000562.

Barton, G. (2007). Viral recognition by Toll-like receptors. *Semin Immunol*, 19(1):33-40.

Bell, J. Askins, J. Hall, P. Davies, D. & Segal, D. (2006). The dsRNA binding site of human Toll-like receptor 3. *Proc Natl Acad Sci U S A*, 103(23):8792-8797.

Beltrame, M. Catarino, S. Goeldner, I. Boldt, A. & de Messias-Reason, I. (2015). The lectin pathway of complement and rheumatic heart disease. *Front Pediatr*, 2:148.

Bisgaard, H. Hermansen, M. Buchvald, F. Loland, L. Halkjaer, L. Bønnelykke, K. . . . Pipper, C. (2007). Childhood asthma after bacterial colonization of the airway in neonates. *N Engl J Med*, 357;1487–1495.

Blomqvist, S. Roivainen, M. Puhakka, T. Kleemola, M. & Hovi, T. (2002). Virological and serological analysis of rhinovirus infections during the first two years of life in a cohort of children. *J Med Virol*, 66(2):263-268.

Blumental, S. Deplano, A. Jourdain, S. De Mendonça, R. Hallin, M. Nonhoff, C. . . . Denis, O. (2013). Dynamic pattern and genotypic diversity of Staphylococcus aureus nasopharyngeal carriage in healthy pre-school children. *J Antimicrob Chemother*, 68(7):1517-1523.

Bogaert, D. van Belkum, A. Sluijter, M. Luijendijk, A. de Groot, R. Rümke, H.;. . . Hermans, P. (2004). Colonisation by Streptococcus pneumoniae and Staphylococcus aureus in healthy children. *Lancet*, 363(9424):1871-1872.

Botos, I. Segal, D. & Davies, D. (2011). The structural biology of Toll-like receptors. *Structure*, 19(4):447-459.

Canton, J. Neculai, D. & Grinstein, S. (2013). Scavenger receptors in homeostasis and immunity. *Nat Rev Immunol*, 13(9):621-634.

Carroll, K. (2002). Laboratory Diagnosis of Lower Respiratory Tract Infections: Controversy and Conundrums. *J Clin Microbiol*, 40(9): 3115–3120.

Casanova, J. Abel, L. & Quintana-Murci, L. (2011). Human TLRs and IL-1Rs in Host Defense: Natural Insights from Evolutionary, Epidemiological and Clinical Genetics. *Annu. Rev. Immunol.*, 29:447-491.

Chen, J. Deng, Y. Zhao, J. Luo, Z. Peng, W. Yang, J. . . . Liu, E. (2010). The Polymorphism of IL-17 G-152A was Associated with Childhood Asthma and Bacterial Colonization of the Hypopharynx in Bronchiolitis. *J Clin Immunol*, 30:539-545.

Chen, Y. & Kirk, M. (2013). Incidence of acute respiratory infections in Australia. *Epidemiol Infect*, 142(7):1355-1361.

Chien, J. Lin, C. Yang, K. Lin, C. Kao, J. & Tsai, Y. (2013). Increased IL-17A secreting CD4+ T cells, serum IL-17 levels and exhaled nitric oxide are correlated with childhood asthma severity. *Clin Exp Allergy*, 43(9):1018-1026.

Chien, Y. Vidal, J. Grijalva, C. Bozio, C. Edwards, K. Williams, J. . . . Klugman, K. (2013). Density interactions among Streptococcus pneumoniae, Haemophilus influenzae and Staphylococcus aureus in the nasopharynx of young Peruvian children. *Pediatr Infect Dis J*, 32(1):72-77.

Choe, J. Kelker, M. & Wilson, I. (2005). Crystal structure of human toll-like receptor 3 (TLR3) ectodomain. *Science*, 309(5734):581-585.

Coomes, S. Pelly, V. & Wilson, M. (2013). Plasticity within the $\alpha\beta^{+}CD4^{+}$ T-cell lineage: when, how and what for? *Open Biol*, 3(1):120157.

Croucher, N. Finkelstein, J. Pelton, S. Mitchell, P. Lee, G. Parkhill, J. . . . Lipsitch, M. (2013). Population genomics of post-vaccine changes in pneumococcal epidemiology. *Nat Genet*, 45(6):656-663.

Daha, M. (2010). Role of complement in innate immunity and infections. *Crit Rev Immunol*, 30(1):47-52.

De Lencastre, H. Kristinsson, K. Brito-Avô, A. Sanches, I. Sá-Leão, R. Saldanha, J. . . . Tomasz, A. (1999). Carriage of respiratory tract pathogens and molecular epidemiology of Streptococcus pneumoniae colonization in healthy children attending day care centers in Lisbon, Portugal. *Microb Drug Resist*, 5(1):19-29.

de Vries, S. Bootsma, H. Hays, J. & Hermans, P. (2009). Molecular aspects of Moraxella catarrhalis pathogenesis. *Microbiol Mol Biol Rev*, 73(3):389-406.

Deban, L. Jaillon, S. Garlanda, C. Bottazzi, B. & Mantovani, A. (2011). Pentraxins in innate immunity: lessons from PTX3. *Cell Tissue Res*, 343(1):237-249.

Dhiman, N. Ovsyannikova, I. Vierkant, R. Ryan, J. Pankratz, V. Jacobson, R. & Poland, G. (2008). Association between SNPs in Toll-like receptors and related intracellular signalling molecules and immune responses to measles vaccine: Preliminary results. *Vaccine*, 26:1731-1736.

Dommett, R. Klein, N. & Turner, M. (2006). Mannose-binding lectin in innate immunity: past, present and future. *Tissue Antigens*, 68(3):193-209.

Dunne, E. Smith-Vaughan, H. Robins-Browne, R. Mulholland, E. & Satzke, C. (2013). Nasopharyngeal microbial interactions in the era of pneumococcal conjugate vaccination. *Vaccine*, 31(19):2333-2342.

Eisen, D. & Minchinton, R. (2003). Impact of mannose-binding lectin on susceptibility to infectious diseases. *Clin Infect Dis*, 37(11):1496-1505.

Endeman, H. Herpers, B. de Jong, B. Voorn, G. Grutters, J. van Velzen-Blad, H. & Biesma, D. (2008). Mannose-binding lectin genotypes in susceptibility to community-acquired pneumonia. *Chest*, 134(6):1135-1140.

Ermini, L. Wilson, I. Goodship, T. & Sheerin, N. (2012). Complement polymorphisms: geographical distribution and relevance to disease. *Immunobiology*, 217(2):265-271.

Erridge, C. (2010). Endogenous ligands of TLR2 and TLR4: agonists or assistants? *J Leukoc Biol*, 87(6):989-999.

Espinoza, J. Takami, A. Nakata, K. Onizuka, M. Kawase, T. Akiyama, H. . . . Program, J. M. (2011). A genetic variant in the IL-17 promoter is functionally associated with acute graft-versus-host disease after unrelated bone marrow transplantation. *PLoS One*, 6 (10): p. e26229.

