Bacterial Colonisation of the Nasal and Nasopharyngeal Cavities in Children

The Generation R Study

Ankie Lebon

Colofon

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Bacteriële kolonisatie van de nasale en
nasofaryngeale holten bij kinderen

Het Generation R onderzoek

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4. Lebon A., Verkaik, N.J., Labout, J.A.M., de Vogel, C.P., Hooijkaas, H., Verbrugh, H. A., Jaddoe, V. W., Hofman, A., van Wamel, W. J., Moll, H. A. and van Belkum, A., Natural antibodies against several pneumoccal virulence proteins in children in the pre-pneumococcal vaccine-era: the Generation R Study. Infect Immun 2011. In press.

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7. Lebon, A., Labout, J. A.M., Verbrugh, H. A., Jaddoe, V. W., Hofman, A., van Wamel, W. J., van Belkum, A. and Moll, H. A., Role of *Staphylococcus aureus* nasal colonisation in atopic dermatitis in infants: the Generation R Study. Arch Pediatr Adolesc Med 2009. 163: 745-749.

8. Lebon, A., Velders, F.P., Hofman, A., Jaddoe, V.W.V, Verbrugh, H.A., van Belkum, A., Moll, H.A., Glucocorticoid receptor polymorphisms are associated with *Staphylococcus aureus* colonisation and atopic dermatitis in childhood: the Generation R Study. Submitted

9. Lebon, A., Labout, J. A.M., de Jongste, J.C., Verbrugh, H. A., Jaddoe, V. W., Hofman, A., van Belkum, A. and Moll, H. A. Nasopharyngeal bacterial colonisation and respiratory symptoms in childhood: the generation R Study. Submitted

Chapter 1

Introduction

GENERAL INTRODUCTION

Bacterial colonisation from a microbiological and epidemiological perspective

Humans are surrounded by microorganisms: viruses, bacteria, fungi and parasites. We can divide these organisms into the following four categories: innocent, beneficial, harmful and dangerous. Most of the times, microorganisms are not harmful and are therefore referred to as non-pathogenic. Innocent microorganisms cause no harm nor do they provide benefits. Beneficial microorganisms even provide a significant advantage for humans by aiding digestion or preventing pathogenic microorganisms to cause infection via colonisation resistance. By colonising the respiratory and/or gastro-intestinal tract, these organisms prevent pathogenic microorganisms to settle and cause harm. Pathogens causing harm in certain cases, but not per definition, are grouped into the harmful category. However, microbes that fall into the dangerous category comprise organisms that cause morbidity and mortality in humans even in those with an intact immune system [1].

Airway pathogens *Streptococcus pneumoniae, Haemophilus influenzae* and *Moraxella catarrhalis* prosper in the nasopharynx of healthy humans [2-5]. The main ecological niche of the opportunistic pathogen *Staphylococcus aureus* is the anterior nares [6-12]. Colonisation with these organisms is harmless most of the time, but it is held responsible for horizontal transmission at population level. Moreover, it increases the risk of endogenous infections [10, 13-25]. Hence their classification as potentially harmful microorganisms. However, they may also be classified as 'beneficial' organisms that prevent other pathogens to colonise the nasal and nasopharyngeal area in their presence; the so-called phenomenon of (bacterial) interference which is referred to as the interaction between different species of bacteria [26-29]. Several factors are involved in the colonisation status of an individual. Firstly, host factors such as the immune system and genetic make-up. Secondly, the pathogens with their tricks to nestle down in the nasal and nasopharyngeal cavities and their interactions with each other play a role in the establishment of the flora. Finally environmental factors play an important role in acquisition and carriage of bacterial microorganisms.

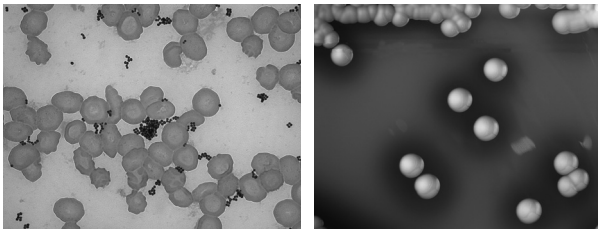
It is important to study bacterial colonisation in young children with an immune system which is still in development since the aetiology of many childhood diseases may originate early in life. Studies of bacterial colonisation in youngsters may provide clues towards development of diseases later in life. Below follows a brief description of four nasopharyngeal opportunists that are relevant to the topic of the studies presented in this thesis.

Staphylococcus aureus

S. aureus is a gram positive, facultatively anaerobic organism. Its name refers to its round morphology (coccus: κόκκος) and the formation of grape like clusters (staphylé: σταφυλή). These can be observed under the microscope and the golden colonies (named after the Greek word *aureus* meaning gold) that grow on solid media [30-31].

Domain	*Bacteria*
Phylum	*Firmicutes*
Class	*Bacilli*
Order	*Bacillales*
Family	*Staphylococcaceae*
Genus	*Staphylococcus*
Species	*S. aureus*

Rosenbach 1884, Skerman 1980, Lapage 1992



a) *S. aureus* on blood agar plate
b) Gram stain *S. aureus* invasion in blood

S. aureus was discovered in Scotland in 1880 by a surgeon named Ogston who observed pus samples from patients with post-operative wound infections and abscesses under a microscope [32]. Although he was also the first person associating the shape with bunches of grapes, he was not the first to isolate and grow the pathogen. Rosenbach, who did so four years later in 1884, named the pathogen *Staphylococcus aureus* [33].

A large fraction of the healthy human population is colonised with *S. aureus*. The most common place where *S. aureus* can be detected is the anterior nares [10, 34-35]. *S. aureus* colonisation is persistent in about 20-30% of the healthy subjects [10, 34, 36]. The same percentage of humans is colonised intermittently whereas half of the human population are so-called non-carriers [10, 37-39]. Recently it was reported that these groups of intermittently colonised individuals and non-carriers were actually indistinguishable [40]. *S. aureus* is a frequent cause of skin infection such as folliculitis, furunculosis, abscesses and post-operative wound infections but may also cause invasive disease such as pneumonia and sepsis [21-22, 24, 34, 41-45]. Prior nasal colonisation is a significant risk for acquiring *S. aureus* infections in both the community and in hospitals. The association between nasal carriage and infection was first reported back in 1931 by Danbolt and more recent studies confirmed this association. This association being causal is supported by the discovery that the strain causing infection is often genotypically indistinguishable from the strain carried by the person. Persistent carriers tend to carry the same strain over time and are at increased risk of auto-infections [16, 46].

These topics were hardly studied in children. Most studies suggest that colonisation rates are higher in childhood and that persistent carriage is observed more frequently among children and adolescents under the age of 20 [7, 10, 34, 47]. It is unknown though at what age persistent carriage is exactly initiated and whether it exists in infancy. A perfect host-pathogen match is needed for persistent carriage, which may still be ill-defined in early childhood. Several deter-

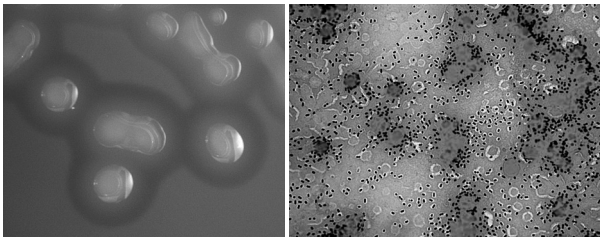
minants were reported to influence *S. aureus* carriage rates in healthy children. The number of older siblings, family size, breast-feeding and passive smoking were found to be positively associated with *S. aureus* nasal carriage in cross-sectionally observed groups of children, determinants of colonisation longitudinally, however, is lacking [12, 26]. Genetic make-up of both host and pathogen may also be important in colonisation status [48-49]. Moreover, microbial interactions with other pathogens including *S. pneumoniae* may play a role in *S. aureus* colonisation rates [26-27]. No vaccine is currently available for this bacterial species.

Streptococcus pneumoniae

S. pneumoniae is another gram positive, alpha-haemolytic, facultatively anaerobic organism. The name refers to its round (coccus: κόκκος) chain like clusters (streptos: στρεπτός) which can be observed under the microscope after growing in liquid media. The initial name that was given to this pathogen was *Diplococcus pneumoniae*, as the cocci present in pairs. Pneumoniae was added to the name for its primary location in or close to the lungs [31].

Domain	*Bacteria*
Phylum	*Firmicutes*
Class	*Bacillie*
Order	*Lactobacillales*
Family	*Streptococcaceae*
Genus	*Streptococcus*
Species	*S. pneumoniae*

Klein 1884, Chester 1901, Skerman 1980



a) Pneumococcus cultivation in mucoid
b) Gram stain pneumococcus in sputum

S. pneumoniae was discovered by Sternberg and Pasteur in 1880. However, it was not considered to be the actual cause of bacterial pneumonia until a few years later [50]. This pathogen, which is also often referred to as pneumococcus, may cause superficial respiratory as well as invasive infections, such as sepsis, meningitis and endocarditis. More commonly, the pneumococcus causes sinusitis, otitis media, bronchitis and pneumonia. Morbidity and mortality are high; worldwide it causes around 11% of all deaths in children below the age of 5 years [51]. Healthy children may be colonised with the pneumococcus in the nasopharynx, the prevalence of which increases in the first year of life from approximately 8% to 45% [52]. The pneumococcus often presents as a commensal, causing no harm due to adequate innate and adaptive immune reactions of the host. Pneumococcal infection is usually preceded by asymptomatic nasopharyngeal colonisation with the homologous strain; hence, pneumococcal colonisation is the first step in infections caused by this pathogen [14]. Since pneumococcal duration of colonisation is usually shorter than a month, the risk of infection is short-lived as well [53]. Beside the risk of (auto-) infection, nasopharyngeal carriage is important for pneumococcal spread at the population level [54-55]. Especially crowding has been shown to enhance the hori-

zontal spread of pneumococci, and was identified as an important risk factor for pneumococcal colonisation in children [52].

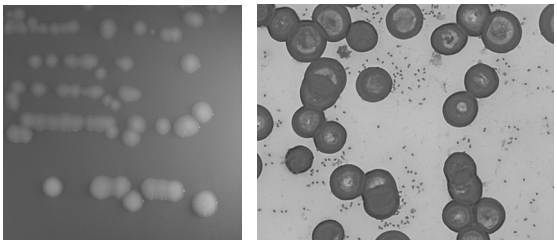
Over 90 different pneumococcal serotypes have been identified on the basis of variability in the capsular polysaccharides. The current vaccines cover only some of these serotypes by inducing antibodies against certain capsular polysaccharides only. Although these antibodies are highly protective against most pneumococcal infections, new strategies to the development of vaccines that cover all pneumococcal serotypes are required to prevent more infections, especially in risk groups such as immunocompromised patients and young children. Recent studies report on vaccination-associated shifts in pneumococcal serotypes causing nasopharyngeal colonisation and infection [56-58]. Moreover, an increase in colonisation rates with other pathogens was observed following the pneumococcal conjugate vaccine implementation in national projects, due to previously explained bacterial interference. The search for novel vaccines with expanded coverage and immunogenicity is urgently needed for optimal prevention of pneumococcal colonisation and infections. Protein-based vaccines may provide a future perspective as these vaccines may broadly prevent pneumococcal colonisation and infections and regardless of serotype [59].

Haemophilus influenzae

H. influenzae, meaning blood–loving in ancient Greek (haima: αιμα, philia: φιλια), is a gram negative, generally aerobic but facultatively anaerobic organism [60]. It was first described in 1892 by Richard Pfeiffer during an influenza pandemic. It was mistaken as the cause of influenza until 1933 when the viral aetiology of the common flu became apparent.

Domain	*Bacteria*
Phylum	*Proteobacteria*
Class	*Gammaproteobacteria*
Order	*Pasteurellales*
Family	*Pasteurellaceae*
Genus	*Haemophilus*
Species	*H. influenzae*

Lehman 1896, Chester 1901



a) *H. influenzae* on chocolate agar plate
b) Gram stain *H. influenzae*

H. influenzae was the first free-living organism to have its entire genome sequenced, which took place in 1995 [61].

Most strains of *H. influenzae* are commensal and may behave opportunistic by causing problems when an opportunity occurs. In infants and young children, *H. influenzae* type b (Hib) causes invasive disease such as epiglotitis, bacteraemia, pneumonia and acute bacterial meningitis. However, this is barely seen these days since the implementation of the Hib vaccination in national vaccination programmes is very successful. Non-encapsulated *H. influ-*

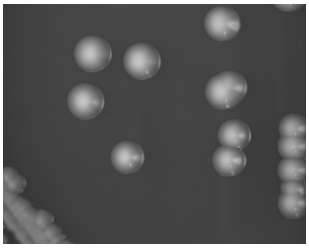
enzae strains (non-typeable) cause otitis media, conjunctivitis and sinusitis in children and may cause more severe diseases including pneumonia as well [17, 20, 62-63]. *H. influenzae* is able to colonise the nasopharynges of healthy subjects without causing harm most of the time [2-3, 55, 64-66]. Microbial interference, as mentioned before, has also been documented for *H. influenzae* colonisation [28, 67].

Moraxella catarrhalis

M. catarrhalis is a gram negative, aerobic, oxidase-positive diplococcus. It was previously named *Branhamella catarrhalis*. *Moraxella* is named after Victor Morax, a Swiss ophtalmologist who first described this genus of bacteria. Catarrhalis (kata: κατά and rhein: ῥεῖν) means 'to flow down' in ancient Greek. This describes the profuse discharge from eyes and nose typically associated with severe inflammation in infections caused by this organism.

Domain	*Bacteria*
Phylum	*Proteobacteria*
Class	*Gammaproteobacteria*
Order	*Pasteurellales*
Family	*Moraxellaceae*
Genus	*Moraxella / Branhamella*
Species	*M. catarrhalis*

Frosh and Kolle 1896, Bovre 1984



a) *M. Catarrhalis* on blood agar plate

During the recent decades, *M. catarrhalis* became to be seen as a common respiratory tract pathogen causing 15-20% of acute otitis media episodes in children [17-18, 68-70]. Before, it was considered to be a harmless commensal microbe for a long time. The widespread use of the pneumococcal conjugate vaccine has led to new colonisation patterns of *M. catarrhalis* due to bacterial interactions [28]; prevalence of both colonisation and disease caused by *M. catarrhalis* has increased following the implementation of the PCV [71]. Currently, there is no vaccine available for this pathogen.

Respiratory tract infection

All bacterial pathogens identified above are capable of causing respiratory tract infections, especially in childhood. Respiratory tract infections provide the most common reason for parents of infants and pre-school children for visits to a physician. Most respiratory tract infections are caused by viruses [72], although acute otitis media is most frequently caused by either *S. pneumoniae,* non-typeable *H. influenzae* or *M. catarrhalis* [73]. Even though these respiratory tract infections have a low mortality in the industrialized world and are often self-limiting, morbidity and the global burden is high to patients, caregivers and the healthcare system. In developing countries with a high prevalence of HIV infections, respiratory tract

infections are a significant cause of mortality. Especially *S. pneumoniae* is a universally leading microbial cause of mortality in children below 5 years of age.

A peak incidence in respiratory tract infections occurs in the children's first two years of life [72]. This is possibly due to a combination of day care attendance in the Western world and an immune system yet too immature to cope with all microbes. Asymptomatic colonisation with respiratory pathogens shows its peak incidence around that same age. As yet it remains unknown whether colonisation results in an increased risk to viral infections or whether viral infections generate a porte d'entrée for bacterial pathogens to colonise the mucosa. There may even be a common risk factor, for example a certain genetic make-up, which renders children more prone to both events.

Atopic diseases

Atopic diseases such as asthma and eczema represent an increasing clinical problem worldwide. Initially and most prevalent in Westernized countries, which embraced the so-called hygiene hypothesis, atopic diseases are now increasing in developing countries as well. According to the hygiene hypotheses, the decreasing incidence of infections and exposure to microbes in Western countries is at the origin of the increasing incidence of atopic diseases. Recent studies on the influence of bacterial microbes on the development of atopic diseases suggest a different kind of hypothesis in which an increase, rather than decrease, in exposure to specific microbes has an effect on the subsequent development of atopic diseases.

Asthma is a leading cause of chronic disease among children. Symptoms and exacerbations are frequently provoked by a wide range of triggers, partially unknown or poorly characterized, including viral respiratory tract infections. Many infants and preschool children experience recurrent episodes of bronchial symptoms, especially wheezing and cough, beginning at a few months of age mainly during a lower respiratory tract infection. A clinical diagnosis of asthma can only be made with some certainty at the age of 5 [74]. Although wheezing symptoms are highly prevalent among preschool children, they are often transient, leaving 60% of children with earlier wheezing complaints symptom-free by school age [75-77]. Even though there is a significant burden associated with wheezing symptoms in early life and asthma later in childhood in children in industrialized countries, the aetiology remains obscure. Viral infections are considered to play an important role, both in wheezing in preschool children as well as in the development and exacerbations of asthma [78-87]. Recently, however, early bacterial colonisation was also suggested to influence the development of asthma in children born to asthmatic mothers [13, 88].

Aside from asthma, other atopic diseases such as atopic dermatitis are generally immune mediated and often chronic, inflammatory diseases [89]. Atopic dermatitis (AD) is a common disease in young children with a prevalence of up to 15% and often presenting in the first few years of life [90]. AD is supposed to be a multifactorial disease; multiple factors such as genetic and environmental determinants may lead to the development of symptoms [91-92]. Genetics

alone or external factors by themselves may not be enough to invoke the clinical symptoms of AD. Candidate gene approach studies revealed several genes to be involved in AD. However, external determinants including dietary allergens, house dust mites, and *S. aureus* colonisation [19, 93-94] are important. *S. aureus* skin colonisation is much more prevalent in patients with AD as compared to controls [19, 95-97]. It is hard to tell, though, whether these patients suffer from AD due to *S. aureus* colonisation or whether *S. aureus* is more capable to colonise the damaged skin of an AD patient as compared to the intact skin of controls. Several studies reported on the phenomenon of IgE-mediated sensitization caused by *S. aureus* enterotoxins [98], which suggests an aetiological role for *S. aureus* in the development of AD.

As the role of microorganisms in the development of atopic diseases becomes more often described, these days the hygiene hypothesis, as explanation for the increase in allergic diseases in the Western world due to absence of early childhood exposure to infectious agents, becomes more and more questioned.

Immune response and bacterial colonisation

In order to stimulate the development of new preventive measures against the burden caused by certain bacterial species, it is important to increase knowledge on the human antibody response. Currently, the 7-valent pneumococcal conjugate vaccine is implemented in many national vaccination programmes in the Western world. Unfortunately the coverage is limited to 7 of the 90 pneumococcal serotypes. Several studies have now shown that this results in replacement of vaccine serotypes by non-vaccine serotypes causing colonisation and infection. Moreover, an increased colonisation rate was observed for other pathogens such as *S. aureus* which potentially leads to more and more serious staphylococcal disease.