Faden, H. Duffy, L. Wasielewski, R. Wolf, J. Krystofik, D. & Tung, Y. (1997). Relationship between nasopharyngeal colonization and the development of otitis media in children. Tonawanda/Williamsville Pediatrics. *J Infect Dis*, 175(6):1440-1445.

Faden, H. Duffy, L. Williams, A. Krystofik, D. & Wolf, J. (1995). Epidemiology of nasopharyngeal colonization with nontypeable Haemophilus influenzae in the first 2 years of life. *J Infect Dis*, 172(1):132-135.

Faden, H. Harabuchi, Y. & Hong, J. (1994). Epidemiology of Moraxella catarrhalis in children during the first 2 years of life: relationship to otitis media. *J Infect Dis*, 169(6):1312-1317.

Farjo, R. Foxman, B. Patel, M. Zhang, L. Pettigrew, M. McCoy, S. . . . Gilsdorf, J. (2004). Diversity and sharing of Haemophilus influenzae strains colonizing healthy children attending day-care centers. *Pediatr Infect Dis J*, 23(1):41-46.

Ferwerda, B. McCall, M. Alonso, S. Giamarellos-Bourboulis, E. Mouktaroudi, M. Izagirre, N. & Syafruddin, D. (2007). TLR4 polymorphisms, infectious diseases, and evolutionary pressure during migration of modern humans. *Proc Natl Acad Sci U S A*, 104(42):16645-16650.

Forrest, M. Skibola, C. Lightfoot, T. Bracc, i. P. Willett, E. Smith, M. . . . Roman, E. (2006). Polymorphisms in innate immunity genes and risk of non-Hodgkin lymphoma. *Br J Haematol*, 134(2):180-183.

Foundation, W. L. (2010). Acute Respiratory Infections Atlas, First edition, World Lung Foundation, New York 2010.

Fowler, V. J. & Proctor, R. (2014). Where does a Staphylococcus aureus vaccine stand? *Clin Microbiol Infect*, 20 Suppl 5:66-75.

Frans, G. Meyts, I. Picard, C. Puel, A. Zhang, S. Moens, L. . . . Bossuyt, X. (2014). Addressing diagnostic challenges in primary immunodeficiencies: laboratory evaluation of Toll-like receptor- and NF-κB-mediated immune responses. *Crit Rev Clin Lab Sci*, 51(2):112-123.

García-Rodríguez, J. & Fresnadillo Martínez, M. (2002). Dynamics of nasopharyngeal colonization by potential respiratory pathogens. *J Antimicrob Chemother*, 50 Suppl S2:59-73.

Garred, P. Brygge, K. Sørensen, C. Madsen, H. Thiel, S. & Svejgaard, A. (1993). Mannan-binding protein--levels in plasma and upper-airways secretions and frequency of genotypes in children with recurrence of otitis media. *Clin Exp Immunol*, 94(1):99-104.

Garred, P. Larsen, F. Seyfarth, J. Fujita, R. & Madsen, H. (2006). Mannose-binding lectin and its genetic variants. *Genes Immun*, 7(2):85-94.

Gern, J. (2002). Rhinovirus respiratory infections and asthma. *Am J Med*, 112 Suppl 6A:19S-27S.

Gessner, B. Mueller, J. & Yaro, S. (2010). African meningitis belt pneumococcal disease epidemiology indicates a need for an effective serotype 1 containing vaccine, including for older children and adults. *BMC Infect Dis*, 10:22.

Giebink, G. (1989). The microbiology of otitis media. *Pediatr Infect Dis J*, 8(1 Suppl):S18-20.

Givon-Lavi, N. Fraser, D. Porat, N. & Dagan, R. (2002). Spread of Streptococcus pneumoniae and antibiotic-resistant S. pneumoniae from day-care center attendees to their younger siblings. *J Infect Dis*, 186:1608-1614.

Glezen, W. Taber, L. Frank, A. & Kasel, J. (1986). Risk of primary infection and reinfection with respiratory syncytial virus. *Am J Dis Child*, 140(6):543-546.

Gorbea, C. Makar, K. Pauschinger, M. Pratt, G. Bersola, J. Varela, J. . . . Bowles, N. (2010). A role for Toll-like receptor 3 variants in host susceptibility to enteroviral myocarditis and dilated cardiomyopathy. *J Biol Chem*, 285(30):23208-23223.

Gunnarsson, R. Holm, S. & Söderström, M. (1998). The prevalence of potential pathogenic bacteria in nasopharyngeal samples from healthy children and adults. *Scand J Prim Health Care*, 16(1):13-17.

Guo, Y. Audry, M. Ciancanelli, M. Alsina, L. Azevedo, J. Herman, M. . . . al., e. (2011). Herpes simplex virus encephalitis in a patient with complete TLR3 deficiency: TLR3 is otherwise redundant in protective immunity. *J Exp Med*, 208(10):2083-2098.

Hall-Stoodley, L. Hu, F. Gieseke, A. Nistico, L. Nguyen, D. Hayes, J. . . . Kerschner, J. (2006). Direct detection of bacterial biofilms on the middle-ear mucosa of children with chronic otitis media. *JAMA*, 296(2):202-211.

Hallström, T. Nordström, T. Tan, T. Manolov, T. Lambris, J. Isenman, D. . . . Riesbeck, K. (2011). Immune evasion of Moraxella catarrhalis involves ubiquitous surface protein A-dependent C3d binding. *J Immunol*, 186(5):3120-3129.

Hamilos, D. (2014). Host-microbial interactions in patients with chronic rhinosinusitis. *J Allergy Clin Immunol*, 133(3):640-653.

Harrison, L. Morris, J. Telford, D. Brown, S. & Jones, K. (1999). The nasopharyngeal bacterial flora in infancy: effects of age, gender, season, viral upper respiratory tract infection and sleeping position. *FEMS Immunol Med Microbiol*, 25(1-2):19-28.

Hassan, F. (2013). Molecular mechanisms of moraxella catarrhalis-induced otitis media. *Curr Allergy Asthma Rep*, 13(5):512-517.

Hausdorff, W. Feikin, D. & Klugman, K. (2005). Epidemiological differences among pneumococcal serotypes. *Lancet Infect Dis*, 5(2):83-93.

Heiskanen-Kosma, T. Korppi, M. & Leinonen, M. (2003). Serologically indicated pneumococcal pneumonia in children: a population-based study in primary care settings. *APMIS*, 111(10):945-950.

Heitzeneder, S. Seidel, M. Förster-Waldl, E. & Heitger, A. (2012). Mannan-binding lectin deficiency - Good news, bad news, doesn't matter? *Clin Immunol*, 143(1):22-38.

Hendley, J. Hayden, F. & Winther, B. (2005). Weekly point prevalence of Streptococcus pneumoniae, Hemophilus influenzae and Moraxella catarrhalis in the upper airways of normal young children: effect of respiratory illness and season. *APMIS*, 113(3):213-220.