Protein-based pneumococcal vaccines and vaccines against *S. aureus* may bring a solution. Increasing our knowledge on the humoral immune response directed to pathogen-specific protein antigens will help to identify putative vaccine candidates. These specific antibodies can be measured by several technologies such as immunodiffusion tests, Western blotting assays or enzyme-linked immunosorbent assays (ELISA's). These days, ELISA is used in common practice. However, ELISA tests require relatively large amounts of serum, are time-consuming and opportunities for multiplex testing are quite limited. These are disadvantages that are resolved by the application of a new, innovative, high-throughput immunological assay that has been developed recently and is known as so-called xMAP® Technology (Luminex Corporation). This allows for simultaneous quantification of antibodies in the same sample directed to different proteins. *S. aureus* and *S. pneumoniae* multiplex assays were developed by using this flow cytometry-based technique. Multiplexing is achieved by means of microspheres, beads that are internally colour-coded. Through precise and balanced concentrations of these dyes, distinctly coloured bead sets can be created, each of which can be coated with a specific protein. After coupling, a mix of the protein-coupled beads is introduced in separate wells of 96-wells plate. Then, in each well, diluted serum is added. The analyser resembles a

flow cytometer with 2 lasers. High-speed digital processors identify each individual bead and quantify the level of antibodies, as based on fluorescent reporter signals. Results are reported in median fluorescence intensity (MFI) values [99-100]. This technique may help us to gain additional insight in the humoral immune response involved in colonisation and disease in childhood, which may enhance development of new preventive measures (i.e. vaccines) against respiratory tract infections and atopic diseases.

AIM OF THIS THESIS

General aim

The general aim of this thesis was to study the dynamics and kinetics of bacterial nasal and nasopharyngeal colonisation and its consequences in early childhood. Although several determinants of colonisation have been investigated in adults and small groups of children, our present study aimed to analyse nasal and nasopharyngeal colonisation longitudinally in a large population-based birth cohort. Moreover, the effects of colonisation have been described in patients, but not as widely in healthy subjects. The second aim of this thesis was to study the consequences of bacterial colonisation on infections and atopic diseases in healthy children.

Associations studied in this thesis

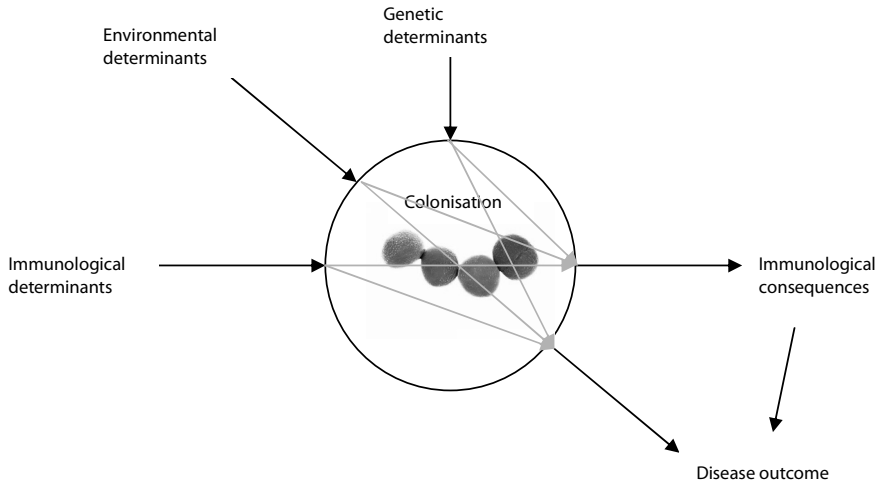


Figure 1. Aims of the study

Specific aims

1. Colonisation

To study the prevalence and dynamics of *S. aureus* and *M. catarrhalis* colonisation in healthy infants.

2. Environmental Determinants → Colonisation

To identify environmental determinants which are associated with nasal *S. aureus* colonisation and nasopharyngeal colonisation with *M. catarrhalis*.
To study whether maternal colonisation with bacterial pathogens is associated with colonisation status of their children and to assess whether direct mother-to-child transmission occurs frequently.

3. Genetic determinants → Colonisation

To study the association between glucocorticoid receptor polymorphisms and *S. aureus* colonisation in childhood.
To study whether the association between staphylococcal colonisation and atopic dermatitis depends on glucocorticoid receptor polymorphisms.

4. Immunological determinants → Colonisation

To study whether the pathogen-specific maternal humoral immune response is associated with nasal colonisation with *S. aureus* and nasopharyngeal colonisation with *S. pneumoniae* in healthy children.
To study whether anti-staphylococcal antibodies produced by the child are associated with *S. aureus* colonisation and atopic dermatitis.
To study whether anti-pneumococcal antibodies produced by the child are associated with *Streptococcus pneumoniae* colonisation and respiratory tract infections.
To study the inverse association between *S. aureus* and *S. pneumoniae* on humoral immune response level.

5. Colonisation → Immunological consequences

To study whether nasal and nasopharyngeal colonisation with *S. aureus* and *S. pneumoniae* induce a humoral immune response.

6. Colonisation → Disease outcome

To study whether *S. aureus* colonisation is associated with atopic dermatitis in young children.
To study whether nasopharyngeal colonisation with the airway pathogens *S. pneumoniae*, *M. catarrhalis* and *H. influenzae* and nasal colonisation with *S. aureus* are associated with wheezing symptoms and respiratory tract infections in childhood.

GENERAL STUDY DESIGN

The studies presented in this thesis were all embedded in the Generation R Study [101-102]. This is a population-based prospective cohort study, following pregnant women and their children from foetal life until young adulthood, in Rotterdam, The Netherlands. Originally, 9,778 women were included in the cohort of whom 8,880 participated from the prenatal phase, all with an expected delivery date between April 2002 and January 2006. Additionally, more detailed assessments of foetal and postnatal growth and development were conducted in a subgroup of 1,232 Dutch pregnant women and their children. This cohort took part in the Generation R Focus Study. To study aetiological research questions, this subgroup is ethnically homogeneous to exclude confounding or effect modification by ethnicity. Hence, pregnant women were selected for this sub-cohort whose parents and the parents of the father were all Dutch. Overall, of all approached, eligible, pregnant women and their partners, 79% participated in the Generation R Focus Study. All children were born between February 2003 and August 2005. After birth, the children participating in the Focus Cohort were invited to visit the research centre at 1.5, 6, 12, 14, 24 and 36 months (Figure 2). During these visits nasal and nasopharyngeal swabs as well as serum was obtained as presented in the Figures 2 and 3 below. Questionnaires were sent out during pregnancy, shortly after birth (at 1.5 month), at 6 months, at 1 year, at 2 years, at 3 years and at 4 years. Information was obtained on disease prevalence including atopic diseases such a wheezing complaints and atopic dermatitis (assessed by using questions from the validated, age-adapted, questionnaire of the International Study on Allergies and Asthma Cohort (ISAAC) [103]) and respiratory tract infections (both diagnoses and symptoms were asked) as well as of many environmental determinants (socioeconomic status, breast-feeding, maternal smoking habits, number of siblings, day-care attendance and so on). The study is still ongoing and currently the 5 year old children from the whole cohort are being invited to the research centre to undergo an extensive investigation.

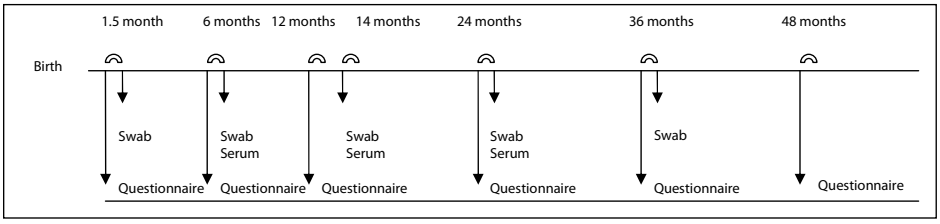
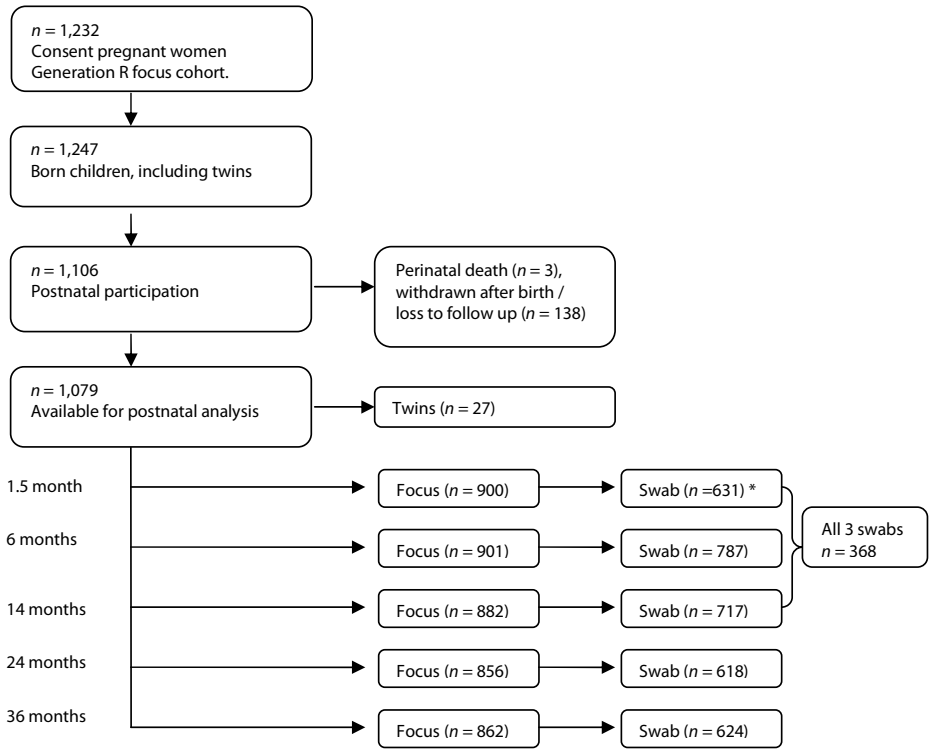


Figure 2. Timeline of data collection



* This subgroup study started in April 2003, data collection on bacterial colonisation started in November 2003, therefore data on bacterial colonisation in the first 224 participants at 1.5 months are missing.

Figure 3. Flowchart of participants

OUTLINE OF THIS THESIS

In **chapter 2** we identify risk factors for bacterial colonisation in childhood. Two studies report on the association between environmental factors such as crowding (day-care attendance and presence of siblings), maternal smoking, breast-feeding with colonisation of *S. aureus* and *M. catarrhalis* in the first year of life (**chapters 2.1** and **2.2**). To describe the dynamics and to assess patterns of *S. aureus* colonisation in our population, we present genotypes from the *S. aureus* strains of children with more than one positive culture in **chapter 2.1.** Additionally, we present the association of colonisation status between mothers and their offspring at the age of 2 (**chapter 2.3**). To assess whether mothers and children carry genotypically identical bacterial strains we present data of genotypes of the strains obtained in mothers and their children. **Chapter 3** describes the humoral immune response preceding and following colonisation, with a binominal focus: protective effect of maternal and child's own produced antibodies against future colonisation and disease and the humoral immune

response enhancement following colonisation (**chapters 3.1** and **3.2**). Moreover, the inverse correlation between *S. aureus* and pneumococcus colonisation is studied at the level of specific IgG (**chapter 3.3**). In **chapter 4** we studied disease outcome following colonisation. We describe the role of *S. aureus* in the development of atopic dermatitis as an environmentally acquired factor triggering genetically susceptible children (**chapters 4.1** and **4.2**). In addition we studied whether frequent colonisation with *S. aureus* and the airway pathogens *S. pneumoniae, H. influenzae and M. catarrhalis* is associated with respiratory tract infections and wheezing complaints in children (**chapter 4.3**). Finally **chapter 5** discusses and interprets the issues reported in this thesis and speculates on some potential future directions. A summary of this thesis is presented in **chapter 7** following a section on Questions and Answers (Q&A) based on the specific aims of this thesis, in **chapter 6**.

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Chapter 2

Prevalence and determinants of bacterial colonisation

Chapter 2.1

Dynamics and determinants of *Staphylococcus aureus* carriage in infancy

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ABSTRACT

Serial nasal swabs were collected at the age of 1.5, 6 and 14 months from 353 infants in the Generation R Study. The objective was to study the dynamics and determinants of *Staphylococcus aureus* nasal carriage in the first year of life. The prevalence of *S. aureus* carriage decreased in the first year of life, from 53.8% at the age of 1.5 months to 11.9% at 14 months. Persistent carriage, defined as continuous carriage of the same *S. aureus* strain at the three sampling moments, was rarely detected in early infancy.

INTRODUCTION

Staphylococcus aureus is a human commensal as well as a cause of a wide range of infections [1-3]. A significant fraction of the human population is colonised with *S. aureus* on epithelial surfaces, of which the anterior nares are the most frequent carriage sites [3-7]. Longitudinal studies distinguish three carriage patterns among healthy adult individuals [8-12]. Persistent carriage occurs in about 20% of the adult population, 30% are intermittent carrier and 50% of the individuals are non-carriers [3, 6, 10, 13]. Persistent carriers usually carry the same strain for extended periods of time, whereas intermittent carriers tend to host different strains over time [3, 6, 11].
Children and adolescents under 20 years of age seem to have higher persistent carriage rates than adults [3, 8, 14-15]. Ten percent of the children from 0 to 9 years old and 24% of the children from 10 to 19 years old were found to be persistent carriers [8]. The highest *S. aureus* carriage rate was observed in infants aged 3 months or younger [16].
Several determinants have been suggested to influence carriage rate in healthy children. The number of older siblings, family size (fewer than five versus five or more people), breastfeeding and passive smoking were found to be associated with *S. aureus* nasal carriage [17-18].
The objective of the present investigation was to study the dynamics of *S. aureus* nasal carriage, as well as its human and microbial determinants, in the first year of life.

METHODS

Study design and population

This project was embedded in the Generation R Study, a population-based prospective cohort study of pregnant women and their children from foetal life onward. Detailed assessments of foetal and postnatal growth and development were conducted with 1,232 Dutch pregnant women and their children. Of all approached pregnant women and their partners, 79% participated. All children were born between February 2003 and August 2005 [19-20].
The Medical Ethics Committee of the Erasmus Medical Center, Rotterdam, has approved the study. Written, informed consent was obtained from all participants. In total 1,232 women were enrolled in the focus cohort study during pregnancy. Three infants died perinatally. The remaining mothers gave birth to 1,244 infants, of whom 138 were not included in the cohort of analysis as the consent was withdrawn after birth. Twins (n = 27) were excluded for this analysis since they are related, leaving 1,079 infants in the group of postnatal participants. The infants visited the Generation R focus study research center at age 1.5 months (n = 900), 6 months (n = 901) and 14 months (n = 882), during these visits 622 had swabs taken at 1.5 month, 774 at 6 months and 706 at 14 months; 758 infants attended all visits and 353 provided us with three swabs to use for longitudinal analysis.

Staphylococcus aureus

Trained research nurses obtained a nasal swab (Copan Dacron Swabs, Brescia, Italy) for *S. aureus* isolation from each infant at each visit. Nasal samples were taken using a sterile transport swab suitable for isolating aerobes and anaerobes. Each swab was rubbed gently through both nostrils, transported in Amies transport medium to the medical microbiology laboratory of the Erasmus MC within 6 h of sampling, put directly in phenol red mannitol broth and kept at 35°C for 5 days. Material from tubes that turned yellow was plated on a blood agar plate with 5% sheep blood for 1 day at 35°C to isolate *S. aureus*. None of the infants used antibiotics in the preceding 48 hours.

S. aureus strains from samples of infants who were positive twice or more were genotyped, using pulsed field gel electrophoresis (PFGE). The plugs for PFGE were prepared in 1% low-melting-point agarose gel and kept for 3 to 4 h at 37ºC in the presence of lysostaphin. The plugs were deproteinised using proteinase K. One sixth of a plug was then put into restriction buffer and incubated for 4 hours with endonuclease *Sma*I (50U). After digestion of the DNA, PFGE, performed using a Chef Mapper (Bio-Rad, Veenendaal, The Netherlands), was used to separate the DNA in fragments in a 1% agarose gel at 14ºC. The gels were stained for 30 minutes with ethidium bromide in distilled water and photographed. A dendrogram was made using BioNumerics (Applied Maths, Belgium) to visualize strain relatedness.

Information related to determinants of carriage, was obtained from midwives, hospital registries (gender, birth weight and gestational age) and parent-retrieved questionnaires at the infant's age of 6 and 12 months (breast-feeding, educational level of the mother, maternal smoking (pre- and postpartum), day-care attendance, and presence of siblings).

Statistical methods

Binary logistic regression analysis was performed to report on the association of *S. aureus* carriage pattern with gender, birth weight, gestational age, breast-feeding, educational level of the mother, maternal smoking (pre- and postpartum), day-care attendance and presence of siblings. We used all variables as determinants of longitudinal carriage and confirmed independence by adjusting for each variable with multivariate binary logistic regression analysis. The statistical analyses were performed using the Statistical Package of Social Sciences version 11.0 for Windows (SPSS Inc, Chicago, IL).

RESULTS

Of all mothers of the non-colonised infants, 172 (62.8%) attended higher education as compared to 53 (69.7%) mothers of the colonised infants. The mean birth weights were 3,565 grams (SD 472) for the non-colonised infants and 3,506 grams (SD 566) for the colonised infants. The mean gestational ages were nearly the same for the non-colonised (40.1, SD 1.3)

and colonised infants (39.9, SD 2.0). Of the non-colonised infants, 129 (46.6%) were male compared to 47 (61.8%) in the colonised group. Of the non-colonised infants, 39.6% (*n* = 103) had at least one sibling compared to 41.3% (*n* = 31) of the colonised group. Of all the infants in the non-colonised group, 29.5% (*n* = 79) received breast-feeding up until 6 months compared to 34.7% (*n* = 26) in the colonised group. One hundred and seventy-seven (76.6%) non-colonised infants attended day-care in the first year of life compared to 47 (69.1%) of the colonised infants (Table 1).