Hernesniemi, J. Raitakari, O. Kähönen, M. Juonala, M. Hutri-Kähönen, N.;Marniemi, J. . . . Lehtimäki, T. (2008). Toll-like receptor 4 gene (Asp299Gly) polymorphism associates with carotid artery elasticity. The cardiovascular risk in young Finns study. *Atherosclerosis*, 198(1):152-159.

Hoebe, K. Georgel, P. Rutschmann, S. Du, X. Mudd, S. Crozat, K. . . . Beutler, B. (2005). CD36 is a sensor of diacylglycerides. *Nature*, 433(7025):523-527.

Hong, J. Leung, E. Fraser, A. Merriman, T. Vishnu, P. & Krissansen, G. (2007). TLR2, TLR4 and TLR9 polymorphisms and Crohn's disease in a New Zealand Caucasian cohort. *J Gastroenterol Hepatol*, 22(11):1760-1766.

Howard, A. Dunkin, K. & Millar, G. (1988). Nasopharyngeal carriage and antibiotic resistance of Haemophilus influenzae in healthy children. *Epidemiol Infect*, 100(2):193-203.

Ishigame, H. Kakuta, S. Nagai, T. Kadoki, M. Nambu, A. Komiyama, Y. . . . Iwakura, Y. (2009). Differential roles of interleukin-17A and -17F in host defense against mucoepithelial bacterial infection and allergic responses. *Immunity*, 30;108–119.

Ishizuka, S. Yamaya, M. Suzuki, T. Takahashi, H. Ida, S. Sasaki, T. . . . Sasaki, H. (2003). Effects of rhinovirus infection on the adherence of Streptococcus pneumoniae to cultured human airway epithelial cells. *J Infect Dis*, 188(12):1928-1939.

Iwaki, D. Mitsuzawa, H. Murakami, S. Sano, H. Konishi, M. Akino, T. & Kuroki, Y. (2002). The extracellular toll-like receptor 2 domain directly binds peptidoglycan derived from Staphylococcus aureus. *J Biol Chem*, 277(27):24315-24320.

Iwakura, Y. Ishigame, H. Saijo, S. & Nakae, S. (2011). Functional specialization of interleukin-17 family members. *Immunity*, 34(2):149-162.

Iwasaki, A. & Medzhitov, R. (2010). Regulation of adaptive immunity by the innate immune system. *Science*, 327(5963):291-295.

Jacobs, M. (2004). Streptococcus pneumoniae: epidemiology and patterns of resistance. *Am J Med*, 117 Suppl 3A:3S-15S.

Jacoby, P. Watson, K. Bowman, J. Taylor, A. Riley, T. Smith, D. & Lehmann, D. (2007). Modelling the co-occurrence of Streptococcus pneumoniae with other bacterial and viral pathogens in the upper respiratory tract. *Vaccine*, 25(13):2458-2464.

Jartti, T. Lehtinen, P. Vuorinen, T. Osterback, R. van den Hoogen, B. Osterhaus, A. & Ruuskanen, O. (2004). Respiratory picornaviruses and respiratory syncytial virus as causative agents of acute expiratory wheezing in children. *Emerg Infect Dis*, 10(6):1095-1101.

Jenkins, K. & Mansell, A. (2010). TIR-containing adaptors in Toll-like receptor signalling. *Cytokine*, 49(3):237-44.

Johansson, N. Kalin, M. Tiveljung-Lindell, A. Giske, C. & Hedlund, J. (2010). Etiology of community-acquired pneumonia: increased microbiological yield with new diagnostic methods. *Clin Infect Dis*, 50(2):202-209.

Jourdain, S. Smeesters, P. Denis, O. Dramaix, M. Sputael, V. Malaviolle, X. . . . Vergison, A. (2011). Differences in nasopharyngeal bacterial carriage in preschool children from different socio-economic origins. *Clin Microbiol Infect*, 17(6):907-14.

Karikó, K. Bhuyan, P. Capodici, J. & Weissman, D. (2004). Small interfering RNAs mediate sequence-independent gene suppression and induce immune activation by signaling through toll-like receptor 3. *J Immunol*, 172(11):6545-6549.

Karikó, K. Ni, H. Capodici, J. Lamphier, M. & Weissman, D. (2004). mRNA is an endogenous ligand for Toll-like receptor 3. *J Biol Chem*, 279(13):12542-12550.

Kawada, M. Okuzumi, K. Hitomi, S. & Sugishita, C. (2003). Transmission of Staphylococcus aureus between healthy, lactating mothers and their infants by breastfeeding. *J Hum Lact*, 19(4):411-417.

Kawai, T. & Akira, S. (2006). TLR signaling. *Cell Death Differ*, 13(5):816-825.

Kawai, T. & Akira, S. (2010). The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. *Nat Immunol*, 11(5):373-384.

Kawasaki, T. & Kawai, T. (2014). Toll-like receptor signaling pathways. *Front Immunol*, 5:461.

Kilpi, T. Herva, E. Kaijalainen, T. Syrjänen, R. & Takala, A. (2001). Bacteriology of acute otitis

media in a cohort of Finnish children followed for the first two years of life. *Pediatr Infect Dis J*, 20(7):654-662.

Kim, B. Lee, S. Berg, R. Simecka, J. & Jones, H. (2013). Interleukin-23 (IL-23) deficiency disrupts Th17 and Th1-related defenses against Streptococcus pneumoniae infection. *Cytokine*, 64(1):375-381.

Kindberg, E. Vene, S. Mickiene, A. Lundkvist, Å. Lindquist, L. & Svensson, L. (2011). A functional Toll-like receptor 3 gene (TLR3) may be a risk factor for tick-borne encephalitis virus (TBEV) infection. *J Infect Dis*, 203(4):523-528.

King, P. (2012). Haemophilus influenzae and the lung (Haemophilus and the lung). *Clin Transl Med*, 1(1):10.

Klein, J. (1997). Role of nontypeable Haemophilus influenzae in pediatric respiratory tract infections. *Pediatr Infect Dis J*, 16(2 Suppl):S5-8.

Kloepfer, K. Lee, W. Pappas, T. Kang, T. Vrtis, R. Evans, M. . . . Gern, J. (2014). Detection of pathogenic bacteria during rhinovirus infection is associated with increased respiratory symptoms and asthma exacerbations. *J Allergy Clin Immunol*, 133(5):1301-1307.

Kluytmans, J. & Wertheim, H. (2005). Nasal carriage of Staphylococcus aureus and prevention of nosocomial infections. *Infection*, 33(1):3-8.

Koch, A. Melbye, M. Sørensen, P. Homøe, P. Madsen, H. Mølbak, K. . . . Garred, P. (2001). Acute respiratory tract infections and mannose-binding lectin insufficiency during early childhood. *JAMA*, 285(10):1316-1321.

Krilov, L. (2011). Respiratory syncytial virus disease: update on treatment and prevention. *Expert Rev Anti Infect Ther*, 9(1):27-32.