TABLE 1. Determinants of *Staphylococcus aureus* carriage in the first year of life

Parameter	Value for infants			
	Not colonised (0-1) (n=292)	Colonised (2>) (n=76)	OR (95% CI)	aOR (95% CI)
Gender				
- Female	148 (53.4%)	29 (38.2%)	1.00	1.00
- Male	129 (46.6%)	47 (61.8%)	1.81 (1.08 – 3.03)*	1.84 (1.00 – 3.41)*
Gestational age	40.1 (1.3)	39.9 (2.0)	0.90 (0.76 – 1.06)	0.87 (0.69 – 1.10)
Birth weight	3,565 (472)	3,506 (566)	1.00 (1.00 – 1.00)	1.00 (1.00 – 1.00)
Breast-feeding at 6 months				
- No	189 (70.5%)	49 (65.3%)	1.00	1.00
- Yes	79 (29.5%)	26 (34.7%)	1.26 (0.73 – 2.16)	1.34 (0.69 – 2.60)
Mother's educational level				
- Higher education	172 (62.8%)	53 (69.7%)	1.00	1.00
- Lower/intermediate education	102 (37.2%)	23 (30.3%)	0.74 (0.43 – 1.27)	0.59 (0.27 – 1.25)
Mother's prenatal smoking				
- No	242 (90.6%)	65 (90.3%)	1.00	1.00
- Yes	25 (9.4%)	7 (9.7%)	1.11 (0.46 – 2.67)	2.78 (0.54 – 14.26)
Mother's postnatal smoking				
- No	201 (88.5%)	59 (89.4%)	1.00	1.00
- Yes	26 (11.5%)	7 (10.6%)	0.98 (0.41 – 2.37)	0.37 (0.07 – 2.00)
Siblings				
- No	157 (60.4%)	44 (58.7%)	1.00	1.00
- Yes	103 (39.6%)	31 (41.3%)	1.05 (0.63 – 1.77)	0.99 (0.52 – 1.90)
Day -care attendance				
- No	54 (23.4%)	21 (30.9%)	1.00	1.00
- Yes	177 (76.6%)	47 (69.1%)	0.70 (0.39 – 1.26)	0.58 (0.29 – 1.18)

Values are means (SD) or absolute numbers (percentage). 353 infants provided nasal swabs at all three collection moments. Data were missing on breast-feeding (n=10), mother's educational level of the (n=3), mother's prenatal smoking (n=14), mother's postnatal smoking (n=60), siblings (n=18), and day-care attendance (n=54). * p-value <0.05

The prevalence of *S. aureus* carriage significantly decreased from 53.8% (190 of 353 infants) at the age of 1.5 months to 22.9% (81 of 353 infants) at the age of 6 months and 11.9% (42 of 353 infants) at the age of 14 months (*P* <0.001). (Figure 1)

One group of infants, 35.7% (126 of 353 infants) of the infants was never found positive for *S. aureus*, 2.8% (10 of 353 infants) were found positive at all three collection moments. The

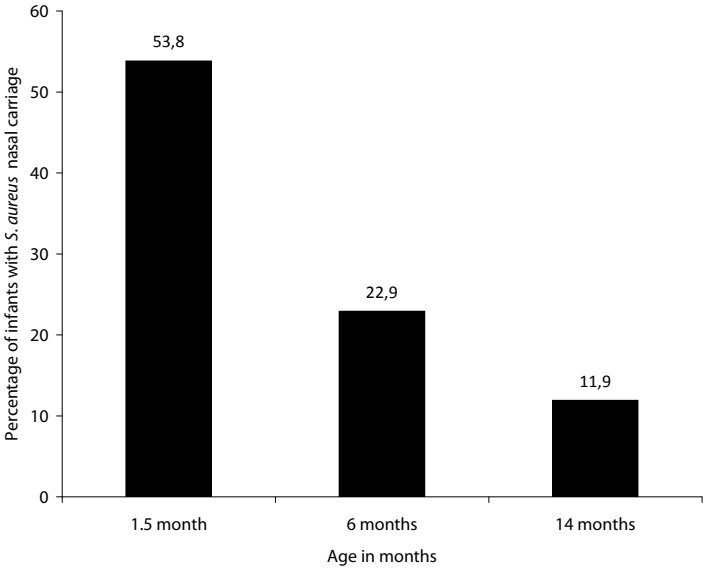
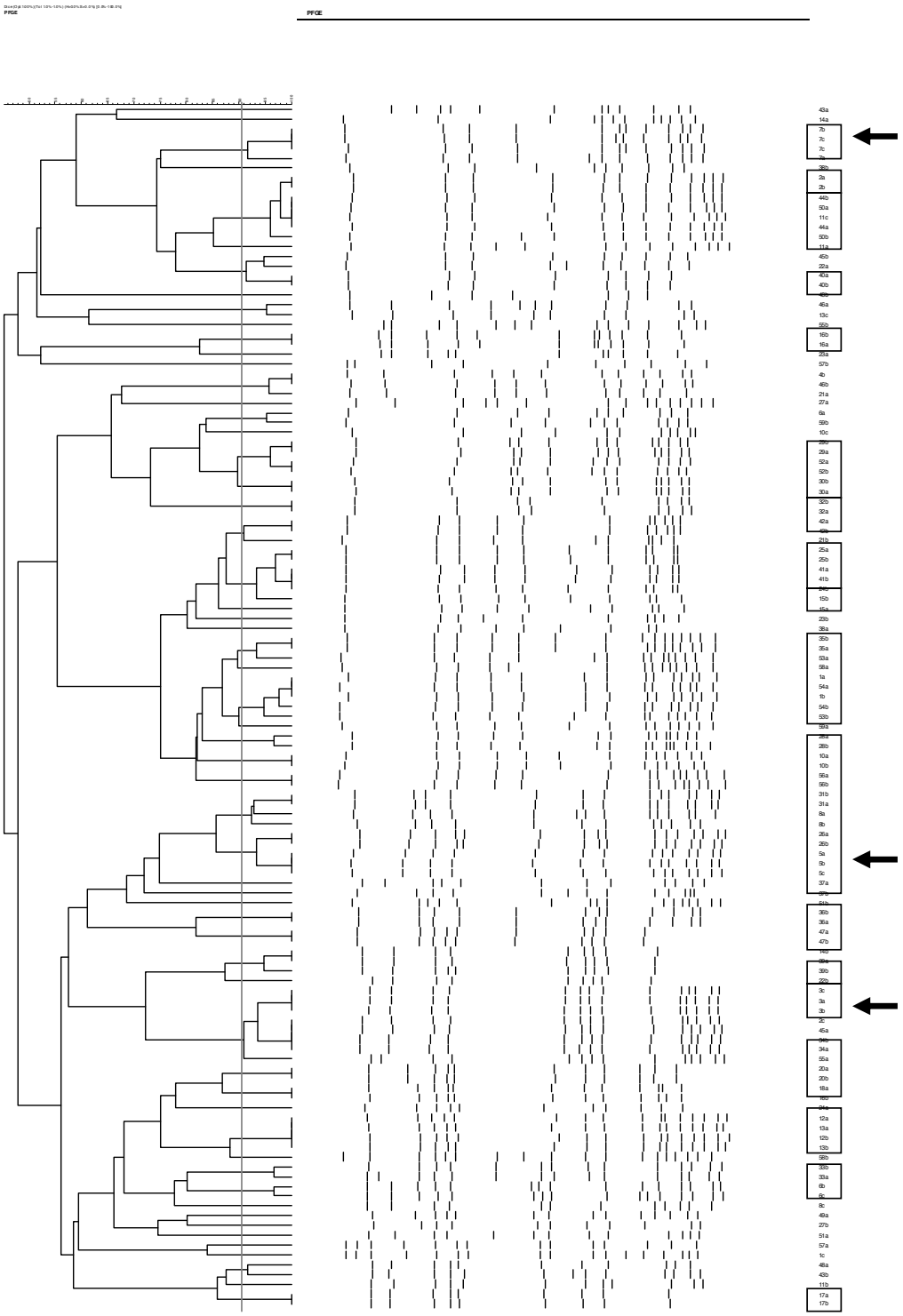


Figure 1. *S. aureus* carriage in the first year of life.
We found a significant decrease in *S. aureus* nasal carriage in the first year of life. P<0.001, for the difference between *S. aureus* carriage rates at 1.5, 6 and 14 months of age.

largest group (42.8%; 151 of 353 infants) consisted of infants with one positive swab. Sixty-six infants (18.7%) had two nasal swabs test positive for *S. aureus.*

We genotyped the *S. aureus* strains from infants with two or more positive swabs. All three strains were available for further research in 10 of 13 infants who were found positive at all three moments (three samples were missing: one lab number was missing, one swab was lost, and one sample did not grow properly). Only 3 of these 10 infants seemed to carry the same *S. aureus* genotype over time; 6 carried two different strains leaving 1 infant with three different strains for each of the three swabs. We genotyped the strains from 45 infants with two positive swabs in a row, of whom 29 (63%) carried the same strain. We did not observe large genetic clusters of *S. aureus*; rather, we observed a great variety of different genotypes (Figure 2). Boys have a significantly higher risk than girls to be positive two or more times (aOR 1.84 95% CI 1.00 - 3.84). We did not find a significant association between *S. aureus* carriage and the presence of siblings (aOR 0.99 95%CI 0.52 - 1.90), or with breast-feeding, day-care attendance, maternal smoking (pre- and postpartum), birth weight or gestational age (Table 1).

Figure 2. *S. aureus* dendrogram.
Pulsed field gel electrophoretic analysis of all strains derived from infants with two or more cultures positive for S. aureus. The banding patterns are shown in the central portion of the figure. On the left, strain relatedness percentage is indicated in the form of a Bionumerics generated dendrogram. A cut off of 90% (vertical solid line) is used to identify similar to identical strains. Strains meeting this criterium and derived from the same infant, are indicated by boxing in the right column which identifies children by study number and a, b and c as the first, second and third culture moment. In three cases strains isolated at the three cultures moments were identical (indicated by an arrow).


PFGE
PFGE
43a
14a
7b
7c
7c
7c
38b
2a
2b
44b
50a
11c
44a
50b
11a
46b
22a
40a
40b
46a
13c
55b
16b
16a
25a
57b
4b
46b
21a
27a
6a
59b
10c
29a
52a
52b
30b
30a
32b
32a
42a
21b
25a
25b
41a
41b
15b
15a
28b
38a
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37a
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36a
47a
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39b
3c
3a
3b
2c
45a
34a
55a
20a
20b
18a
12a
13a
12b
13b
58b
33b
33a
6b
6c
8c
49a
27b
51a
57a
1c
48a
48b
11b
17a
17b

DISCUSSION

We documented a significant decrease in the prevalence of *S. aureus* carriage in the first year of life (p-value < 0.001), which is in line with literature data [17-18]. A possible explanation for this drop may be found in the competition between *S. aureus* and *Streptococcus pneumoniae*. Bogaert et al. found an inverse prevalence of these pathogens in slightly older children; the same could occur in young infants [17]. In our cohort, Labout et al. found an increased level of pneumococcal carriage in the first year of life [21]. The significant decrease in the prevalence of *S. aureus* carriage in the first year of life might be explained by pneumococcal competition or bacterial interference with other organisms present in the nasopharynges of these children (unpublished data).

In our study, persistent nasal carriage as defined by bacterial genotyping of *S. aureus* is extremely rare in infancy. In the small group of infants with two or more positive swabs in a row we rarely found infants carrying the same strain over time. Previous studies show a higher prevalence of persistent carriage among older children and adolescents up to the age of 20 than among adults [8, 14-15]. However, we studied infants in the first year of life and found the majority of them to be intermittent carriers. The apparent close match between pathogen and host as documented for adult persistent carriers may be an explanation as to why there are barely any persistent carriers among infants: the optimal match between pathogen and host may still be absent. Extensive staphylococcal dynamics seems to occur in the nasal cavity of infants, with staphylococcal elimination rather than acquisition as the main feature. In adults, by contrast, persistent carriers host the same strain over time by definition. Redefining the nature of carriage may be necessary to describe the dynamics in the anterior nares during infancy.

Of the 45 infants with two positive swabs in a row, 29 (63%) carried the same strain. This suggests that active colonisation with a new genotype during the first year happens less frequent among these infants. This rather high frequency of 63% of the infants might also be explained by re-colonisation with the strain from the mother or other family members, who might be persistent carriers in 20% of the cases [13, 22].

The main difference between our finding and the studies on determinants of carriage by Bogaert et al. and Peacock et al, is our failure to identify family size, passive smoking or breast-feeding as significant determinants of carriage [17-18]. However, w our data on breast-feeding seems to be more precise than those of the earlier studies, with very little missing data; furthermore, our data cover a larger cohort of children in the same age group (first year of life), than do the two previously mentioned studies.

We conclude that *S. aureus* carriage among young infants is clearly different from that among adults. Long-term persistent carriage rarely occurs among infants and the incidence of carriage drops enormously in the first year of life. Whether these differences are a result of immunomodulation or other biological phenomena is subject to further investigation.

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Chapter 2.2

Determinants of *Moraxella catarrhalis* colonisation in healthy Dutch children during the first 14 months of life

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ABSTRACT

Moraxella catarrhalis is an established bacterial pathogen, previously thought to be an innocent commensal of the respiratory tract of children and adults. The objective of this study was to identify significant risk factors associated with *M. catarrhalis* colonisation in the first year of life in healthy Dutch children.

This study investigated a target cohort group of 1,079 children forming part of the Generation R Study, a population-based prospective cohort study following children from foetal life until young adulthood, conducted in Rotterdam, The Netherlands. Nasopharyngeal swabs for *M. catarrhalis* culture were obtained at 1.5, 6 and 14 months of age, with all three swabs being available for analyses from 353 children. Data on risk factors possibly associated with *M. catarrhalis* colonisation were obtained by questionnaires at 2, 6 and 12 months.

M. catarrhalis colonisation increased from 11.7% to 30.9% and finally to 30.0% at the age of 1.5, 6 and 14 months, respectively. Two significantly important colonisation risk factors were found: the presence of siblings and day-care attendance, which both increased the risk of being positive for *M. catarrhalis* colonisation on two or more occasions within the first year of life. Colonisation with *M. catarrhalis* was not associated with gender, educational level of the mother, maternal smoking, breast-feeding, or antibiotic use.

Apparently, crowding is an important risk factor for early and frequent colonisation by *M. catarrhalis* in the first year of life.

INTRODUCTION

Moraxella catarrhalis is an acknowledged respiratory tract pathogen [1]. The bacterium has the capacity to colonise the nasopharynx, and may be isolated in pure culture or together with other bacterial pathogens e.g. *Staphylococcus aureus*, *Streptococcus pneumoniae* and/or *Haemophilus influenzae* [2]. Although colonisation does not always result in disease, it may be the first step towards invasive disease, and a source of horizontal spread of *M. catarrhalis* in the community [3]. Children are frequently colonised with *M. catarrhalis* and frequent colonisation with *M. catarrhalis* has been reported to increase the risk of otitis media (OM) [4]. Risk factors associated with *M. catarrhalis* colonisation have been previously studied in several countries and age groups, and the findings have indicated that crowding and contact with children are risk factors for colonisation, not only for *M. catarrhalis* but also for *S. pneumoniae* and *H. influenzae* [5]. Other reported risk factors are genetics [6-7], smoking [8], social economic status [9], synergy and interference with other micro organisms [10-11], frequency and location of sampling [12], season [12], gender [13] and vaccination [14]. However, medical and living conditions in The Netherlands may be quite different from those in other countries. For example, there exists a restrictive prescription policy for antibiotic usage. These differences could lead to differences in *M. catarrhalis* epidemiology and colonisation rate in The Netherlands, as compared with other countries.
The objective of this study was to assess risk factors for *M. catarrhalis* colonisation in the first year of life in healthy Dutch infants within a geographically restricted environment (Generation R Focus cohort, living in Rotterdam, The Netherlands).

METHODS

Study design and population

This study was embedded in the Generation R Study, a population-based prospective cohort study following children from foetal life until young adulthood, conducted in Rotterdam, The Netherlands [15-16]. As part of the Generation R Study, detailed assessments of foetal and postnatal growth and development were conducted in 1,232 Dutch pregnant women and their children (Generation R Focus cohort), who were born between February 2003 and August 2005. The Medical Ethics Committee of the Erasmus Medical Centre, Rotterdam, The Netherlands, has approved the study. Written informed consent was obtained from the parents of the children who were included. The mothers gave birth to 1,244 infants, of whom 138 were excluded from the study analysis due to the fact that consent was withdrawn after birth. Twins ($n = 27$) were also excluded from the analysis because of genetic relatedness, leaving a final total of 1,079 infants. The infants visited the Generation R focus study research center at age 1.5 months ($n = 900$), 6 months ($n = 901$) and 14 months ($n = 882$), during these visits 631 had swabs taken at 1.5 month, 787 at 6 months and 717 at 14 months; Seven hundred and

fifty eight infants attended all visits and 353 provided us with three swabs for use in longitudinal analysis. None of the infants used antibiotics in the preceding 48 hours.

Moraxella catarrhalis

Nasopharyngeal samples were taken using a sterile transport swab suitable for aerobes and anaerobes by a trained research nurse using Amies transport medium. Swabs were processed within the medical microbiology laboratory of the Erasmus MC, Rotterdam, The Netherlands, within 6 hours of sampling, using blood agar plates containing 5% sheep blood at an incubation temperature of 35°C in 5% CO_2 for 48 hours. Plates were examined daily for growth of *M. catarrhalis*.

Statistical methods

Information related to determinants of colonisation (birth weight, gestational period, gender, educational level of the mother, breast-feeding, maternal smoking (pre- and postnatal), day-care attendance, presence of siblings and antibiotic usage) was obtained from midwives, hospital registries and via questionnaires at 2, 6 and 12 months of age. Binary logistic regression analysis was used to determine significant associations between the parameters described above and *M. catarrhalis* colonisation. Additionally, multivariate analysis was performed to adjust for confounding factors. All variables from the univariate analysis were included in the multivariate model. For all determinants the missing values were modeled as a separate category and thus adjusted for in all analyses. Statistical analyses were performed using the Statistical Package of Social Sciences version 15.0 for Windows (SPSS Inc, Chicago, IL, USA).

RESULTS

Characteristics of the study participants are shown in Table 1. The prevalence of *M. catarrhalis* increased from 11.7% at the age of 1.5 months to 30.9% at the age of 6 months and 30.0% at the age of 14 months. The corresponding cumulative colonisation rates were 12% (73/622), 22% (312/1396), and 25% (524/2102). The presence of siblings at the age of 1.5 month (aOR 2.36, 95% CI 1.37 – 4.06) and 14 months (aOR 1.73, 95% CI 1.02 – 2.91) and day-care attendance at the age of 6 months (aOR 2.57, 95% CI 1.35 – 4.89) and 14 months (aOR 5.39, 95% CI 2.30 – 12.64) were found to be significant risk factors for colonisation by *M. catarrhalis*. However, gender, educational level of the mother, maternal smoking and breast-feeding were found to be non-significant (Table 2). Additionally, children who were colonised twice or more for *M. catarrhalis* were significantly more likely to have siblings (aOR 2.22, 95% CI 1.20 – 4.13) and attend day-care centres (aOR 5.84, 95% CI 1.67 – 20.41) (Table 3).