Kumar, H. Kawai, T. & Akira, S. (2011). Pathogen recognition by the innate immune system. *Int Rev Immunol*, 30(1):16-34.

Lagström, H. Rautava, P. Kaljonen, A. Räihä, H. Pihlaja, P. Korpilahti, P. . . . Niemi, P. (2013). Cohort Profile: Steps to the Healthy Development and Well-being of Children (the STEPS Study). *Int. J. Epidemiol 42 (5)*, 1273-1284.

Larsen, F. Madsen, H. Sim, R. Koch, C. & Garred, P. (2004). Disease-associated mutations in human mannose-binding lectin compromise oligomerization and activity of the final protein. *J Biol Chem*, 279(20):21302-21311.

Leach, A. Boswell, J. Asche, V. Nienhuys, T. & Mathews, J. (1994). Bacterial colonization of the nasopharynx predicts very early onset and persistence of otitis media in Australian aboriginal infants. *Pediatr Infect Dis J*, 13(11):983-989.

Lee, C. Avalos, A. & Ploegh, H. (2012). Accessory molecules for Toll-like receptors and their function. *Nat Rev Immunol*, 12(3):168-179.

Lee, S. Kok, K. Jaume, M. Cheung, T. Yip, T. Lai, J. . . . Peiris, J. (2014). Toll-like receptor 10 is involved in induction of innate immune responses to influenza virus infection. *Proc Natl Acad Sci U S A*, 111(10):3793-3798.

Lichtman, A. & Abbas, A. (1997). T-cell subsets: recruiting the right kind of help. *Curr Biol*, 7(4):R242-244.

Lorenz, E. Mira, J. Cornish, K. Arbour, N. & Schwartz, D. (2000). A novel polymorphism in the toll-like receptor 2 gene and its potential association with staphylococcal infection. *Infect Immun*, 68(11):6398-6401.

Lorenz, E. Mira, J. Frees, K. & Schwartz, D. (2002). Relevance of mutations in the TLR4 receptor in patients with gram-negative septic shock. *Arch Intern Med*, 162(9):1028-1032.

Lowy, F. (1998). Staphylococcus aureus Infections. *N Engl J Med*, 339:520-532.

Lozano, R. Naghavi, M. Foreman, K. Lim, S. Shibuya, K. Aboyans, V. & Abraham, J. (2012). Global and regional mortality from 235 causes of death for 20 age groups in 1990 and 2010: a systematic analysis for the Global Burden of Disease Study 2010. *Lancet*, 380(9859):2095-2128.

Lu, Y. Gross, J. Bogaert, D. Finn, A. Bagrade, L. Zhang, Q. . . . Malley, R. (2008). Interleukin-17A mediates acquired immunity to pneumococcal colonization. *PLoS Pathog*, 4(9):e1000159.

Lynch, J. 3. & Zhanel, G. (2010). Streptococcus pneumoniae: epidemiology and risk factors, evolution of antimicrobial resistance, and impact of vaccines. *Curr Opin Pulm Med*, 16(3):217-225.

Löfgren, J. Marttila, R. Renko, M. Rämet, M. & Hallman, M. (2010). Toll-like receptor 4 Asp299Gly polymorphism in respiratory syncytial virus epidemics. *Pediatr Pulmonol*, 45(7):687-692.

Ma, X. Liu, Y. Gowen, B. Graviss, E. Clark, A. & Musser, J. (2007). Full-exon resequencing reveals toll-like receptor variants contribute to human susceptibility to tuberculosis disease. *PLoS One*, 2(12):e1318.

Madore, D. (1996). Impact of immunization on Haemophilus influenzae type b disease. *Infect Agents Dis*, 5(1):8-20.

Madsen, H. Garred, P. Thiel, S. Kurtzhals, J. Lamm, L. Ryder, L. & Svejgaard, A. (1995). Interplay between promoter and structural gene variants control basal serum level of mannan-binding protein. *J Immunol*, 155(6):3013-3020.

Marguet, C. Lubrano, M. Gueudin, M. Le Roux, P. Deschildre, A. Forget, C. . . . Freymuth, F. (2009). In very young infants severity of acute bronchiolitis depends on carried viruses. *PLoS One*, 4(2):e4596.

Maródi, L. (2006). Innate cellular immune responses in newborns. *Clin Immunol*, 118(2-3):137-144.

Marom, T. Alvarez-Fernandez, P. Jennings, K. Patel, J. McCormick, D. & Chonmaitree, T. (2014). Acute bacterial sinusitis complicating viral upper respiratory tract infection in young children. *Pediatr Infect Dis J*, 33(8):803-808.

McDonald, D. (2012). TH17 deficiency in human disease. *J Allergy Clin Immunol*, 129(6):1429-1435.

Medzhitov, R. Preston-Hurlburt, P. & Janeway, C. J. (1997). A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. *Nature*, 388(6640):394-397.

Midulla, F. Scagnolari, C. Bonci, E. Pierangeli, A. Antonelli, G. De Angelis, D. . . . Moretti, C. (2010). Respiratory syncytial virus, human bocavirus and rhinovirus bronchiolitis in infants. *Arch Dis Child*, 95(1):35-41.

Miller, E. Lu, X. Erdman, D. Poehling, K. Zhu, Y. Griffin, M. . . . Network., N. V. (2007). Rhinovirus-associated hospitalizations in young children. *J Infect Dis*, 195(6):773-781.

Miller, E. Williams, J. Gebretsadik, T. Carroll, K. Dupont, W. Mohamed, Y. . . . Hartert, T. (2011). Host and viral factors associated with severity of human rhinovirus-associated infant respiratory tract illness. *J Allergy Clin Immunol*, 127(4):883-891.

Miller, S. Ernst, R. & Bader, M. (2005). LPS, TLR4 and infectious disease diversity. *Nat Rev Microbiol*, 3(1):36-46.

Mirsaeidi, M. (2014). Pneumococcal vaccines: understanding centers for disease control and prevention recommendations. *Ann Am Thorac Soc*, 11(6):980-985.

Mitchell, A. & Mitchell, T. (2010). Streptococcus pneumoniae: virulence factors and variation. *Clin Microbiol Infect*, 16(5):411-418.

Mizgerd, J. (2006). Lung infection--a public health priority. *PLoS Med*, 3(2):e76.

Moresco, E. LaVine, D. & Beutler, B. (2011). Toll-like receptors. *Curr Biol*, 21(13):R488-493.

Mosmann, T. & Coffman, R. (1989). TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. *Annu Rev Immunol*, 7:145-173.

Mukhopadhyay, S. & Gordon, S. (2004). The role of scavenger receptors in pathogen recognition and innate immunity. *Immunobiology*, 209(1-2):39-49.

Murphy, T. Brauer, A. Grant, B. & Sethi, S. (2005). Moraxella catarrhalis in chronic obstructive pulmonary disease: burden of disease and immune response. *Am J Respir Crit Care Med*, 172(2):195-199.

Murphy, T. Faden, H. Bakaletz, L. Kyd, J. Forsgren, A. Campos, J. . . . Pelton, S. (2009). Nontypeable Haemophilus influenzae as a pathogen in children. *Pediatr Infect Dis J*, 28(1):43-48.

Musher, D. & Thorner, AR. (2014). Community-acquired pneumonia. *N Engl J Med*, 371(17):1619-1628.

Nahum, A. Dadi, H. Bates, A. & Roifman, C. (2011). The L412F variant of Toll-like receptor 3 (TLR3) is associated with cutaneous candidiasis, increased susceptibility to cytomegalovirus, and autoimmunity. *J Allergy Clin Immunol*, 127(2):528-531.

Nair, H. Nokes, D. Gessner, B. Dherani, M. Madhi, S. Singleton, R. . . . Campbell, H. (2010). Global burden of acute lower respiratory infections due to respiratory syncytial virus in young children: a systematic review and meta-analysis. *Lancet*, 375(9725):1545-1555.

Nair, H. Simões, E. Rudan, I. Gessner, B. Azziz-Baumgartner, E. Zhang, J. & Feikin, D. (2013). Global and regional burden of hospital admissions for severe acute lower respiratory infections in young children in 2010: a systematic analysis. *Lancet*, 381(9875):1380-1390.

Najmi, N. Kaur, G. Sharma, S. & Mehra, N. (2010). Human Toll-like receptor 4 polymorphisms TLR4 Asp299Gly and Thr399Ile influence susceptibility and severity of pulmonary tuberculosis in the Asian Indian population. *Tissue Antigens*, 76(2):102-109.

Narbutt, J. Wojtczak, M. Zalińska, A. Salinski, A. Przybylowska-Sygut, K. Kuna, P. . . . Lesiak, A. (2014). The A/A genotype of an interleukin-17A polymorphism predisposes to increased severity of atopic dermatitis and coexistence with asthma. *Clin Exp Dermatol*, doi: 10.1111/ced.12438. [Epub ahead of print].

Niemelä, M. Uhari, M. Möttönen, M. & Pokka, T. (1999). Costs arising from otitis media. *Acta Paediatr*, 88(5):553-556.

Nordang, G. Viken, M. Hollis-Moffatt, J. Merriman, T. Førre, Ø. Helgetveit, K. . . . Lie, B. (2009). Association analysis of the interleukin 17A gene in Caucasian rheumatoid arthritis patients from Norway and New Zealand. *Rheumatology (Oxford)*, 48(4):367-370.

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

O'Brien, K. Wolfson, L. Watt, J. Henkle, E. Deloria-Knoll, M. McCall, N. . . . Team., H. a. (2009). Burden of disease caused by Streptococcus pneumoniae in children younger than 5 years: global estimates. *Lancet*, 374(9693):893-902.

Ocejo-Vinyals, J. de Mateo, E. Hoz, M. Arroyo, J. Agüero, R. Ausín, F. & Fariñas, M. (2013). The IL-17 G-152A single nucleotide polymorphism is associated with pulmonary tuberculosis in northern Spain. *Cytokine*, 64(1):58-61.

Ohto, U. Yamakawa, N. Akashi-Takamura, S. Miyake, K. & Shimizu, T. (2012). Structural analyses of human Toll-like receptor 4 polymorphisms D299G and T399I. *J Biol Chem*, 287(48):40611-40617.

Omrane, I. Baroudi, O. Kourda, N. Bignon, Y. Uhrhammer, N. Desrichard, A. . . . Bougatef, K. (2014). Positive link between variant Toll-like receptor 4 (Asp299Gly and Thr399Ile) and colorectal cancer patients with advanced stage and lymph node metastasis. *Tumour Biol*, 35(1):545-551.

Omrane, I. Marrakchi, R. Baroudi, O. Mezlini, A. Ayari, H. Medimegh, I. . . . Benammar-Elgaaied, A. (2014). Significant association between interleukin-17A polymorphism and colorectal cancer. *Tumor Biol.*, 35(7):6627-6632.

Oosting, M. Cheng, S. Bolscher, J. Vestering-Stenger, R. Plantinga, T. Verschueren, I. & Arts, P. (2014). Human TLR10 is an anti-inflammatory pattern-recognition receptor. *Proc Natl Acad Sci U S A*, 111(42):E4478-84.

Organization, W. H. (2014). Pneumonia. *http://www.who.int/mediacentre/factsheets/fs331/en/*, Accessed January 5, 2015.

Palaniyar, N. Nadesalingam, J. Clark, H. Shih, M. Dodds, A. & Reid, K. (2004). Nucleic acid is a novel ligand for innate, immune pattern recognition collectins surfactant proteins A and D and mannose-binding lectin. *J Biol Chem*, 279(31):32728-32736.

Palikhe, N. Kim, S. Kim, J. Losol, P. Ye, Y. & Park, H. (2011). Role of Toll-like Receptor 3 Variants in Aspirin-Exacerbated Respiratory Disease. *Allergy Asthma Immunol Res*, 3(2):123-127.

Park, B. & Lee, J. (2013). Recognition of lipopolysaccharide pattern by TLR4 complexes. *Exp Mol Med*, 45:e66.

Pavlovic, V. Dimic, A. Milenkovic, S. & Krtinic, D. (2014). Serum levels of IL-17, IL-4, and INFγ in Serbian patients with early rheumatoid arthritis. *J Res Med Sci*, 19(1):18-22.

Peacock, S. Justice, A. Griffiths, D. de Silva, G. Kantzanou, M. Crook, D. . . . Day, N. (2003). Determinants of acquisition and carriage of Staphylococcus aureus in infancy. *J Clin Microbiol*, 41(12):5718-5725.

Peltola, V. & McCullers, J. (2004). Respiratory viruses predisposing to bacterial infections: role of neuraminidase. *Pediatr Infect Dis J*, 23(1 Suppl):S87-97.

Peltola, V. Waris, M. Osterback, R. Susi, P. Hyypiä, T. & Ruuskanen, O. (2008). Clinical effects of rhinovirus infections. *Journal of Clinical Virology 43(4)*, 43(4):411-4.

Peri, F. Piazza, M. Calabrese, V. Damore, G. & Cighetti, R. (2010). Exploring the LPS/TLR4 signal pathway with small molecules. *Biochem Soc Trans*, 38(5):1390-1395.

Pericone, C. Overweg, K. Hermans, P. & Weiser, J. (2000). Inhibitory and bactericidal effects of hydrogen peroxide production by Streptococcus pneumoniae on other inhabitants of the upper respiratory tract. *Infect Immun*, 68(7):3990-3997.

Pettigrew, M. Gent, J. Revai, K. Patel, J. (2008). Microbial interactions during upper respiratory tract infections. *Emerg Infect Dis*, 14(10):1584-1591.