From the 1,079 children enrolled in the study (all of whom were swabbed at least once), 93 children received antibiotics in the first 6 months after birth. Two hundred twenty-seven

TABLE 1. Infant characteristics

Parameter	Value for infants
	n = 1,079
Gender	
- Male	521 (48.3%)
- Female	558 (51.7%)
Gestational age	40.0 (1.7)
Birth weight	3.509 (538)
Breast-feeding at 2 months	
- No	332 (33.5%)
- Yes	659 (66.5%)
Breast-feeding at 6 months	
- No	714 (70.6%)
- Yes	297 (29.4%)
Mother educational level	
- Higher education	683 (64.1%)
- Lower/intermediate education	382 (35.9%)
Mother prenatal smoking	
- No	885 (87.5%)
- Yes	127 (12.5%)
Mother postnatal smoking	
- No	606 (87.1%)
- Yes	90 (12.9%)
Siblings	
- No	583 (61.1%)
- Yes	371 (38.9%)
Day-care attendance	
- No	164 (20.0%)
- Yes	654 (80.0%)
M. catarrhalis at 1.5 month	
- No	553 (88.2%)
- Yes	74 (11.8%)
M. catarrhalis at 6 months	
- No	583 (70.1%)
- Yes	249 (29.9%)
M. catarrhalis at 14 months	
- No	532 (70.3%)
- Yes	225 (29.7%)

Values are means (SD) and absolute numbers (percentage).

children received antibiotics between 6 and 12 months of age and 109 children received antibiotics in the first 12 months after birth. Two hundred seventy-six did not receive any antibiotics in their first year of life. There was no significant association between antibiotic use in the first 6 months after birth and *M. catarrhalis* colonisation at 6 months, or antibiotic use in the first year of life and *M. catarrhalis* colonisation at 14 months (data not shown)

TABLE 2. Determinants of *Moraxella catarrhalis* colonisation in the first year of life

	Value for infants					
Parameter	*M. catarrhalis* 1.5 months		*M. catarrhalis 6* months		*M. catarrhalis* 14 months	
	OR (95%CI)	aOR (95%CI)	OR (95%CI)	aOR (95%CI)	OR (95%CI)	aOR (95%CI)
Gender						
- Male	1.00	1.00	1.00	1.00	1.00	100
- Female	1.38 (0.84 – 2.25)	1.43 (0.86 – 2.38)	1.07 (0.79 – 1.44)	1.29 (0.85 – 1.97)	0.84 (0.61 – 1.16)	0.73 (0.45 – 1.21)
Gestational age	0.99 (0.85 – 1.16)	0.96 (0.79 – 1.18)	1.04 (0.94 – 1.14)	1.02 (0.86 – 1.22)	1.15 (1.03 – 1.28)*	1.09 (0.88 – 1.35)
Birth weight	1.13 (0.70 – 1.83)	1.08 (0.59 – 1.99	1.11 (0.83 – 1.48)	1.27 (0.76 – 2.14)	1.59 (1.16 – 2.19)*	1.40 (0.76 – 1.35)
Breast-feeding at 2 months						
- No	1.00	1.00	1.00	1.00	1.00	1.00
- Yes	1.18 (0.69 – 2.01)	1.33 (0.76 – 2.35)	0.83 (0.59 – 1.16)	0.73 (0.43 – 1.23)	0.86 (0.60 – 1.24)	0.92 (0.49 – 1.71)
Breast-feeding at 6 months						
- No	N.A.	N.A.	1.00	1.00	1.00	1.00
- Yes			0.90 (0.64 – 1.27)	1.26 (0.75 – 2.10)	0.93 (0.65 – 1.33)	1.24 (0.67 – 2.32)
Mother educational level						
- Higher education	1.00	1.00	1.00	1.00	1.00	1.00
- Lower/intermediate education	0.76 (0.44 – 1.30)	0.65 (0.37 – 1.17)	0.75 (0.54 – 1.03)	0.92 (0.55 – 1.52)	0.75 (0.53 – 1.06)	1.39 (0.78 – 2.49)
Mother prenatal smoking						
- No	1.00	1.00	1.00	1.00	1.00	1.00
- Yes	0.94 (0.41 – 2.16)	0.92 (0.34 – 2.51)	0.77 (0.47 – 1.25)	1.11 (0.44 – 2.81)	0.54 (0.30 – 0.97)*	1.41 (0.50 – 3.97)
Mother postnatal smoking						
- No	0.76 (0.29 – 1.99)	0.81 (0.27 – 2.41)	1.01 (0.57 – 1.79)	1.08 (0.45 – 2.59)	0.91 (0.50 – 1.67)	0.91 (0.33 – 2.49)
- Yes						
Siblings						
- No	1.00	1.00	1.00	1.00	1.00	1.00
- Yes	2.40 (1.41 – 4.09)*	2.36 (1.37 – 4.06)*	1.06 (0.76 – 1.48)	1.27 (0.81 – 2.00)	1.61 (1.15 – 2.27)*	1.73 (1.02 – 2.91)*
Day-care attendance						
- No	N.A.	N.A.	1.00	1.00	1.00	1.00
- Yes			2.75 (1.65 – 4.57)*	2.57 (1.35 – 4.89)*	2.38 (1.44 – 3.94)*	5.39 (2.30 – 12.64)*

TABLE 2. Continue

	Value for infants					
Parameter	*M. catarrhalis* 1.5 months		*M. catarrhalis 6* months		*M. catarrhalis* 14 months	
	OR (95%CI)	aOR (95%CI)	OR (95%CI)	aOR (95%CI)	OR (95%CI)	aOR (95%CI)
M. catarrhalis at 1.5 months						
- No	N.A.	N.A.	1.00	1.00	1.00	1.00
- Yes			1.09 (0.58 – 2.02)	0.81 (0.41 – 1.60)	1.79 (0.98 – 3.27)	1.38 (0.63 – 3.02)
M. catarrhalis at 6 months						
- No	N.A.	N.A.	N.A.	N.A.	1.00	1.00
- Yes					1.52 (1.04 – 2.23)*	1.06 (0.61 – 1.84)

Values represent crude odds ratios (OR) and adjusted odds ratios (aOR) including 95% confidence intervals. Birth weight and gestational period were included in the models as continuous variables; all other parameters were included as binary variables. * = p-value <0.05

TABLE 3. Determinants of frequent (≥twice) *Moraxella catarrhalis* colonisation in the first year of life

Parameter	Value for infants	
	OR (95%CI)	aOR (95%CI)
Gender		
- Male	1.00	1.00
- Female	1.17 (0.67 – 2.06)	1.13 (0.62 – 2.04)
Gestational age	1.14 (0.93 – 1.41)	1.08 (0.83 – 1.41)
Birth weight	1.58 (0.87 – 2.87)	1.27 (0.60 – 1.41)
Breast-feeding at 6 months		
- No	1.00	1.00
- Yes	1.81 (0.91 – 3.58)	1.72 (0.82 – 3.60)
Mother educational level		
- Higher education	1.00	1.00
- Lower/intermediate education	0.86 (0.47 – 1.56)	1.17 (0.59 – 2.31)
Mother prenatal smoking		
- No	1.00	1.00
- Yes	0.93 (0.34 – 2.53)	1.22 (0.35 – 4.17)
Mother postnatal smoking		
- No	1.00	1.00
- Yes	0.74 (0.25 – 2.21)	0.82 (0.22 – 3.03)
Siblings		
- No	1.00	1.00
- Yes	2.31 (1.29 – 4.14)*	2.22 (1.20 – 4.13)*
Day-care attendance		
- No	1.00	1.00
- Yes	6.55 (1.98 – 21.69)*	5.84 (1.67 – 20.41)*

Values represent crude odds ratios (OR) and adjusted odds ratios (aOR) including 95% confidence intervals. Incomplete data parameters included: breast-feeding (*n* = 10), educational level of the mother (*n*=3), prenatal smoking mother (*n*=14), postnatal smoking mother (*n*=60), siblings (*n*=18) and day-care attendance (*n*=54). * = p-value <0.05

DISCUSSION

In this study of a large number of Dutch children, born in Rotterdam between February 2003 and August 2005, an increase in *M. catarrhalis* colonisation prevalence was observed from 11.7% at 1.5 months of age up to 30% at the ages of 6 and 14 months, where a "plateau" phase of colonisation was seen. The corresponding cumulative acquisition rates were 12%, 22%, and 25%, respectively. The figure of 12% at 1.5 months is almost identical to that of an American study dating from 1997, though in this latter study, the cumulative colonisation rates at the ages of 6 and 12 months were approximately 55% and 70%, respectively [4]. A reason for the difference in the cumulative colonisation rate between The Netherlands and the USA is not available, though geographical and/or chronological differences in the genetic background of both bacteria and the human hosts cannot be ruled out. Interestingly, both studies indi-

cated a peak in cumulative colonisation rates at approximately 6 months of age (though this peak was much lower in the Rotterdam study). It appears therefore, that geographically and/or chronologically distinct child populations remain susceptible to *M. catarrhalis* colonisation up to approximately 6 months of age, whereas after this time, the cumulative acquisition and elimination rates of *M. catarrhalis* remain constant. This phenomenon may be due to the development of a more effective immune response against *M. catarrhalis* colonisation, possibly as a consequence of children acquiring and eliminating genotypically distinct strains [17-18].

Of the environmental factors investigated in this study, only the presence of siblings within the family, and day-care attendance, were found to be significantly associated with the risk of acquiring *M. catarrhalis*. Moreover, both risk factors were also significantly associated with an increased frequency of colonisation. Previous studies in France and Sweden have also identified both of these environmental factors as significantly contributing to *M. catarrhalis* colonisation [19-20]. Taken together, it appears likely that crowding is a global risk factor in facilitating *M. catarrhalis* colonisation. Further, this phenomenon is not only confined to the home environment, but has also been found to be related to the hospital environment, as a recent publication indicated that multi-bed wards provided a significant risk factor for patient-to-patient *M. catarrhalis* transmission in adults [21].

No significant association between the use of antibiotics and colonisation with *M. catarrhalis* at 6 and 14 months of age was observed in this study. This means that there was no significant increase, or decrease, in colonisation rate in children receiving antibiotics compared with children who had received no antibiotics. In contrast, Varon *et al.* (2000), studied 629 children with respiratory tract infections in France and cultured nasopharyngeal swabs before and after antibiotic treatment [19]. In this study, a significant decrease in the nasopharyngeal carriage of *M. catarrhalis* was observed after antibiotic treatment. However, the antibiotics used included amoxicillin/clavulanate, cefixime, erythromycin/sulfisoxazole and cefpodoxime, antibiotics that are particularly effective against *M. catarrhalis*. In another study, Molstad *et al.* (1992) showed that increasing use of antibiotics was associated with an increase in beta-lactam resistance in *M. catarrhalis* [22]. This effect may be reduced by enforcing strict national antibiotic prescription policies, as demonstrated by data obtained from Denmark [23]. Unfortunately, our study did not have access to individual antibiotic prescription patterns. Further, The Netherlands has a strict policy on (limiting) antibiotic usage and the number and type of antibiotics that may be prescribed, though the use of amoxicillin/clavulanic acid is increasing in children [24]. In any case, antibiotic resistance rates in bacteria obtained from the nasopharynx of children who have not previously been treated with antibiotics may be relatively high. For example, Faden *at al.* (1994) indicated *M. catarrhalis* resistance rates of 90% for amoxicillin, and 19% for trimethoprim-sulfamethoxazole, in children who had not previously been treated with such antibiotics [25].

Prenatal and postnatal smoking was not found to be a significant risk factor for *M. catarrhalis* colonisation, which is in contrast with the study of Brook and Gober (2008), who found

that the nasopharynx of healthy children of smokers harbours a high number of pathogens that are similar to the bacterial flora found in their parents when compared to healthy children and non-smoking parents ($P<0.005$) [8]. In a previous study by Principi *et al.* (1999), it was shown that passive smoking was not a significant risk factor for *M. catarrhalis* colonisation, which agrees with our findings if we consider that children whose parents smoke are likely to be passively exposed to cigarette smoke.

Breast-feeding was not a significant risk factor. Breast-feeding has been found to be beneficial in reducing the risk for respiratory infections, but appears not to be significant with respect to bacterial nasopharyngeal colonisation rates [26-28].

In conclusion, the presence of siblings and day-care attendance are important independent risk factors for *M. catarrhalis* colonisation in infants in The Netherlands. Consequently, crowding is an important risk factor for early (and frequent) colonisation. The *M. catarrhalis* cumulative colonisation rate increases until a peak is reached at 6 months of age, whereupon a colonisation plateau is observed. This plateau could be a consequence of clearing of maternal antibodies or the development of a more effective immune response against the many different *M. catarrhalis* genotypes circulating in the first year of life, though this hypothesis requires further investigation.

Further, this study was set up purely to investigate the genetic and environmental factors associated with nasopharyngeal carriage of respiratory bacterial pathogens in infancy. Although information about (presumed) otitis media episodes is available, the diagnosis otitis media is based on parental reports (postal questionnaires at the infant' age of 6, 12 and 24 months). Mothers were asked whether their children suffered from fever in the prior period, and whether this period of fever was accompanied by earache and if they had visited a general practitioner. The diagnosis (presumed) OM was defined as having at least one period of fever accompanied by earache for which a doctor was visited. Using these criteria, no association was found between the frequency of colonisation and otitis media (personal communication J.A.M. Labout). Studies have shown that the use of parental reports for the definition of a clinical symptom may influence the outcome of epidemiological research [29-30]. In order to collect more definite data, OM should be diagnosed by a medical practitioner in future studies [4]. Additional studies will be necessary in order to investigate the effect of (multiple) colonisation events on the prevalence of upper respiratory tract disease in infants.

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Chapter 2.3

Correlation of bacterial colonisation status between mother and child

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For this thesis we added two extra figures (figure 1 and 2)

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ABSTRACT

Determinants of bacterial colonisation in children have been described. In the Generation R Study, a population-based cohort study, we determined whether colonisation statuses of mothers and children are correlated. Such a correlation was observed for *Staphylococcus aureus* and *Haemophilus influenzae*. Direct transmission, genetic susceptibility and/or unidentified environmental factors may play a role here.

INTRODUCTION

Although colonisation with bacterial microorganisms in the anterior nares and the nasopharynx is mostly asymptomatic, it may indirectly cause morbidity by increasing the risk of autoinfection [1-3]. The nasopharynx may be colonised by potential pathogens including *Streptococcus pneumoniae, Haemophilus influenzae* and *Moraxella catarrhalis* [4-5]. The opportunistic pathogen *Staphylococcus aureus* is often found in the anterior nares [6]. Pneumococcal disease is usually preceded by nasopharyngeal colonisation with the causative strain [1, 7-8] and for *S. aureus* also, most infections result from endogenous nasal colonisation as well [9].
S. pneumoniae and *M. catarrhalis* often colonise children but are not frequently found in adults, whereas *S. aureus* is more commonly found in adults [7, 10-12]. About 25%-37% of the healthy adult population carries *S. aureus* in the anterior nares [13-14]. The *S. aureus* colonisation rate steeply decreases in the first year of life [15-16]. Forty-four percent to 64% of children up to the age of 3 years have been colonised with or infected by *H. influenzae* at least once [7, 11, 17]. Little is known on bacterial colonisation in healthy family settings. Peacock et al. showed that mothers were the usual source for colonising *S. aureus* isolates in infants. They found a striking degree of concordance for *S. aureus* colonisation between mothers and infants in the first 6 months of life [18]. Another study on pneumococcal colonisation speculated that newborns mostly obtained their strains from other children rather than from their parents, as the serotype distribution in infants differed from that in adults [19]. A Costa Rican study revealed that very few mother-infant pairs carried identical airway pathogens simultaneously [12].
We determined whether nasopharyngeal colonisation with *S. pneumoniae, H. influenzae* and *M. catarrhalis* and nasal colonisation with *S. aureus* of the mother is independently correlated with the colonisation status of their healthy children at the age of 2 years.

METHODS

Study design and population

This study was conducted in the population-based prospective Generation R Focus Cohort (20-21). When the children were 24 months old, 836 (78%) children visited the research centre and a nasopharyngeal swab was taken from 71% of the children (n = 596) and 62% of the mothers (n = 515).

Bacterial cultures

A nasal swab for *S. aureus* isolation and a nasopharyngeal swab for *H. influenzae, S. pneumoniae and M. catarrhalis* isolation were obtained. Methods of sampling were described in detail previously [10, 16]. Bacterial genotyping was performed for the strains derived from infants and mothers colonised with the same pathogen. *S. aureus* genotyping using pulsed field gel elec-

trophoresis (PFGE) has been described previously [16]. In case of non-typable strains (ST398), PFGE was performed with *Srf*9I (20U), a neoschizomer of *Sma*I. *H. influenzae* strains were genotyped by PFGE as described previously by Hashida et al. [22]. PFGE data were inspected visually for banding pattern identity.

Statistical methods

Information on potential confounding variables (socioeconomic status, someone in the home smoking, and presence of siblings) was obtained by postnatal questionnaires when the infant was 2, 6 and 24 months old.

Univariate binary logistic regression analysis was performed to report on the association of maternal colonisation with the four bacterial microorganisms and colonisation with the same agents in their children. Subsequently, we adjusted for potential confounding variables (socioeconomic status, someone in the home smoking, and presence of siblings) with multivariate binary logistic regression analysis. We corrected for previous swab result of the child at 14 months. We thereby correct for earlier colonisation status of the child and the effect that that may have on maternal colonisation. Missing data in the confounding variables were accounted for in the analyses by adding them in the model as separate category. Measures of association are presented by crude odds ratios (OR) and adjusted odds ratios (aOR) with their 95% confidence interval (95% CI). The statistical analyses were performed using the Statistical Package of Social Sciences version 15.0 for Windows (SPSS Inc, Chicago, IL).

RESULTS

A total of 836 children visited the Generation R research centre when they were 2 years of age, and swabs were obtained from 618 children (73.9%). In total, 511 mother-child pairs were available. There were no differences in the confounding variables among the children from the mother-child pairs as compared to the total cohort. The children who did not provide a swab sample more often had missing data on confounding variables, in particular postnatal smoking of the mother and presence of siblings. Of the 511 children selected, 47.0% were female. The mean gestational age was 40.0 weeks (SD 1.6) with a mean birth weight of 3511 grams (SD 541 gram). Of this group, 195 children had older siblings (38.1%). The mothers were overall highly educated (61.4% completed high school and further education). In 14.1% of the households someone smoked cigarettes at home (n = 72). Data were missing on educational level of the mother in 3 pairs, on presence of a smoker at home in 35 pairs, and on the presence of siblings in 41 pairs.