Pletz, M. Maus, U. Krug, N. Welte, T. & Lode, H. (2008). Pneumococcal vaccines: mechanism of action, impact on epidemiology and adaption of the species. *Int J Antimicrob Agents*, 32(3):199-206.

Poehling, K. Lafleur, B. Szilagyi, P. Edwards, K. Mitchel, E. Barth, R. . . . Griffin, M. (2004). Population-based impact of pneumococcal conjugate vaccine in young children. *Pediatrics*, 114(3):755-761.

Poltorak, A. He, X. Smirnova, I. Liu, M. Van Huffel, C. Du, X. & Birdwell, D. (1998). Defective LPS

signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. *Science*, 282(5396):2085-2088.

Principi, N. Marchisio, P. Schito, G. & Mannelli, S. (1999). Risk factors for carriage of respiratory pathogens in the nasopharynx of healthy children. Ascanius Project Collaborative Group. *Pediatr Infect Dis J*, 18(6):517-23.

Prymula, R. (2006). Pneumococcal capsular polysaccharides conjugated to protein D for prevention of acute otitis media caused by both Streptococcus pneumoniae and non-typable Haemophilus influenzae: a randomised double-blind efficacy study. *Lancet*, 367(9512):740-748.

Pullan, C. & Hey, E. (1982). Wheezing, asthma, and pulmonary dysfunction 10 years after infection with respiratory syncytial virus in infancy. *Br Med J (Clin Res Ed)*, 284(6330):1665-1669.

Ramensky, V. Bork, P. & Sunyaev, S. (2002). Human non-synonymous SNPs: server and survey. *Nucleic Acids Res*, 30(17):3894-900.

Ranjith-Kumar, C. Miller, W. Sun, J. Xiong, J. Santos, J. Yarbrough, I. . . . Kao, C. (2007). Effects of single nucleotide polymorphisms on Toll-like receptor 3 activity and expression in cultured cells. *J Biol Chem*, 282(24):17696-17705.

Rantala, A. Lajunen, T. Juvonen, R. Bloigu, A. Silvennoinen-Kassinen, S. Peitso, A. . . . Leinonen, M. (2008). Mannose-binding lectin concentrations, MBL2 polymorphisms, and susceptibility to respiratory tract infections in young men. *J Infect Dis*, 198(8):1247-53.

Raphael, I. Nalawade, S. Eagar, T. & Forsthuber, T. (2014). T cell subsets and their signature cytokines in autoimmune and inflammatory diseases. *Cytokine*, pii: S1043-4666(14)00539-0.

Regev-Yochay, G. Dagan, R. Raz, M. Carmeli, Y. Shainberg, B. Derazne, E. . . . Rubinstein, E. (2004). Association between carriage of Streptococcus pneumoniae and Staphylococcus aureus in Children. *JAMA*, 292(6):716-720.

Regev-Yochay, G. Trzcinski, K. Thompson, C. Malley, R. & Lipsitch, M. (2006). Interference between Streptococcus pneumoniae and Staphylococcus aureus: In vitro hydrogen peroxide-mediated killing by Streptococcus pneumoniae. *J Bacteriol*, 188(13):4996-5001.

Revai, K. McCormick, D. Patel, J. Grady, J. Saeed, K. & Chonmaitree, T. (2006). Effect of pneumococcal conjugate vaccine on nasopharyngeal bacterial colonization during acute otitis media. *Pediatrics*, 117(5):1823-1829.

Reynolds, J. Angkasekwinai, P. & Dong, C. (2010). IL-17 family member cytokines: Regulation and function in innate immunity. *Cytokine Growth Factor Rev*, 21;413–423.

Rock, F. Hardiman, G. Timans, J. Kastelein, R. & Bazan, J. (1998). A family of human receptors structurally related to Drosophila Toll. *Proc Natl Acad Sci U S A*, 95(2):588-593.

Rodgers, G. & Klugman, K. (2011). The future of pneumococcal disease prevention. *Vaccine*, 29 Suppl 3:C43-8.

Rodrigues, F. Foster, D. Nicoli, E. Trotter, C. Vipond, B. Muir, P. . . . Finn, A. (2013). Relationships between rhinitis symptoms, respiratory viral infections and nasopharyngeal colonization with Streptococcus pneumoniae, Haemophilus influenzae and Staphylococcus aureus in children attending daycare. *Pediatr Infect Dis J*, 32(6):707.

Roos, A. Dieltjes, P. Vossen, R. Daha, M. & de Knijff, P. (2006). Detection of three single nucleotide polymorphisms in the gene encoding mannose-binding lectin in a single pyrosequencing reaction. *J Immunol Methods*, 309: 108–114.

Ruohola, A. Meurman, O. Nikkari, S. Skottman, T. Salmi, A. Waris, M. . . . Ruuskanen, O. (2006). Microbiology of acute otitis media in children with tympanostomy tubes: prevalences of bacteria and viruses. *Clin Infect Dis*, 43(11):1417-1422.

Rupa, V. Isaac, R. Jalagandeeswaran, R. Manoharan, A. & Rebekah, G. (2014). Epidemiology of nasopharyngeal colonization by S. pneumoniae in Indian infants in the first 2 years of life. *Int J Pediatr Otorhinolaryngol*, 78(10):1701-1706.

Sanders, V. (2006). Epigenetic regulation of Th1 and Th2 cell development. *Brain Behav Immun*, 20(4):317-324.

Schlapbach, L. Mattmann, M. Thiel, S. Boillat, C. Otth, M. Nelle, M. . . . Aebi, C. (2010). Differential role of the lectin pathway of complement activation in susceptibility to neonatal sepsis. *Clin Infect Dis*, 51(2):153-162.

Schreibelt, G. Tel, J. Sliepen, K. Benitez-Ribas, D. Figdor, C. Adema, G. & de Vries, I. (2010). Toll-like receptor expression and function in human dendritic cell subsets: implications for dendritic cell-based anti-cancer immunotherapy. *Cancer Immunol Immunother*, 59(10):1573-1582.

Schröder, N. & Schumann, R. (2005). Single nucleotide polymorphisms of Toll-like receptors and susceptibility to. *Lancet Infect Dis*, 5:156-64.

Schröder, N. Diterich, I. Zinke, A. Eckert, J. Draing, C. von Baehr, V. . . . Schumann, R. (2005). Heterozygous Arg753Gln polymorphism of human TLR-2 impairs immune activation by Borrelia burgdorferi and protects from late stage Lyme disease. *J Immunol*, 175(4):2534-2540.

Selva, L. Viana, D. Regev-Yochay, G. Trzcinski, K. Corpa, J. Lasa, I. . . . Penadés, J. (2009). Killing niche competitors by remote-control bacteriophage induction. *Proc Natl Acad Sci U S A*, 106(4):1234-1238.

Selvaraj, K. Chinnakali, P. Majumdar, A. & Krishnan, I. (2014). Acute respiratory infections among under-5 children in India: A situational analysis. *J Nat Sci Biol Med*, 5(1):15-20.

Sender, V. & Stamme, C. (2014). Lung cell-specific modulation of LPS-induced TLR4 receptor and adaptor localization. *Commun Integr Biol*, 7:e29053.

Seppänen, M. Lokki, M. Lappalainen, M. Hiltunen-Back, E. Rovio, A. Kares, S. . . . Aittoniemi, J. (2009). Mannose-binding lectin 2 gene polymorphism in recurrent herpes simplex virus 2 infection. *Hum Immunol*, 70(4):218-221.

Shiri, T. Nunes, M. Adrian, P. Van Niekerk, N. Klugman, K. & Madhi, S. (2013). Interrelationship of Streptococcus pneumoniae, Haemophilus influenzae and Staphylococcus aureus colonization within and between pneumococcal-vaccine naïve mother-child dyads. *BMC Infect Dis*, 13:483. doi: 10.1186/1471-2334-13-483.

Sigurs, N. Bjarnason, R. Sigurbergsson, F. & Kjellman, B. (2000). Respiratory syncytial virus bronchiolitis in infancy is an important risk factor for asthma and allergy at age 7. *Am J Respir Crit Care Med*, 161(5):1501-1507.

Sironi, M. Biasin, M. Cagliani, R. Forni, D. De Luca, M. Saulle, I. . . . Clerici, M. (2012). A common polymorphism in TLR3 confers natural resistance to HIV-1 infection. *J Immunol*, 188(2):818-823.

Smith, K. Samet, J. Romieu, I. & Bruce, N. (2000). Indoor air pollution in developing countries and. *Thorax*, 55:518-532.

Song, X. Gao, H. & Qian, Y. (2014). Th17 differentiation and their pro-inflammation function. *Adv Exp Med Biol*, 841:99-151.

Speshock, J. Doyon-Reale, N. Rabah, R. Neely, M. & Roberts, P. (2007). Filamentous influenza A virus infection predisposes mice to fatal septicemia following superinfection with Streptococcus pneumoniae serotype 3. *Infect Immun*, 75(6):3102-3111.

Steffensen, R. Thiel, S. Varming, K. Jersild, C. & Jensenius, J. (2000). Detection of structural gene mutations and promoter polymorphisms in the mannan-binding lectin (MBL) gene by polymerase chain reaction with sequence-specific primers. *J Immunol Methods*, 241(1-2):33-42.

Strunk, T. Currie, A. Richmond, P. Simmer, K. & Burgner, D. (2011). Innate immunity in human newborn infants: prematurity means more than immaturity. *J Matern Fetal Neonatal Med*, 24(1):25-31.

Syrjänen, R. Kilpi, T. Kaijalainen, T. Herva, E. & Takala, A. (2001). Nasopharyngeal carriage of Streptococcus pneumoniae in Finnish children younger than 2 years old. *J Infect Dis*, 184(4):451-459.

Takeuchi, O. & Akira, S. (2010). Pattern recognition receptors and inflammation. *Cell*, 140(6):805-820.

Takeuchi, O. Hoshino, K. Kawai, T. Sanjo, H. Takada, H. Ogawa, T. . . . Akira, S. (1999). Differential roles of TLR2 and TLR4 in recognition of gram-negative and gram-positive bacterial cell wall components. *Immunity*, 11(4):443-51.

Tal, G. Mandelberg, A. Dalal, I. Cesar, K. Somekh, E. Tal, A. . . . Amariglio, N. (2004). Association between common Toll-like receptor 4 mutations and severe respiratory syncytial virus disease. *J Infect Dis*, 189(11):2057-2063.

Tan, T. Forsgren, A. & Riesbeck, K. (2006). The respiratory pathogen moraxella catarrhalis binds to laminin via ubiquitous surface proteins A1 and A2. *J Infect Dis*, 194(4):493-497.

Tano, K. Grahn-Håkansson, E. Holm, S. & Hellström, S. (2000). Inhibition of OM pathogens by alpha-hemolytic streptococci from healthy children, children with SOM and children with rAOM. *Int J Pediatr Otorhinolaryngol*, 56(3):185-190.

Tano, K. Olofsson, C. Grahn-Håkansson, E. & Holm, S. (1999). In vitro inhibition of S. pneumoniae, nontypable H. influenzae and M. catharralis by alpha-hemolytic streptococci from healthy children. *Int J Pediatr Otorhinolaryngol*, 47(1):49-56.

Tellería-Orriols, J. García-Salido, A. Varillas, D. Serrano-González, A. & Casado-Flores, J. (2014). TLR2-TLR4/CD14 polymorphisms and predisposition to severe invasive infections by Neisseria meningitidis and Streptococcus pneumoniae. *Med Intensiva*, 38(6):356-362.

Thada, S. Valluri, V. & Gaddam, S. (2013). Influence of Toll-like receptor gene polymorphisms to tuberculosis susceptibility in humans. *Scand J Immunol*, 78(3):221-229.

Toivonen, L. Schuez-Havupalo, L. Rulli, M. Ilonen, J. Pelkonen, J. Melen, K. . . . Waris, M. (2015). Blood MxA protein as a marker for respiratory virus infections in young children. *J Clin Virol*, 62:8-13.

Trejo-de la, O. A. Hernández-Sancén, P. & Maldonado-Bernal, C. (2014). Relevance of single-nucleotide polymorphisms in human TLR genes to infectious and inflammatory diseases and cancer. *Genes Immun*, 15(4):199-209.

Uehara, Y. Nakama, H. Agematsu, K. Uchida, M. Kawakami, Y. Abdul Fattah, A. & Maruchi, N. (2000). Bacterial interference among nasal inhabitants: eradication of Staphylococcus aureus from nasal cavities by artificial implantation of Corynebacterium sp. *J Hosp Infect*, 44(2):127-133.

Wald, E. (1998). Microbiology of acute and chronic sinusitis in children and adults. *Am J Med Sci*, 316(1):13-20.

Walker, J. Barlow, J. & McKenzie, A. (2013). Innate lymphoid cells--how did we miss them? *Nat Rev Immunol*, 13(2):75-87.

van de Pol, A. van der Gugten, A. van der Ent, C. Schilder, A. Benthem, E. Smit, H. . . . Damoiseaux, R. (2013). Referrals for recurrent respiratory tract infections including otitis media in young children. *Int J Pediatr Otorhinolaryngol*, 77(6):906-910.

van der Graaf, C. Kullberg, B. Joosten, L. Verver-Jansen, T. Jacobs, L. Van der Meer, J. & Netea, M. (2005). Functional consequences of the Asp299Gly Toll-like receptor-4 polymorphism. *Cytokine*, 30(5):264-268.

van Rijn, B. Roest, M. Franx, A. Bruinse, H. & Voorbij, H. (2004). Single step high-throughput determination of Toll-like receptor 4 polymorphisms. *J Immunol Methods*, 289(1-2):81-7.