Table 1 shows the prevalence of bacterial colonisation in these children and their mothers. *S. pneumoniae* was most frequent in children (n = 180), whereas hardly any of the mothers carried this pathogen (n = 14). Similarly, *M. catarrhalis* was found to be more frequent in children

TABLE 1. Correlation between maternal swab result and child swab result

Species	Prevalence of bacterial colonisation:			OR (95% CI)	aOR (95% CI)
	Mother	Child	Mother and Child		
Staphylococcus aureus	129 (25.2%)	69 (13.5%)	25 (4.9%)	1.85 (1.08 – 3.16) *	2.04 (1.18 – 3.56) *
Streptococcus pneumoniae	14 (2.7%)	180 (35.2%)	5 (1.0%)	1.02 (0.34 – 3.10)	1.05 (0.34 – 3.23)
Moraxella catarrhalis	6 (1.2%)	150 (29.4%)	3 (0.6%)	2.44 (0.49 – 12.21)	3.40 (0.64 – 17.98)
Haemophilus influenzae	60 (11.7%)	135 (26.4%)	23 (4.5%)	1.88 (1.07 – 3.30) *	2.02 (1.14 – 3.60) *

Results are presented as crude odds ratios (OR) and adjusted odds ratios (aOR) with a 95 % confidence interval (CI). Adjusted odds ratios are corrected for socio-economic status, smoking at home, siblings and previous swab result of the child. * = p-value <0.05

(29.4%, *n* = 150 versus 1.2%, *n* = 6). *S. aureus* was carried more frequently by the mothers than by their children (25.2% versus 13.5% in the children), whereas *H. influenzae* was carried more frequently by the children (26.4% versus 11.7%). A small number of mother and child pairs were colonised with *S. pneumoniae* and *M. catarrhalis*. PFGE on samples for the mother and child pairs colonised with S. pneumoniae and M. catarrhalis was not conducted (*n* = 3 and *n* = 5, respectively). *H. influenzae* colonisation in both mother and child occurred in 23 pairs. We found a significantly increased risk for infants to be colonised with this pathogen at the age of two when the mother was culture positive (aOR 2.02 95% CI 1.14 – 3.60). We found 25 mother-child pairs positive for *S. aureus*. There was also a significantly increased risk for infants to be colonised with *S. aureus* when the mother was positive (aOR 2.04 95% CI 1.18 – 3.56)

Of the 25 mother and child pairs with positive *S. aureus* cultures, 24 pairs were genotyped (1 missing pair). Of these 24 pairs, 75% (*n* =18 pairs) were colonised with a genotypically indistinguishable strain (figure 1). There was one mother and child pair with a non-typeable strain, a non-methicillin resistant multi locus sequence type (MLST) ST398 strain. No methicillin resistant *S. aureus* was detected in the 48 genotyped samples. Of the 23 mother-child pairs where both the mother and the child had a positive *H. influenzae* culture, 13% (*n* = 3 pairs) were colonised with a genotypically indistinguishable strain (figure 2).

DISCUSSION

We show that colonisation with *S. aureus* and *H. influenzae* in mothers and children are correlated, which was not the case for *S. pneumoniae* and *M. catarrhalis*. When the child is two years old, the mother and child hold a 2-fold increased risk to be simultaneously colonised with *S. aureus* or *H. influenzae*. This effect remained significant after adjusting for parental smoking, presence of other children in the household and socioeconomic status. The effect remained significant after correction for previous culture result of the child, which took place 10 months prior – when the child was 14 months. Accounting for earlier colonisation of the

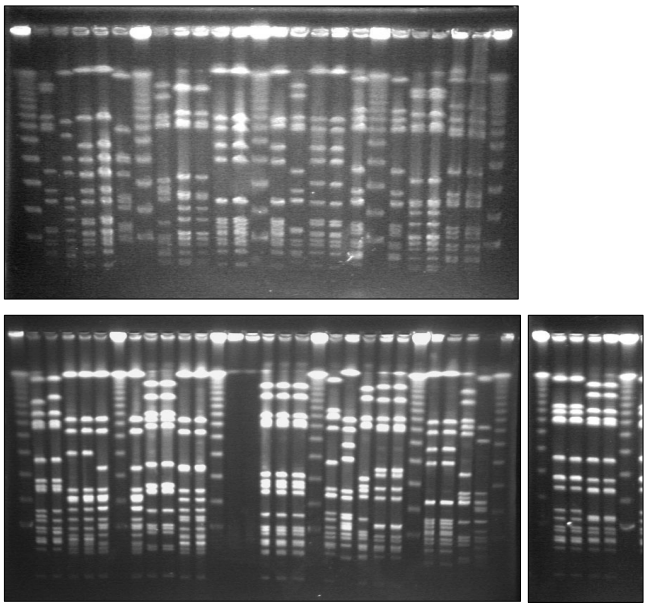

Figure 1. PFGE *S. aureus* colonising strains from mothers and children.
The first strain is obtained from the child; the one next to it was cultured from the anterior nares of the mothers.
Of 24 mother and child couples, 18 couples carried genetically indistinguishable strains.

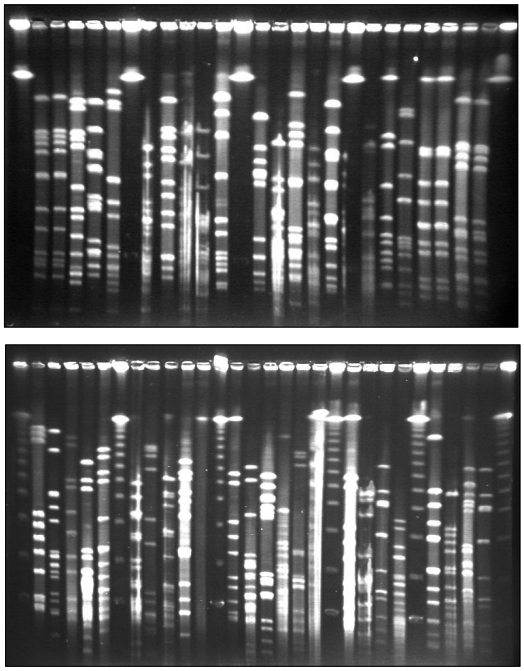

Figure 2. PFGE of *H. influenzae* colonising strains from mothers and children.
The first strain is obtained from the child; the one next to it was cultured from the nasopharynx of the mothers.
Of the 23 mother and child couples, 3 couples carried a genetically indistinguishable strain.

child provides some evidence that colonisation status of the child does not affect the correlation between mother and child colonisation a year after, as the results remain statistically significant.

Genotypic analyses of the *S. aureus* strains isolated from the mothers and children when both were colonised with the same microorganism identify 75% genotypically indistinguishable pairs. This suggests direct transmission. Conversely, there is little overlap in the *H. influenzae* genotypes colonising mothers and children. Eighty seven percent (20 pairs) are colonised with genotypically distinct strains. This may suggests that household spread of *S. aureus* occurs more frequently and perhaps easier than transmission of *H. influenzae*.

An explanation for these statistically significant correlations could be genetic host components that make both mother and child more prone to bacterial colonisation. No literature is available on genetic susceptibility towards *H. influenzae* colonisation. Van den Akker et al. described the effect of polymorphisms of the glucocorticoid receptor gene and *S. aureus* nasal colonisation [23]. A recent study identified additional polymorphisms [24]. Peacock et al. also speculated about the importance of genetic components. On the basis of colonisation concordance, they concluded that a strong influence from a shared environment and/or common host genetics can be expected [18].

We can not provide evidence of what occurs first: maternal or child colonisation. We also do not present colonisation data of all members of the household. Since children are in close contact with others beside their mothers, this would have strengthened our analyses.

In conclusion, we show a correlation between bacterial carriage status of mothers and their children. Direct transmission of *S. aureus* occurs between mothers and children. Other phenomena must play a role in the correlation of simultaneous *H. influenzae* colonisation. Genetic susceptibility may be an important factor and should be assessed in future research.

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Chapter 3

Specific humoral immune response and bacterial colonisation

Chapter 3.1

Natural antibodies against pneumococcal virulence proteins in children in the pre-pneumococcal conjugate vaccine-era

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ABSTRACT

The currently available pneumococcal vaccines do not protect against all serotypes of *Streptococcus pneumoniae*. A shift towards non-vaccine serotypes causing colonisation and invasive disease has occurred and studies on protein-based vaccines are undertaken. We assessed the association between specific antibodies against pneumococcal virulence proteins and colonisation and respiratory tract infections (RTI). Additionally, we assessed to what extend colonisation induces a humoral immune response.

Nasopharyngeal swabs were cultured for pneumococcus at 1.5, 6, 14 and 24 months of age. Serum samples were obtained at birth, 6, 14 and 24 months (n=57 children providing 177 serum samples). Data were collected prior to the pneumococcal vaccine-era. IgG, IgA and IgM levels against seventeen pneumococcal protein vaccine candidates were measured using a bead based flow cytometry technique (xMAP®, Luminex Corporation). Information regarding RTI was questionnaire-derived.

IgG levels to all proteins were high in cord blood, decreased in the first 6 months and increased again thereafter, contrary to the course of IgA and IgM levels. Specific antibodies were induced upon colonisation. Increased levels of IgG against BVH-3, NanA and SP1003 at 6 months, NanA, PpmA, PsaA, SlrA, SP0189 and SP1003 at 14 months and SlrA at 24 months were associated with a decreased number of RTI in the 3rd year of life but not with colonisation. Maternal anti-pneumococcal antibodies did not protect against pneumococcal colonisation and infection.

Certain antibodies against pneumococcal virulence proteins, some of which are induced by colonisation, are associated with a decreased number of RTIs in children. This should be taken into account for future pneumococcal vaccine studies.

INTRODUCTION

Streptococcus pneumoniae (pneumococcus) is a commensal but also a pathogen that plays an important role in the pathogenesis of respiratory tract infections (RTIs) such as pneumonia and otitis media in infants and young children. In addition, the pneumococcus may also cause invasive diseases such as meningitis and sepsis [1]. Morbidity and mortality in infants and young children worldwide is frequently caused by this pathogen. The World Health Organization generated estimates on child mortality due to invasive pneumococcal infection range from 700,000 to more than a million children per annum [1]. Healthy children may be colonised with the pneumococcus in the nasopharynx the frequency of which increases in the first year of life from approximately 8% to 45% [2]. This pathogen often presents as a commensal, causing no harm due to adequate innate and adaptive immune reactions of the host. However, asymptomatic nasopharyngeal carriage is the primary source for pneumococcal infection [3].
Over 90 different pneumococcal serotypes have been identified on the basis of variability in the capsular polysaccharides. The current vaccines are based on antibodies against these polysaccharides, hence only a part of these serotypes is covered. These days, significant research is focused on improving pneumococal vaccines in order to generate broader protection against pneumococcal disease. The current 7-valent pneumococcal conjugate vaccine (PCV-7), which has now been introduced in national immunization programs in most developed countries, is up to 90% effective in reducing vaccine-serotype specific invasive pneumococcal disease. However, the net vaccine benefit was negatively affected by a 71% increased rate of non-vaccine serotype invasive pneumococcal disease [4]. Recently, a 13-valent pneumococcal conjugate vaccine (PCV-13) was developed to improve further protection [5-6]. However this still does not cover most pneumococal serotypes. The increase in carriage of non-vaccine serotypes, and the associated increase in invasive disease, could ultimately outweigh the benefit of the current PCV [7]. Since PCV is also quite expensive and therefore not extensively used in developing countries where it is needed the most, there is an urgent need to develop alternative pneumococcal vaccines to cover these gaps. The search for novel vaccines with expanded coverage and immunogenicity is urgently needed for optimal prevention of pneumococcal infections. Most promise is held by adding protein-based vaccines to the current PCV, which may provide protection regardless of serotype [8]. Several protein antigens have been identified as vaccine candidates such as Ply, CbpA, PspA, PsaA, PiaA, PhtB, PhtE (BVH-3) and NanA [9-20]. There is evidence that immunization with certain combinations of virulence proteins provides additive or even synergistic protection [21-22]. Especially the combination of Ply, PspA and CbpA successfully provided protection in mouse models [11, 23-24]. These murine studies have demonstrated a protective effect of immunoglobulines directed against these primarily but not exclusively surface-located pneumococcal proteins. However, prospective studies on protectiveness of antibodies against pneumococcal proteins in humans are lacking. Since infection with *S. pneumoniae* is supposed to start with colonisation it seems

rational to aim for prevention of colonisation and thus search for anti-pneumococcal antibodies providing protection against colonisation [25].

Our primary objective was to assess, in children from the pre-pneumococcal vaccine-era, the level of protection provided by antibodies against seventeen pneumococcal proteins on infant pneumococcal colonisation and RTI. Additionally, we assessed to what extend colonisation induces a humoral immune response.

METHODS

Study design and population

This study was part of the Generation R study. The Generation R Study is a population-based prospective cohort study following pregnant women and their children. Further details on this cohort study were described previously [26-27]. The present study was performed in a subgroup of 1,079 Dutch women and their single born children. Detailed assessments were conducted in this subgroup. All of these children were born between February 2003 and August 2005. This was prior to introduction of the pneumococcal vaccination in The Netherlands in 2006. The Medical Ethics Committee of the Erasmus Medical Center, Rotterdam, approved the study and written informed consent was obtained.

Streptococcus pneumoniae

A cord blood sample was obtained after delivery and infant blood samples were obtained during the visits at the research centre at the ages of 6, 14 and 24 months. Of the 1,079 infants in the postnatal cohort of analyses, 57 were selected for this particular study. They were selected on the basis of availability of biological samples. Seventeen pneumococcal proteins were selected based on importance as indicated by current scientific literature, their potential role in vaccine development and availability. Antigens included putative protease maturation protein A (PpmA), pneumococcal surface adhesin A (PsaA), pneumococcal surface protein A (PspA) and choline binding protein A (PspC /CbpA), as well as neuraminidase (NanA), pneumolysin (PLY), a double mutant of pneumolysin (PdbD), the pneumococcal histidine triad (Pht) family (BVH-3), streptococcal lipoprotein rotamase A (SlrA), alpha-enolase (eno), IgA-1 protease (IgA-1 protease), hyaluronidase (Hyl), and *Streptococcus pneumoniae* (SP) proteins; SP0189 (hypothetical protein), SP0376 (response regulator, intracellular location), SP1003 (PhtD/ BVH11-2, histidine triad protein), SP1633 (response regulator, intracellular location) and SP1651 (thiol peroxidase, intracellular location). Isolation and purification methods were as described previously [28]. Table 2 explains the function of the studied proteins [29]. The levels of IgG, IgA and IgM against these proteins were measured using a recently described 17-plex based on pneumococcal proteins [30] with the (bead) based flow cytometry technique (xMAP®, Luminex Corporation, Austin, Texas, USA). Here we used this novel multiplex assay.

The median fluorescence intensity (MFI) values, reflecting semi-quantitative antibody levels, were averaged. Tests were performed in independent duplicates and control beads (where no protein was coupled) were included to determine non-specific binding. In case of non-specific binding, the a-specific MFI values were subtracted from the antigen-specific results. During the visits at the ages of 1.5, 6, 14 and 24 months nasopharyngeal swabs for isolation of *S. pneumoniae* were obtained. Methods of sampling were as described previously [2].

Respiratory tract infections

Parentally retrieved questionnaires were obtained at 12, 24, 36 and 48 months. Questions regarding doctor visits (never, once or twice, three or four times, more than four times) because of fever and respiratory tract complaints was used to assess the burden of RTIs. We defined three subgroups: child has not been to a doctor with fever and cough/runny or blocked nose/ear ache in the preceding year, child has been to a doctor with fever and cough/runny or blocked nose/ear ache once or twice in the preceding year and child has been to a doctor with fever and cough/runny or blocked nose/ear ache three times or more in the preceding year. For the analyses we compared children who frequently visited the doctor (at least three times) with the children who never visited the doctor for RTIs. Children scoring three or four times or four times or more on number of doctor visits were classified as at least three times. Additionally, children scoring once or twice on doctor visits for at least two different symptoms (e.g. once or twice for fever with ear ache and once or twice for fever with a cough) were scored as at least 3 doctor visits as well. This latter category may comprise children with only two doctor visits as we can not distinguish between two and three or four doctor visits in this group.

Statistical methods

Wilcoxon Signed Rank tests were used to compare anti-pneumococcal antibody levels in the group of children at the four different ages. We compared IgG, IgM and IgA levels between 0-6 months, 6-14 months, 14-24 months and overall between 0-24 months.

Mann-Whitney *U* tests were used to compare differences in maternal antibody levels for colonised and non-colonised infants in the first year of life and to compare differences in maternal antibody levels for children with and without frequent RTIs in the first year of life. Moreover, to assess whether levels of antibodies protect against later colonisation, we used the Mann-Whitney *U* test to compare differences in antibody levels at the ages of 6, 14 and 24 months for later colonised and non-colonised children. Additionally, we used this test to compare differences in antibody levels at the ages of 6, 14 and 24 months for children with and without frequent RTIs in the 3rd year of life. At age 14 months and older the results will not be blurred by maternal antibodies. Finally, Mann Whitney *U* tests were used to compare differences in antibody levels between previously colonised and non-colonised children at the different measurement moments to assess whether these specific antibodies are induced

upon colonisation. The Wilcoxon Signed Rank tests and Mann Whitney *U* tests were used for the same type of analyses before [31].

Results were presented as MFI values. $P<0.05$ was considered statistically significant. The statistical analyses were performed using the Statistical Package of Social Sciences version 17.0 for Windows (SPSS Inc, Chicago, IL, USA).

RESULTS

Of the 57 children included for this study, 51 provided three serum samples and four samples were obtained in 6 children. This adds up to a total of 177 serum samples. Of these 177 samples, 54 (31%) were cord blood samples, 32 samples (18%) were obtained at 6 months, 46 (26%) at 14 months and 45 (25%) at 24 months. Nasopharyngeal swabs were available for 40 children (70%) at 1.5 month, 49 (86%) at 6 months, 50 (88%) at 14 months and 48 (89%) at 24 months. At 1.5 month, 17.5% of the children ($n = 7$) were colonised with *S. pneumoniae*, which increased at 6, 14 and 24 months to 28.6% ($n = 14$), 36.0% ($n = 18$) and 39.6% ($n = 19$), respectively. In the first year of life, 7 infants (12.3%) visited a doctor at least 3 times for putative RTIs, this number increased to 13 children (22.8%) in the second year of life and decreased thereafter to 5 children (9.4%) in the third year of life. General population characteristics are presented in table 1.

TABLE 1. Child characteristics

Parameter	Value for children
	N=57
Gestational age (weeks)	40.2 (1.39)
Birth weight (grams)	3677 (489)
Gender female	28 (49.1%)
Positive for colonisation	
- 1.5 month	7 (17.5%)
- 6 months	14 (28.6%)
- 14 months	18 (36.0%)
- 24 months	19 (39.6%)
- 36 months	8 (19.5%)
Frequent respiratory tract infections (>3x)	
- 1st year	7 (12.3%)
- 2nd year	13 (22.8%)
- 3rd year	5 (9.4%)

Values are presented as mean (sd) or absolute number (%).