Wang, J. Kwon, H. & Jang, Y. (2009). Rhinovirus enhances various bacterial adhesions to nasal epithelial cells simultaneously. *Laryngoscope*, 119(7):1406-1411.

Wang, W. Zhou, A. Zhang, X. Xiang, Y. Huang, Y. Wang, L. . . . He, Y. (2014). Interleukin 17A promotes pneumococcal clearance by recruiting neutrophils and inducing apoptosis through a p38 mitogen-activated protein kinase-dependent mechanism in acute otitis media. *Infect Immun*, 82(6):2368-2377.

Welliver, R. (2003). Review of epidemiology and clinical risk factors for severe respiratory syncytial virus (RSV) infection. *J Pediatr*, 143(5 Suppl):S112-117.

Wenger, J. (1993). Impact of Haemophilus influenzae type b vaccines on the epidemiology of bacterial meningitis. *Infect Agents Dis*, 2(5):324-332.

Verdu, P. Barreiro, L. Patin, E. Gessain, A. Cassar, O. Kidd, J. . . . Quintana-Murci, L. (2006). Evolutionary insights into the high worldwide prevalence of MBL2 deficiency alleles. *Hum Mol Genet*, 15(17):2650-2658.

Verduin, C. Hol, C. Fleer, A. van Dijk, H. & van Belkum, A. (2002). Moraxella catarrhalis: from emerging to established pathogen. *Clin Microbiol Rev*, 15(1):125-144.

Verhaegh, S. Lebon, A. Saarloos, J. Verbrugh, H. Jaddoe, V. Hofman, A. . . . van Belkum, A. (2010). Determinants of Moraxella catarrhalis colonization in healthy Dutch children during the first 14 months of life. *Clin Microbiol Infect*, 16(7):992-997.

Verhaegh, S. Snippe, M. Levy, F. Verbrugh, H. Jaddoe, V. Hofman, A. . . . Hays, J. (2011). Colonization of healthy children by Moraxella catarrhalis is characterized by genotype heterogeneity, virulence gene diversity and co-colonization with Haemophilus influenzae. *Microbiology*, 157(Pt 1):169-178.

Wertheim, H. Melles, D. Vos, M. van Leeuwen, W. van Belkum, A. Verbrugh, H. & Nouwen, J. (2005). The role of nasal carriage in Staphylococcus aureus infections. *Lancet Infect Dis*, 5(12):751-762.

Weyrich, P. Staiger, H. Stančáková, A. Machicao, F. Machann, J. Schick, F. . . . Häring, H. (2010). The D299G/T399I Toll-like receptor 4 variant associates with body and liver fat: results from the TULIP and METSIM Studies. *PLoS One*, 5(11):e13980.

White, A. Gompertz, S. & Stockley, R. (2003). Chronic obstructive pulmonary disease . 6: The aetiology of exacerbations of chronic obstructive pulmonary disease. *Thorax*, 58(1):73-80.

Williams, B. Gouws, E. Boschi-Pinto, C. Bryce, J. & Dye, C. (2002). Estimates of world-wide distribution of child deaths from acute respiratory infections. *Lancet Infect Dis*, 2(1):25-32.

Woehrle, T. Du, W. Goetz, A. Hsu, H. Joos, T. Weiss, M. . . . Marion Schneider, E. (2008). Pathogen specific cytokine release reveals an effect of TLR2 Arg753Gln during Candida sepsis in humans. *Cytokine*, 41:322-329.

von Linstow, M. Schønning, K. Hoegh, A. Sevelsted, A. Vissing, N. & Bisgaard, H. (2013). Neonatal airway colonization is associated with troublesome lung symptoms in infants. *Am J Respir Crit Care Med*, 188(8):1041-1042.

World Health Organization, W. (2006). Pneumonia: The forgotten killer of children. *http://whqlibdoc.who.int/publications/2006/9280640489_eng.pdf?ua=1*.

Worthley, D. Bardy, P. & Mullighan, C. (2005). Mannose-binding lectin: biology and clinical implications. *Intern Med J*, 35(9):548-555.

Wright, A. Bangert, M. Gritzfeld, J. Ferreira, D. Jambo, K. Wright, A. . . . Gordon, S. (2013). Experimental human pneumococcal carriage augments IL-17A-dependent T-cell defence of the lung. *PLoS Pathog*, 9(3):e1003274.

Vu-Thien, H. Dulot, C. Moissenet, D. Fauroux, B. & Garbarg-Chenon, A. (1999). Comparison of randomly amplified polymorphic DNA analysis and pulsed-field gel electrophoresis for typing of Moraxella catarrhalis strains. *J Clin Microbiol*, 37:450–452.

Xiong, Y. Song, C. Snyder, G. Sundberg, E. & Medvedev, A. (2012). R753Q polymorphism inhibits Toll-like receptor (TLR) 2 tyrosine phosphorylation, dimerization with TLR6, and recruitment of myeloid differentiation primary response protein 88. *J Biol Chem*, 287(45):38327-38337.

Xu, Q. Almudervar, A. Casey, J. & Pichichero, M. (2012). Nasopharyngeal bacterial interactions in children. *Emerg Infect Dis*, 18(11):1738-1745.

Yamamoto, M. & Takeda, K. (2010). Current views of toll-like receptor signaling pathways. *Gastroenterol Res Pract*, 2010:240365.

Yang, C. Raftery, M. Hamann, L. Guerreiro, M. Grütz, G. Haase, D. . . . Scheibenbogen, C. (2012). Association of TLR3-hyporesponsiveness and functional TLR3 L412F polymorphism with recurrent herpes labialis. *Hum Immunol*, 73(8):844-851.

Yang, Z. Dai, Q. Gu, Y. Guo, Q. & Gong, L. (2012). Cytokine and chemokine modification by Toll-like receptor polymorphisms is associated with nasopharyngeal carcinoma. *Cancer Sci*, 103(4):653-658.

Yu, L. Wang, L. & Chen, S. (2010). Endogenous toll-like receptor ligands and their biological significance. *J Cell Mol Med*, 14(11):2592-2603.

Zareparsi, S. Buraczynska, M. Branham, K. Shah, S. Eng, D. Li, M. . . . Swaroop, A. (2005). Toll-like receptor 4 variant D299G is associated with susceptibility to age-related macular degeneration. *Hum Mol Genet*, 14(11):1449-1455.

Zhang, S. Herman, M. Ciancanelli, M. Pérez de Diego, R. Sancho-Shimizu, V. Abel, L. & Casanova, J. (2013). TLR3 immunity to infection in mice and humans. *Curr Opin Immunol*, 25(1):19-33.

Zhang, S. Jouanguy, E. Ugolini, S. Smahi, A. Elain, G. Romero, P. . . . (2007). TLR3 deficiency in patients with herpes simplex encephalitis. *Science*, 317(5844):1522-1527.

Zhang, Z. Clarke, T. & Weiser, J. (2009). Cellular effectors mediating Th17-dependent clearance of pneumococcal colonization in mice. *J Clin Invest*, 119(7):1899-1909.