Data is missing for colonisation status at 1.5 month (n=17), at 6 months (n=8), at 14 months (n=7), at 24 months (n=9), at 36 months (n=16) and for respiratory tract infection in 2nd (n=1) and 3rd year of life (n=4)

TABLE 2. Functions of the selected seventeen pneumococcal proteins

Pneumococcal virulence proteins	Main role
BVH-3 (PhtE)	Pneumococcal histidine triad, possibly a role in complement inhibition.
PspC (CbpA)	Binds to human secretory component on a polymeric Ig receptor during the first stage of translocation across the epithelium.
PdbD	Double mutant of Ply
Enolase (Eno)	Binds to plasminogen and is subsequently activated to the serine protease plasmin by tPA or uPA.
Hyaluronidase (Hyl)	Breaks down hyaluronan-containing extracellular matrix components.
IgA-1protease	Cleaves human IgA1
NanA	Removes sialic acids and cleaves terminal sugars from various glycoconjugates, which might reveal receptors for adherence.
Ply	Pneumolysin. Cytolytic toxin that also activates complement. An important determinant of virulence in *in vivo* models of disease. Wide range of effects on host immune components at sub-lytic concentrations.
PpmA	Induces opsonophagocytosis *in vitro*.
PsaA	Component of the ABC transport system, which is involved in resistance to oxidative stress and transport of Mn2+.
PspA	Prevents binding of C3 onto pneumococcal surface. Also binds lactoferrin.
SlrA	Cyclophilin-type PPIase can catalyze the *cis-trans* isomerization of proline containing tetrapeptides. Modulates the biological function of important virulence proteins.
SP0189	Hypothetical protein
SP0376	Response regulator (intracellular location)
SP1003 (BVH-11-2 / PhtD)	PhtD (histidine triad protein)
SP1633	Response regulator (intracellular location)
SP1651	Thiol peroxidase (intracellular location)

Dynamics of anti-pneumococcal antibodies

Levels of IgG, IgM and IgA directed against pneumococcal proteins showed a dynamic process over the first two years of life (Figure 1). There was extensive variability in serum responsiveness over time for each pneumococcal protein. Overall, IgG levels against pneumococcal proteins tended to be high in cord blood but these levels significantly decreased in the first 6 months of life. This holds true for all anti-pneumococcal antibodies except for anti-PsaA, anti-SP0189, anti-SP1633 and anti-SP1651 antibodies. The latter anti-SP antibodies were low at birth and showed neither a significant increase nor a notable decrease. Apparently, these proteins are poorly immunogenic. A significant increase was observed after the first six months for all proteins except for Eno, Hyl, PspA and the SP-proteins. Low values were obtained for IgA and IgM in the cord blood samples, increasing significantly in the first two years of life (p-values <0.001 for all proteins except for IgM against PspC).

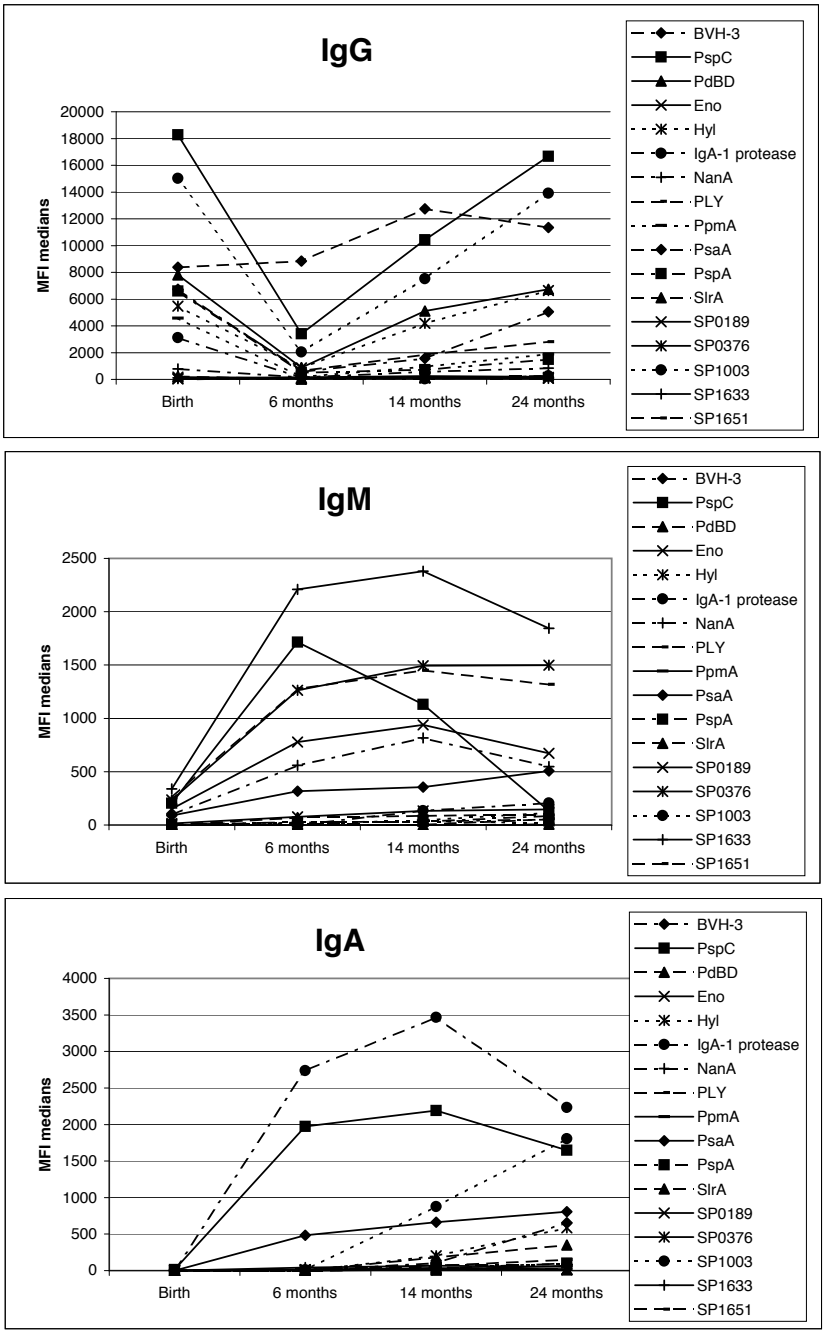


Figure 1. Dynamics of IgG, IgM and IgA in the first two years of life.

Median MFI values, averaged for all children (*n*=57) are presented by age (1.5, 6, 14 and 24 months). High levels of placentally transferred IgG are observed at 1.5 months, which decreases in the first 6 months. After 6 months an increase of IgG is observed. Low levels of IgM and IgA in serum was observed after birth, but this increases in the first year of life.

Maternal anti-pneumococcal antibodies

For 54 infants, cord blood samples for analyses of maternal anti-pneumococcal antibodies were available. Anti-pneumococcal IgA and IgM levels in cord blood were low because maternal IgA and IgM are not transported across the placenta. Hence, we only studied maternal IgG levels in relation to infant colonisation and infection. Maternal IgG levels in cord blood were on average higher in children with higher colonisation prevalence in the first year of life. Elevated levels of maternal anti-BVH-3, NanA and SP1651 IgG were significantly associated with enhanced child colonisation rates at 1.5 month (BVH-3, p-value 0.003) and 14 months (BVH-3, p-value 0.049 and NanA, p-value 0.047). IgG levels against BVH-3 were also significantly increased in children frequently colonised with *S. pneumoniae* in the first year of life (p-value 0.003). This indicates that these antibodies are not able to protect the child against colonisation. In contrast, these antibodies seem to be facilitating colonisation. There were no maternal IgG antibodies found to provide protection against colonisation. Moreover, we did not observe a protective effect of maternal IgG antibody levels and RTIs in the first year of life.

Anti-pneumococcal antibodies and colonisation

To study whether antibodies of the child protect against future colonisation with *S. pneumoniae*, we focused on antibody levels at 14 and 24 months as the samples at 6 months may still contain maternal antibodies. At the age of 24 months, IgG level against PspC were significantly increased in children non-colonised at the age of 36 months as compared to the IgG levels in children colonised at this same age. No other protective associations between protein-specific anti-pneumococcal antibody levels and colonisation were observed.

However, pneumococcal colonisation does induce an antibody response close to the time nearest to the colonisation moment as can be seen in table 3. This may be due to colonisation itself or clinical or sub-clinical infections. Colonisation at 1.5 months induces both an IgG and IgM response against several pneumococal proteins at 6 months and an IgA response against PsaA. IgG levels at 6 months are elevated in the colonised children as compared to the children whom were non-colonised at 1.5 month (Medians: BVH-3 942 versus 392, NanA 287 versus 112, PpmA 1076 versus 180, PsaA 13000 versus 6406, SlrA 176 versus 16). At 14 months, only IgG level against SP0189 was significantly elevated in children earlier colonised with the pneumococcus at 1.5 months (Table 3).

Colonisation at 6 months is correlated to elevated levels of IgG and IgA against several pneumococal proteins at the time of colonisation and later on at 14 months, but is barely correlated to IgM levels. Children who were colonised at 14 months had higher levels of IgG and IgA to several pneumococcal proteins at 14 months than non-colonised children. Few differences between colonised and non-colonised children at 14 months were noted in the antibody levels at 24 months (Table 3). Children with frequent colonisation in the first 14 months (at least twice) barely showed differences in antibody levels at 24 months compared to the children with no colonisation in the first 14 months. Only the level of IgG against PpmA antigens at 24 months

TABLE 3. Anti-pneumococcal antibodies following pneumococcal colonisation

	6 months			14 months			24 months		
	IgG	IgM	IgA	IgG	IgM	IgA	IgG	IgM	IgA
Colonisation at 1.5 month	BVH-3 NanA PpmA PsaA SlrA	PspC Eno NanA SP0189 SP0376	PsaA	SP0189	-	-	N.A.	N.A.	N.A.
Colonisation at 6 months	BVH-3 Hyl PpsA SP1003	PsaA	Hyl PsaA PspA SP1003	PspC Eno	PspC ¶ SlrA	BVH-3 NanA PsaA SP1003	N.A.	N.A.	N.A.
Colonisation at 14 months	N.A.	N.A.	N.A.	BVH-3 PspC PdbD NanA PLY¶ PspA SP1003	PspC ¶	PspC PdbD NanA PLY PspA SP1003	NanA	PLY¶	-

Anti-pneumococcal antibodies in this table are significantly ($p<0.05$) increased until one year after colonisation.
Note: the antibody levels at 6 months might be obscured by the presence of maternal antibodies.
¶ significantly decreased levels following colonisation, p-value<0.05.
N.A. = not applicable, either as the determinant occurs after the outcome (colonisation at 14 months and antibody levels at 6 months), or because of a long time span between determinant and outcome (colonisation at 1.5 and 6 months and antibody levels at 24 months).

was elevated in children with frequent colonisation in the first 14 months (data not shown). This may be due to a long time span between colonisation and antibody level at 24 months.

Anti-pneumococcal antibodies and respiratory tract infections.

Besides colonisation, we studied the correlation between systemic antibody levels and the number of doctor visits for RTIs. Table 4 shows all correlations between levels of specific anti-pneumococcal antibodies and RTIs in the third year of life with p-values below 0.08.
Increased levels of IgG against BVH-3, NanA and SP1003 at 6 months, IgG directed against NanA, PpmA, PsaA, SlrA, SP0189 and SP1002 at 14 months and IgG against SlrA at 24 months were observed in children with a lack of doctor visits for RTIs in the 3rd year of life compared to children with at least 3 visits (Table 4 and Figure 2). The same significant effect was observed for increased levels of IgA against NanA and SP0376 at 6 months and against SP1003 at 14 months. IgM levels at 6 months were not associated with RTI in the 3rd year of life. At 14 months, however, IgM against IgA-1 protease and BVH-3 was significantly increased in children with a lack of doctor visits for RTIs in the third year (Table 4).

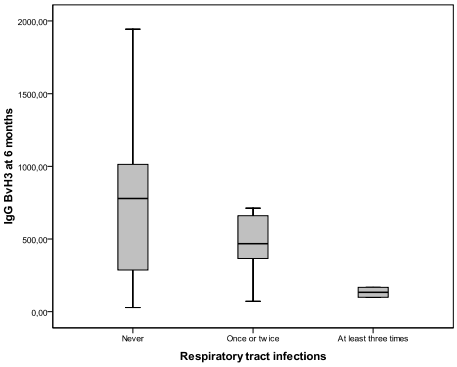
TABLE 4. Correlation antibodies and RTI's in children

	Number of respiratory tract infections in the 3rd year of life		
	Never (n=29)	At least 3 times (n=5)	p-value
6 months			
IgG			
BVH-3	779 (42 – 1,931)	133 (99 – 168)	0.050 *
SP1003	2,138 (344 – 15,291)	826 (448 – 1,205)	0.050 *
NanA	173 (38 – 654)	40 (37 – 43)	0.038 *
IgA			
Eno	10 (0 – 217)	1 (0 – 2)	0.063
IgA-1 protease	2,859 (846 – 6,454)	1,190 (1,061 – 1,319)	0.064
NanA	21 (0 – 146)	0 (0 – 0)	0.022 *
SP0376	18 (1 – 329)	3 (2 – 3)	0.049 *
SP1003	11 (0 – 1102)	0 (0 – 0)	0.061
14 months			
IgG			
NanA	701 (46 – 3,019)	252 (9 – 865)	0.045 *
PpmA	985 (0 – 12,962)	0 (0 – 296)	0.015 *
PsaA	13,531 (833 – 16,944)	4091 (769 – 11,533)	0.021 *
SlrA	58 (0 – 2,550)	9 (3 – 20)	0.032 *
SP0189	127 (31 – 1,512)	58 (22 – 96)	0.030 *
SP1003	9,823 (53 – 18,717)	522 (27 – 7,307)	0.021 *
IgM			
IgA-1 protease	202 (0 – 2,690)	0 (0 – 97)	0.026 *
BVH-3	67 (0 – 1,187)	0 (0 – 9)	0.041 *
IgA			
PpmA	14 (0 – 1,454)	0 (0 – 62)	0.074
SP1003	870 (1 – 7,807)	298 (0 – 571)	0.021 *
24 months			
IgG			
PpmA	2,047 (0 – 8,113)	518 (0 – 1,101)	0.065
SlrA	335 (15 – 3,975)	53 (34 – 74)	0.049 *
SP1003	14,161 (2,018 – 18,743)	8,007 (81 – 11,630)	0.075
IgM			
PspA	40 (0 – 1,817)	154 (98 – 234)	0.066 ¶
IgA			
SlrA	20 (0 – 979)	0 (72 – 712)	0.059

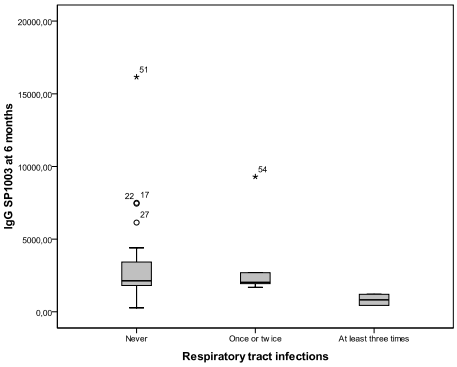
Values represent median MFI levels (5-95% range), reflecting antigen-specific IgG, IgM or IgA levels.

All correlations with a p-value <0.08 are shown. * p-value <0.05

¶ Increased levels in children with frequent RTI's


IgG BvH3 at 6 months
2000,00
1500,00
1000,00
500,00
0,00
Never
Once or twice
At least three times
Respiratory tract infections


IgG NanA at 6 months
600,00
400,00
200,00
0,00
38
Never
Once or twice
At least three times
Respiratory tract infections


IgG SP1003 at 6 months
20000,00
15000,00
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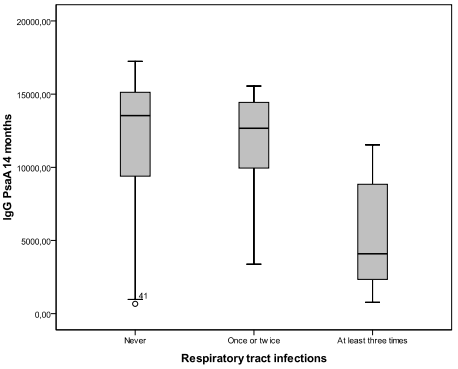
0,00
51
54
22
17
27
Never
Once or twice
At least three times
Respiratory tract infections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20000,00
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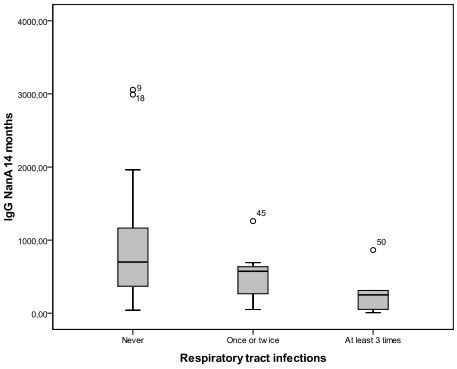
0,00
41
Never
Once or twice
At least three times
Respiratory tract infections


IgG NanA 14 months
4000,00
3000,00
2000,00
1000,00
0,00
9
18
45
50
Never
Once or twice
At least 3 times
Respiratory tract infections


IgG SP1003 at 14months
20000,00
15000,00
10000,00
5000,00
0,00
48
Never
Once or twice
At least three times
Respiratory tract infections

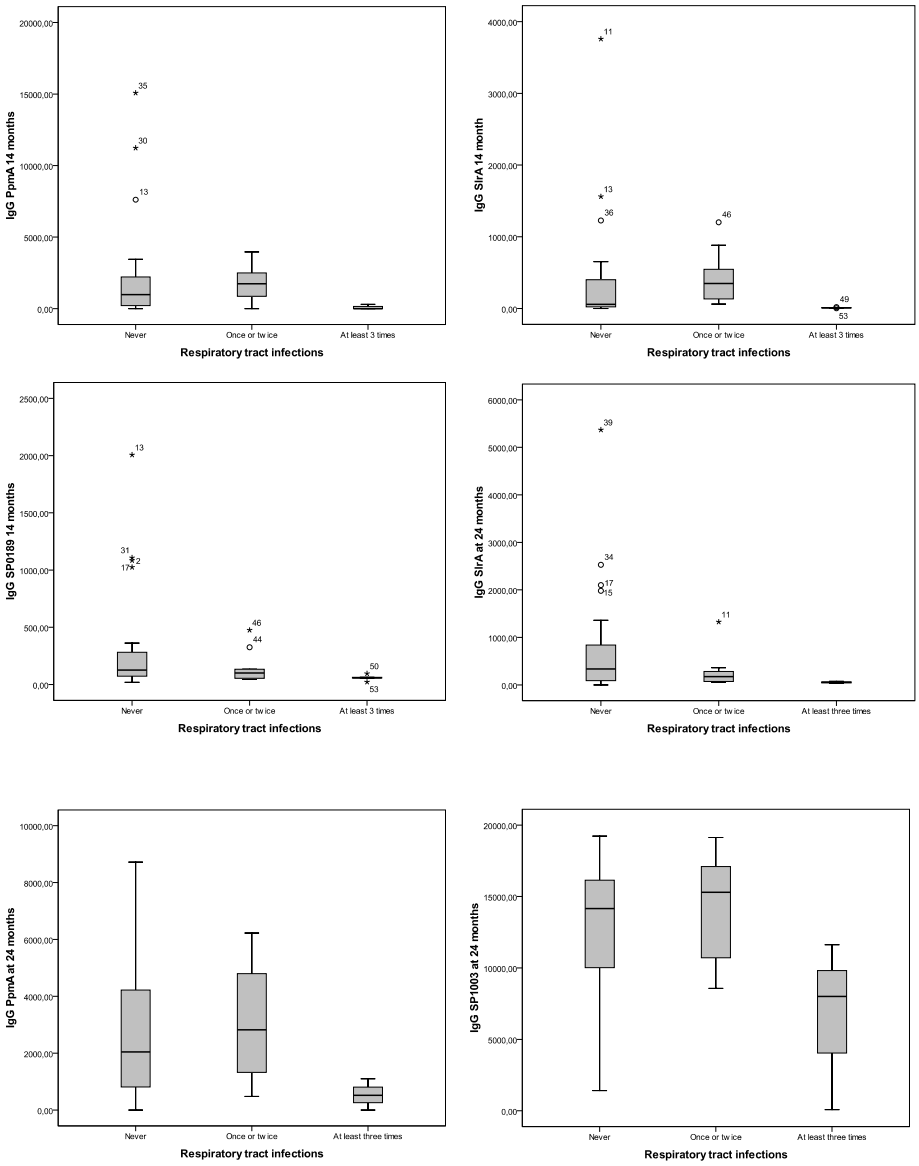


Figure 2. Association between certain antipneumococcal IgG levels and respiratory tract infections in the third year of life.

IgG levels at 6, 14 and 24 months by number of doctor visits for respiratory tract infections in the 3rd year of life. Higher levels of IgG against certain pneumococcal proteins were correlated to no doctor visits for RTI (first box in every box plot). The median level of IgG against certain pneumococcal proteins was lower in children with at least 3 doctor visits for RTI (third box in every box plot). This was statistically significant for the pneumococcal proteins presented in these box plots, except for IgG against PpmA and SP1003 at 24 months (p-value 0.065 and 0.075, respectively). P-values are presented in table 3. Values are presented as median MFI levels, with an interquartile (25-27%) box, a 5-95% range, outliers (○) and extreme outliers (*).

DISCUSSION

Our study demonstrates that several anti-pneumococcal protein antibodies are induced upon colonisation, possibly due to clinical or subclinical infection during colonisation and that some of these specific antibody levels are also associated with reduced number of doctor visits for RTIs. Because of the change in pneumococcal serotypes causing colonisation and infection following the implementation of the polysaccharide-based vaccine, novel protein-based vaccines are needed for prevention of pneumococcal infections. However, data on antibodies against pneumococcal virulence proteins in relation with human colonisation and infection are lacking. Our observations are relevant in the context of future pneumococcal protein vaccine development.

We described that IgG antibodies against BVH-3, NanA, PpmA, PsaA, SlrA, SP0189 and SP1003 were increased in children who suffered less respiratory infections in the third year of life, suggesting that these antibodies are either protective or markers of other protective agents. This is in line with data presented by Bogaert et al, who also found anti-PpmA IgG antibodies to significantly protect against RTIs [32].

We did not find evidence for protection against pneumococcal colonisation in young children by any of our anti-pneumococcal antibodies (except for IgG levels against PspC), which is in line with experimental studies conducted in mice [33].

However, colonisation does induce a humoral immune response against several pneumococcal proteins, some of which are also associated with a lack of doctor visits for RTIs. This suggests a potential protective role of colonisation against RTIs in the long run. Pneumococcal colonisation may increase the risk of clinical or subclinical pneumococcal infection, inducing an immune response which protects against RTIs in the long run.

Some studies document that maternal anti-polysaccharide antibodies prevent colonization and infection in infants and thus propose active immunization of pregnant women [(34-35)]. The largest effect of such maternal antibodies would be expected to occur at young age. We did not find any short term effects of protection by maternal anti-pneumococcal virulence protein antibodies on both nasopharyngeal colonization and RTI's in children. Although it is known that maternal IgG can cross epithelial barriers and can reach significant levels at the nasopharyngeal mucosal surface, these specific antibodies are not capable of preventing colonization and infection in youngsters as was previously described for anti-polysaccharide antibodies. It has been shown that pneumococcal elimination by vaccines may lead to elevated colonisation levels for other bacterial species. For example, it was demonstrated that prevention of pneumococcal otitis media was counterbalanced by increasing numbers of cases caused by *Staphylococcus aureus* [36]. It is unlikely that anti-protein vaccines against the pneumococcus will be different in this respect. However, in our study we do present anti-pneumococcal antibodies associated with a decreased RTIs rate but not associated with

protection against colonisation. If colonisation with *S. pneumoniae* persisted, while RTIs are prevented, other species may remain outside.
Some limitations of our study should be discussed. RTIs of the children were obtained through parentally reported questionnaires, which may represent an over report of complaints; parents with high concerns may report more infections contrary to a doctor's diagnosis. Furthermore, we do not have specific information on the exact timing of RTIs in the children and as described in the methods section a misclassification may have occurred due to grouping the children by number of RTIs. However, if this misclassification has occurred this will most likely lead to an underestimation of the effect. We studied antibodies in serum. A suggestion for future studies would be to study IgA levels in saliva, which may play an important role in colonisation as well. Moreover, we can not distinguish whether serum antibodies protect directly or indirectly as part of a more extensive immune response in which protection can be generated by other immunological factors as well. Alternatively, there may also be diffusion to mucosal surface or the antibodies may be produced locally following colonisation. In other words, the anti-pneumococcal antibodies may protect directly, or they may be markers of alternative responses, or they may result from a combination of these possibilities. In addition, it is possible that the findings presented here simply reflect immune system maturation. Those children with a more mature immune system may have high antibody levels and may remain healthier. Unfortunately at the basis of the current study we can not decide for one or the other scenario. Finally, our study was conducted in 57 children. The results should be confirmed in a larger sample size.

In conclusion, levels of antibodies against diverse pneumococcal virulence proteins are correlated with a reduced frequency of doctor visits for RTIs in children. This was not due to protection of the specific antibodies against asymptomatic colonisation, antibodies rather resulted from colonisation. This study adds to the discussion on improvement of the current preventive strategies against pneumococcal disease. Future studies should put effort in developing protein-based vaccines. In particular the effect of combination of several pneumococcal proteins and their correlate of protection in humans should be studied.

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Chapter 3.2

Induction of antibodies by *Staphylococcus aureus* nasal colonisation in young children

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ABSTRACT

In order to develop novel anti-staphylococcal strategies, understanding the determinants of carriage and how humans respond to *Staphylococcus aureus* exposure is essential. Here, the primary *S. aureus*-specific humoral immune response and its association with nasal colonisation was studied in young children.

Sera from 57 colonised or non-colonised children, serially collected at birth and at 6, 14 and 24 months, were analysed for IgG, IgA and IgM binding to 19 staphylococcal proteins using a flow cytometry-based technology.

The antibody responses showed extensive inter-individual variability. On average, the levels of anti-staphylococcal IgA and IgM increased from birth until the age of 2 years (P-value <0.05), whereas the levels of IgG decreased (P-value <0.001). Placentally transferred maternal IgG did not protect against nasal colonisation. In colonised children, IgG and IgA levels for a number of proteins were higher than in non-colonised children. At both 14 and 24 months, the levels of IgG against chemotaxis inhibitory protein of *S. aureus* (CHIPS; at 24 months, median fluorescence intensity 4928 vs. 24, *P*<0.05), extracellular fibrinogen-binding protein (Efb; 987 vs. 604, P-value <0.05) and iron-responsive surface determinant H (IsdH; 62 vs. 5, P-value <0.05) were significantly higher in colonised children. The levels of IgA against CHIPS, IsdA and IsdH were higher (P-value <0.05). Therefore, CHIPS, Efb, IsdA and IsdH seem to play a role in nasal colonisation of young children.

INTRODUCTION

Staphylococcus aureus efficiently colonises human skin and, most frequently, nasal mucosa [1]. Approximately 20-30% of adults carry *S. aureus* persistently and ~70-80% of adults carry *S. aureus* never or intermittently [2-3]. *S. aureus* is carried by 10-35% of children [4]. During the first 2 months of life, the prevalence of colonisation is 40-50%. Then, the prevalence rapidly decreases to ~20% by 6 months and to ~10% by 14 months [5-6]. How nasal carriage is established and maintained is still largely unknown [2, 7], although the involvement of bacterial components such as teichoic acid, catalase, hydroperoxide reductase, iron-responsive surface determinant (Isd) A, *S. aureus* surface protein G (SasG) and clumping factor (Clf) B has been demonstrated [8-13].

Carriage of *S. aureus* can result in serious endogenous infections. Because of the increasing antibiotic-resistance of *S. aureus*, novel approaches concerning the prevention and therapy of staphylococcal disease are urgently needed. In order to develop such new strategies, understanding the determinants of carriage and understanding how humans respond to *S. aureus* exposure is essential. Here, we provide insights into the anti-staphylococcal humoral immune response in young children. Studying their immune response will allow us to distinguish the bacterial factors that are expressed *in vivo* during early colonisation. This may lead to the discovery of novel determinants of colonisation.

METHODS

Study design and population

This project was performed with a subgroup of the Generation R Study, a population-based prospective cohort study of pregnant women and their children from foetal life onwards [14-15]. The infants were presented at the Generation R research center at the ages of 1.5 months, 6 months, 14 months and 24 months.

Staphylococcus aureus

Research nurses obtained a nasal swab for *S. aureus* isolation from each infant at each visit whenever possible. The methods of nasal sampling and identification of *S. aureus* were as described previously [5]. Serum samples were collected from cord blood and through venapuncture at 6 months, 14 months and 24 months whenever possible. Included in this study were 57 healthy children, from each of whom 3 or 4 serial serum samples were collected. Of the 177 samples that were obtained, 54 (31%) were cord blood samples, 32 samples (18%) were obtained at 6 months, 46 (26%) at 14 months and 45 (25%) at 24 months. The Medical Ethics Committee of the Erasmus Medical Center Rotterdam approved the study. Written informed consent was obtained from the parents of all participating children.

S. aureus colonisation data were available at 1.5, 6, 14 and 24 months for 40 (70%), 49 (86%), 50 (88%) and 48 (84%) children, respectively. Children were classified as colonised if at least one of the nasal swab cultures was positive for *S. aureus*. Children were classified as non-colonised if all swab cultures were negative. Children with a culture moment missing at one time-point, and with the other nasal swab cultures negative, were classified as non-colonised as well. None of the children suffered from apparent staphylococcal infection. The levels of anti-staphylococcal antibodies directed against 3 important groups of *S. aureus* proteins, 'microbial surface components recognizing adhesive matrix molecules' (MSCRAMMs), staphylococcal enterotoxins (SEs) and immune-modulating proteins, were determined. The proteins have been described previously [16]. MSCRAMMs are generally considered to be important for host colonisation [12, 17]. The recombinant MSCRAMMs ClfA, ClfB, SasG, IsdA, IsdH, fibronectin-binding protein (Fnbp) A and B and serine-aspartate repeat protein (Sdr) D and E were used. SEs are superantigens and, therefore, potent pro-inflammatory agents [18]. The recombinant proteins SEA, SEB, SEI, SEM, SEO, SEQ and toxic shock syndrome toxin (TSST)-1 were used. In addition, the immune-modulating proteins staphylococcal complement inhibitor (SCIN), extracellular fibrinogen-binding protein (Efb) and chemotaxis inhibitory protein of *S. aureus* (CHIPS) were used. Efb and SCIN are complement inhibitors that lead to a reduction of bacterial phagocytosis and killing by human neutrophils [19-20]. CHIPS impairs the response of neutrophils and monocytes to formylated peptides and complement factor C5a [21].

The levels of antigen-specific IgG, IgA and IgM were quantified using a bead-based flow cytometry technique (xMAP®, Luminex Corporation). The methods used were as described previously [16, 22-23]. Tests were performed as independent duplicates and the median fluorescence intensity (MFI) values, reflecting antibody levels semi-quantitatively, were averaged. In each experiment, control beads (no protein coupled) were included to determine non-specific antibody binding. In cases of non-specific binding, these non-specific MFI values were subtracted from the antigen-specific values. Human pooled serum was used as a standard.

Statistical methods

Statistical analyses were performed with SPSS version 15.0. The Wilcoxon signed rank test was used to compare the anti-staphylococcal antibody levels between different age groups. Mann-Whitney *U* tests were used to compare differences in antibody levels between colonised and non-colonised children. Binary logistic regression analysis was used to determine the relationship between maternal IgG levels and the dichotomous outcome colonisation. A *P*-value ≤0.05 was considered statistically significant.

RESULTS

Dynamics of the anti-staphylococcal antibody response

The changes in anti-staphylococcal IgG, IgA and IgM levels during the first 2 years of life were determined (Figure 1, data shown for TSST-1, ClfB and Efb). The levels of antigen-specific IgG, IgA and IgM showed extensive inter-individual variability over time. For all *S. aureus* proteins tested, the level of antigen-specific IgG in cord blood was significantly higher than the anti-staphylococcal IgG level at 6 months ($P<0.001$). This was due to the presence of maternal IgG at birth and catabolism of maternal IgG thereafter. In the time interval from 6 to 14 months, the levels of IgG directed to CHIPS, SCIN and SEB decreased further ($P<0.05$). As for the other proteins, no significant changes in the IgG levels were noted in this period.

Anti-staphylococcal IgA and IgM levels in cord blood were low because maternal IgA and IgM are not transported across the placenta. In the first 2 years of life, IgA levels remained low, which is a well-known fact [24-25]. However, for both IgA and IgM a significant increase from birth up to the age of 24 months was noted, for 18 of 19 *S. aureus* proteins in the case of IgA ($P<0.05$, with the exception of anti-SCIN IgA) and for all proteins in the case of IgM ($P<0.01$). It must be emphasized that not every infant developed an antigen-specific IgA or IgM response to each protein in the first 2 years of life. Within one individual, the level of IgG, IgA or IgM directed against one protein was not correlated with the level of IgG, IgA or IgM directed against another protein.

Relationship between colonisation and anti-staphylococcal antibody levels

It was determined whether maternal anti-staphylococcal IgG levels were predictive of the *S. aureus* colonisation state of the infant and whether the colonisation state determines the level of anti-staphylococcal antibodies. Levels of *S. aureus*-specific IgG in cord blood were not predictive of the colonisation state at 1.5 and 6 months (P-value >0.05). This implies that the large amounts of placentally transferred maternal IgG do not protect children from becoming nasally colonised with *S. aureus*.

For 45 of 46 (98%) children from whom serum samples were obtained at 14 months, the colonisation status was known. For 1 child, the colonisation status could not be determined, because 2 nasal swab cultures were missing. In the first year of life, 24 (53%) children were colonised at least once and 21 children (47%) were not colonised. Colonised children had significantly higher levels of IgG directed against CHIPS, Efb, ClfB, SdrD and IsdH than non-colonised children (P-value <0.05; Figure 2). In addition, their levels of IgA directed against CHIPS, IsdA and IsdH were higher (P-value <0.05). Incidentally, high levels of antibodies were detectable in non-colonised children, probably owing to exposure to *S. aureus* that was not recorded during this study. For 42 of 45 (93%) children from whom serum samples were obtained at 24 months, the colonisation status was known. In the first 2 years of life, 24 (57%) children were colonised at least once and 18 (43%) were not colonised. Colonised children

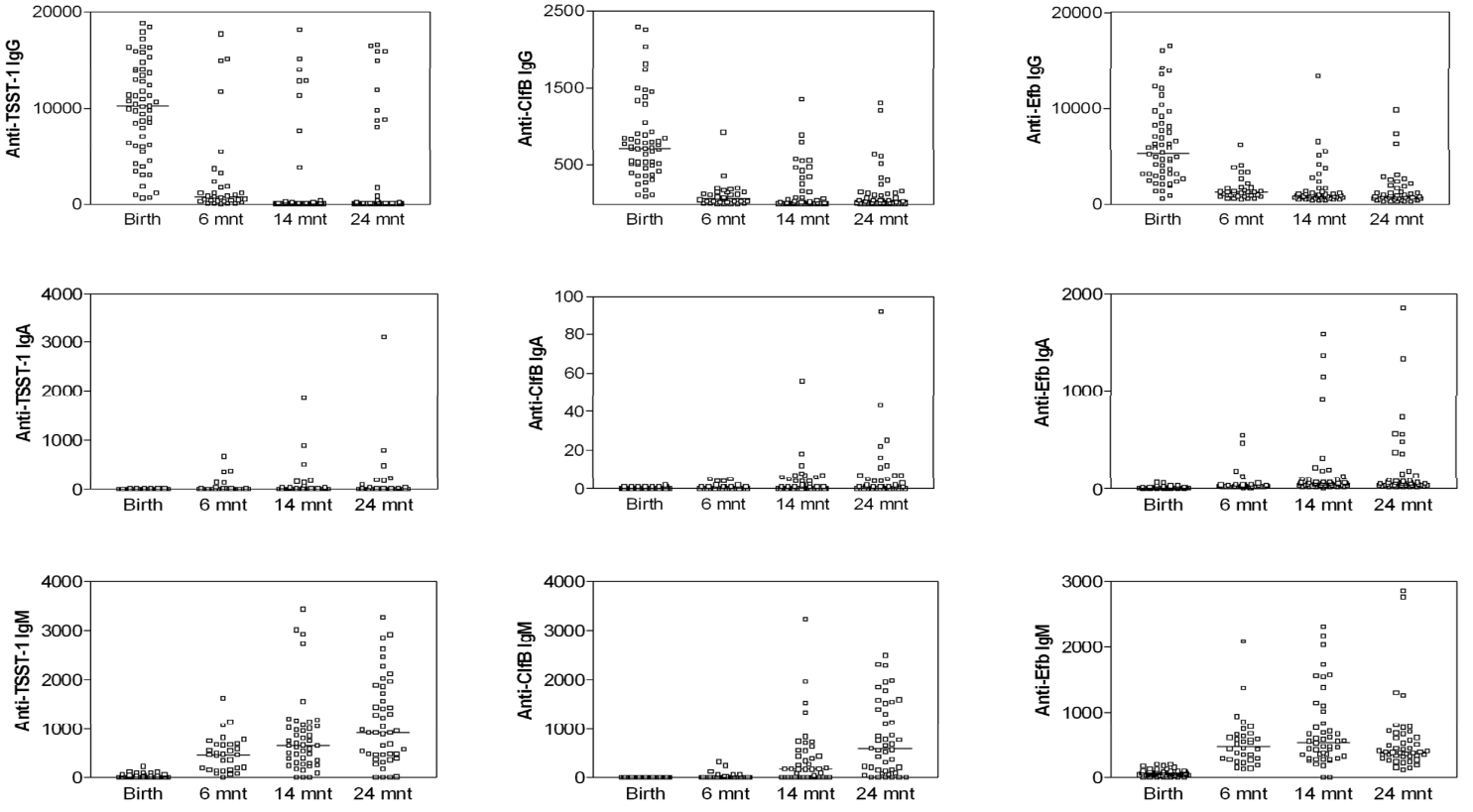

Figure 1 Specific staphylococcal antibodies in infancy.

IgG, IgA and IgM levels directed to toxic shock syndrome toxin-1 (TSST-1), Clumping factor B (ClfB) and Extracellular fibrinogen-binding protein (Efb) in 57 children at birth, 6 months, 14 months and 24 months. Antibody levels are reflected by Median fluorescence intensity (MFI) values. Each dot represents a serum sample. Median values are indicated by horizontal lines.

had higher levels of IgG directed against CHIPS, SCIN, Efb, IsdA, IsdH and SEB at 24 months than non-colonised children (P-value <0.05; Figure 2). Their levels of IgA directed against CHIPS, IsdA and IsdH at 24 months were higher as well (P-value <0.05). The level of IgM did not differ significantly between colonised and non-colonised children (P-value >0.05).

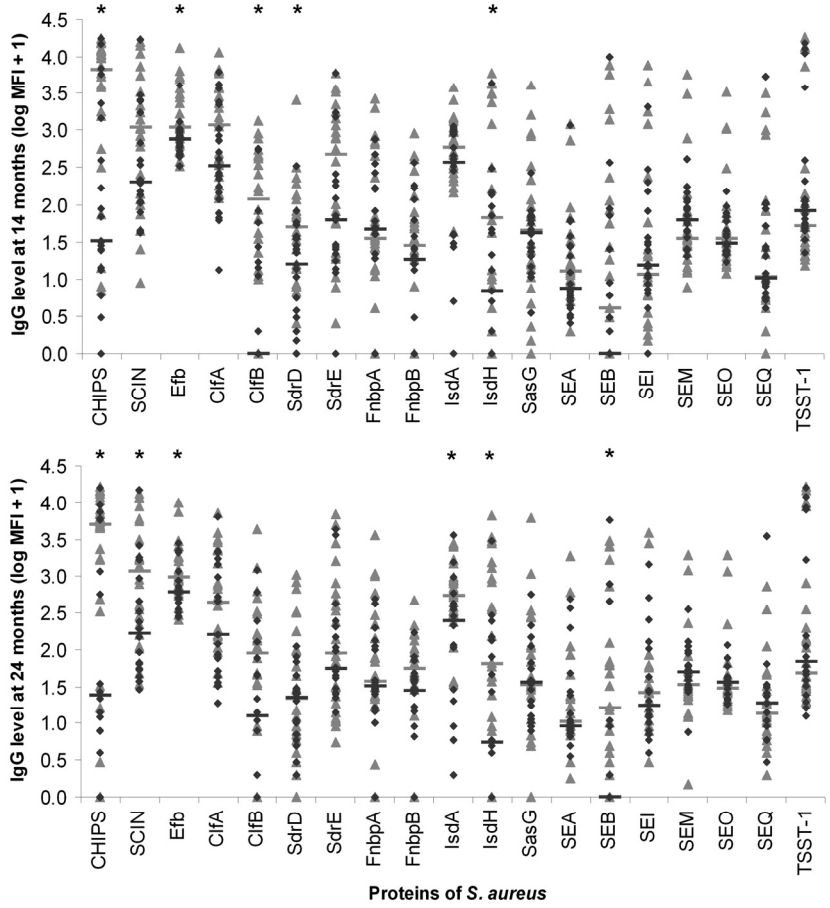

Figure 2:

A Relation between *S. aureus* colonisation in the first year of life and level of anti-staphylococcal IgG at 14 months. This is reflected by Median fluorescence intensity (MFI) value, at 14 months. Each symbol represents a single child. Triangles represent colonised children and diamonts represent non-colonised children. Median values are indicated by horizontal lines.

B Relation between *S. aureus* colonisation in the first two years of life and the level of IgG at 24 months.

Abbreviations: CHIPS, Chemotaxis Inhibitory Protein of *S. aureus*; SCIN, Staphylococcal Complement Inhibitor; Efb, Extracellular fibrinogen-binding protein; Clf, Clumping factor; Sdr, Serine-aspartate dipeptide repeat protein; Fnbp, Fibronectin-binding protein; Isd, Iron-responsive surface determinant; SasG, *S. aureus* surface protein G; SE, staphylococcal enterotoxin; TSST-1, toxic shock syndrome toxin.

DISCUSSION

Understanding the determinants of carriage and how humans respond to *S. aureus* exposure is important for the development of novel anti-staphylococcal measures. We show that, despite extensive inter-individual variability, the levels of IgG and IgA directed against a number of *S. aureus* proteins were significantly higher in colonised, more exposed children than in non-colonised children. In both the first and second year of life anti-CHIPS, anti-Efb and anti-IsdH IgG levels were higher in colonised children. Furthermore, anti-CHIPS, anti-IsdA and anti-IsdH IgA levels were higher. This indicates that these proteins are expressed *in vivo* and that they might be determinants for colonisation in early childhood. A potential role of IsdA in colonisation was demonstrated in a previous study [10].

Furthermore, we show that maternally derived IgG antibodies specifically directed against a series of staphylococcal antigens do not seem to protect the young infant against *S. aureus* nasal colonisation in the first months of life. Thus, although it is known that maternal IgG can cross epithelial barriers and can reach significant levels at the nasal mucosal surface [26-27], these antibodies are not capable of preventing nasal colonisation. In healthy adults, the considerable levels of anti-staphylococcal antibodies that are found do not seem to protect against nasal colonisation either. Carriers even have higher levels of antibodies than non-carriers [16]. These observations suggest that attempts to prevent mucosal colonisation by *S. aureus* through passive immunization approaches are not likely to succeed. Whether this also applies to active immunization remains to be elucidated.

In summary, in healthy children, the antistaphylococcal IgG, IgA and IgM levels show extensive inter-individual variability. On average, the levels of antistaphylococcal IgA and IgM increase from birth until the age of 2 years, whereas the levels of antistaphylococcal (maternal) IgG decrease. Placentally transferred maternal IgG antibodies do not protect against nasal colonisation. CHIPS, Efb, IsdA and IsdH are expressed *in vivo*, and therefore, seem to play a role in nasal colonisation of young children.

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Chapter 3.3

The inverse correlation between *Staphylococcus aureus* and *Streptococcus pneumoniae* colonisation in infants is not explained by differences in serum antibody levels

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For this thesis we added two extra tables (table 2 and 3)

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ABSTRACT

Colonisation rates of S*treptococcus pneumoniae* and *Staphylococcus aureus* are inversely correlated in infants. Several studies have searched for determinants of this negative association. In healthy children, from the pre-pneumococcal vaccine-era, we studied the association between anti-pneumococcal antibodies with *Staphylococcus aureus* colonisation and the association between anti-staphylococcal antibodies with pneumococcal colonisation. In the first year of life, no association between maternal IgG levels and colonisation was seen. In addition, no association between the levels of IgG- and IgA levels from the child versus colonisation status was seen.

INTRODUCTION

Colonisation rates of S*treptococcus pneumoniae* (pneumococcus) and *Staphylococcus aureus* in the first year of life show a mirror image trend [1-4]. *S. aureus* nasal colonisation is very common among newborns and but this colonisation rate decreases rapidly during the first year, while pneumococcal colonisation rates are low at birth and increase significantly in the first year of life [1-2]. Both pathogens are common inhabitants of the upper airways and frequently cause infections in humans; *S. pneumoniae* occupies the nasopharyngeal region of young children, while *S. aureus* primarily nestles in the anterior nares. The pneumococcus is most common in children and is essentially absent in adults, which is contrary to the situation for *S. aureus* that is found in the nares of half of the adult population [5]. Frequent colonisation with these commensal pathogens is associated with bacterial spread at the population level and an increased risk of auto-infection including respiratory tract infections and atopic dermatitis [6-9]. In two studies dating from the pre-pneumococcal vaccine-era, pneumococcal colonisation with vaccine-type strains was negatively associated with *S. aureus* colonisation, suggesting interference between the two pathogens [3-4]. Following the implementation of the pneumococcus conjugate vaccine, a shift has occurred not only towards non-vaccine *S. pneumoniae* serotypes, but also toward higher *S. aureus* carriage rates in children [10-11]. Several studies have looked for determinants of this negative association. Regev-Yochay et al. found hydrogen peroxide produced by the pneumococcus to have bactericidal activity towards *S. aureus* [12]. A more recent study from the same group reports on the importance of the presence of the pneumococcal pilus which decreases the odds of co-colonisation [13]. The negative association was found to be independent on bacterial genotype; no specific *S. aureus* genotypes were found to be correlated to certain *S. pneumoniae* genotypes [14]. The aim of our study was to assess the effect of the humoral immune response on the negative association between *S. pneumoniae* and *S. aureus* in longitudinally followed healthy Dutch children from the pre-pneumococcal vaccine-era.

METHODS

Study design and population

This study was embedded in the Generation R study, a population-based prospective cohort study following pregnant women and their children. Further details on this cohort study were described previously [15]. The Medical Ethics Committee of the Erasmus Medical Centre, Rotterdam, has approved the study and written informed consent was obtained. A cord blood sample was obtained and infant blood samples were obtained during the visits at the research centre at the ages of 6 and 14 months. Of the 1,079 infants in the postnatal cohort, the so-called Generation R Focus Cohort, 57 were selected for this particular study on the basis of availability of

biological samples. All of these children were born between February 2003 and August 2005, prior to introduction of the pneumococcal vaccination in The Netherlands in 2006.

Staphylococcus aureus and Streptococcus pneumoniae

The following seventeen pneumococcal protein-antigens were selected: PspC (CbpA), choline-binding protein A; Eno, enolase; Hyl, Hyaluronidase; IgA1-protease, immunoglobulin A1 protease; NanA, neuraminidase; PLY, pneumolysin; PdBD, a double mutant of pneumolysin; PmpA, putative proteinase maturation protein A; PsaA, pneumococcal surface adhesin A; PspA, pneumococcal surface protein A; the pneumococcal histidine triad (Pht) family (BVH-3 and SP1003); SlrA, streptococcal lipoprotein rotamase; SP, *Streptococcus pneumoniae* proteins; SP0189 (hypothetical protein), SP0376 (response regulator, intracellular location), SP1633 (response regulator, intracellular location) and SP1651 (thiol peroxidase, intracellular location) and the following nineteen staphylococcal proteins: CHIPS, chemotaxis inhibitory protein of *S. aureus*; Clf, clumping factor; Efb, extracellular fibrinogen-binding protein; Fnbp, fibronectin-binding protein; Isd, iron-responsive surface determinant; Sas, *S. aureus* surface protein; SCIN, staphylococcal complement inhibitor; Sdr, serine-aspartate repeat protein; SE, staphylococcal enterotoxin, TSST, toxic shock syndrome toxin. IgG and IgA levels against these proteins were measured using the bead-based flow cytometry technique (xMAP, Luminex Corporation, Austin, Texas, USA) as described previously [16-19]. Tests were performed in independent duplicates and the median fluorescence intensity (MFI) values, reflecting semi-quantitative antibody levels, were averaged. In each experiment, control beads (no protein coupled) were included to determine non-specific binding. In case of non-specific binding, these non-specific MFI values were subtracted from the antigen-specific results. Human pooled serum (HPS) was used as an internal standard. During the visits at the ages of 1.5, 6 and 14 months nasopharyngeal and nasal swabs for isolation of *S. pneumoniae* and *S. aureus* were obtained. Methods of sampling were as described previously [1-2].

Statistical methods

First, we conducted Mann Whitney U tests to assess differences in the levels of antibodies between colonised and non-colonised children at different ages. The association between the levels of maternal IgG antibodies as a continuous variable and the dichotomous outcome of bacterial colonisation at 1.5 and 6 months and colonisation frequency (0-1 versus 2-3 positive swabs) was assessed by binary logistic regression analysis to assess the risk of colonisation following a certain antibody level. These same tests were used to assess the association between the level of IgG and IgA antibodies in the child at 6 and 14 months and colonisation status at 6 and 14 months. A p-value of <0.025 (p-value 0.05 divided by the two pathogens that are tested) was used to adjust for multiple testing and considered statistically significant. The statistical analyses were performed using the Statistical Package for the Social Sciences version 17.0 for Windows (SPSS Inc, Chicago, IL, USA).

RESULTS

Figure 1 shows the mirror image-like graphs of *S. aureus* and pneumococcal colonisation in childhood that forms the basis of our study. Maternal IgG directed against the nineteen staphylococcal proteins did not protect against nor increase the risk of pneumococcal colonisation in the first 6 months of life. Similarly, the seventeen anti-pneumococcal maternal IgG antibodies were not significantly associated with *S. aureus* colonisation in the first 6 months of life. Additionally, there was no effect of maternal IgG levels on the frequency of colonisation in the first year of life (table 1).

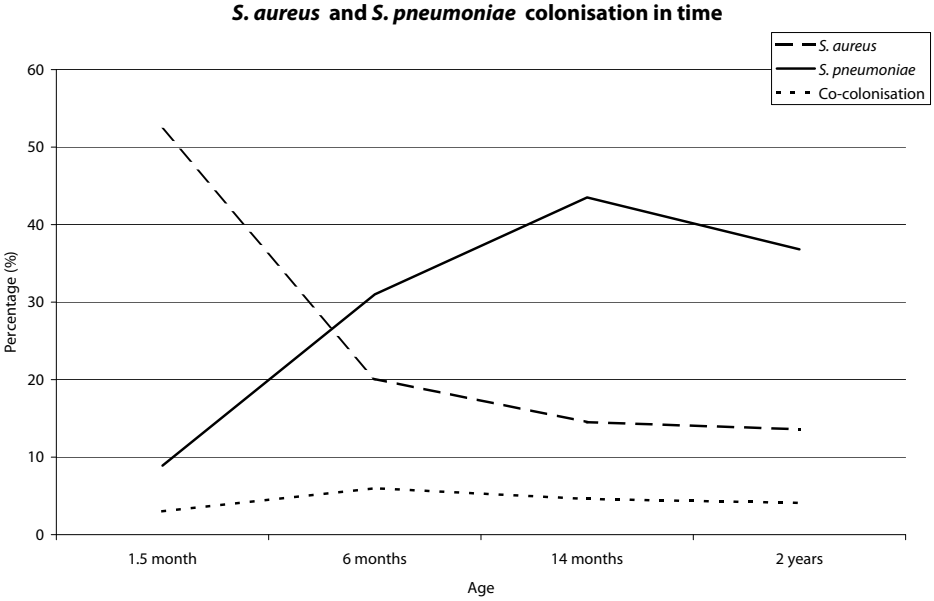

Figure 1. *S. aureus* and *S. pneumoniae* colonisation rates in childhood. Mirror image graphs of pneumococcal and *S. aureus* colonisation rates in 1,079 infants from the Generation R Focus Cohort. *S. pneumoniae* colonisation rates increase in the first year of life from 8.9% to 43.5% of the children and decrease again after the first year of life to 38.8%, while *S. aureus* colonisation rates decrease in the first year of life from 52.3% to 14.5% and slightly decreases the year after to 13.6%. Co-colonisation with the two pathogens exists, however the prevalence is very low (~5%) and stable over time.

We did not study the effect of IgA levels in cord blood on later colonisation since IgA does not get transported across the placenta. Hence, low levels of IgA were measured in cord blood. We were not able to detect an association between serum levels of IgG at 6 months and colonisation status at 6 and 14 months (data not shown). To avoid a mixture of maternal antibodies with antibodies produced by the child him- or herself we explicitly studied levels of IgG at 14 months to assess the effect of antibodies produced by the child on colonisation.

TABLE 1. Maternal antibodies and infant colonisation

Maternal pneumococcal antibodies	*S. aureus* colonisation		
	1.5 month	6 months	≥ twice in the first year
BVH-3	0.91 (0.74 – 1.11)	0.89 (0.69 – 1.15)	0.89 (0.70 – 1.13)
PspC	1.02 (0.86 – 1.21)	0.95 (0.78 – 1.16)	0.92 (0.76 – 1.12)
PdBD	0.82 (0.59 – 1.13)	0.78 (0.55 – 1.11)	0.63 (0.41 – 0.98) *
Eno	0.79 (0.50 – 1.25)	0.77 (0.28 – 2.10)	0.03 (0.00 – 9.10)
Hyl	0.94 (0.75 – 1.19)	0.93 (0.72 – 1.21)	0.74 (0.51 – 1.08)
IgA-1 protease	1.00 (0.75 – 1.31)	1.00 (0.73 – 1.38)	0.97 (0.70 – 1.35)
NanA ¶	0.85 (0.70 – 1.03)	0.94 (0.77 – 1.15)	0.92 (0.74 – 1.14)
PLY	0.94 (0.76 – 1.15)	0.95 (0.78 – 1.17)	0.94 (0.75 – 1.18)
PpmA	0.95 (0.78 – 1.15)	0.81 (0.61 – 1.09)	0.87 (0.66 – 1.15)
PsaA	0.94 (0.82 – 1.09)	1.00 (0.85 – 1.18)	0.89 (0.73 – 1.07)
PspA	1.01 (0.86 – 1.18)	0.77 (0.59 – 1.00) *	0.80 (0.62 – 1.02)
SlrA	1.42 (0.63 – 3.23)	0.81 (0.25 – 2.62)	1.72 (0.60 – 4.98)
SP0189	1.80 (0.26 – 12.40)	-	-
SP0376 ¶	0.78 (0.46 – 1.32)	0.96 (0.53 – 1.72)	1.01 (0.59 – 1.72)
SP1003	1.02 (0.80 – 1.31)	0.75 (0.54 – 1.04)	0.90 (0.68 – 1.18)
SP1633 ¶	1.14 (0.77 – 1.68)	1.04 (0.82 – 1.32)	1.01 (0.78 – 1.30)
SP1651 ¶	1.08 (0.84 – 1.38)	0.71 (0.33 – 1.55)	0.52 (0.16 – 1.68)
Maternal staphylococcal antibodies	*S. pneumoniae* colonisation		
	1.5 month	6 months	≥ twice in the first year
ClfA	0.84 (0.55 – 1.29)	0.99 (0.77 – 1.27)	1.01 (0.75 – 1.38)
ClfB ¶	0.99 (0.85 – 1.16)	0.95 (0.84 – 1.08)	0.99 (0.85 – 1.16)
SasG ¶	0.99 (0.92 – 1.07)	1.03 (0.98 – 1.07)	0.99 (0.92 – 1.06)
IsdA	0.42 (0.13 – 1.39)	1.14 (0.81 – 1.60)	1.04 (0.66 – 1.64)
IsdH	1.00 (0.62 – 1.62)	1.09 (0.79 – 1.51)	1.18 (0.79 – 1.77)
FnbpA	1.05 (0.48 – 2.30)	0.96 (0.54 – 1.69)	0.67 (0.30 – 1.50)
FnbpB ¶	0.94 (0.70 – 1.26)	1.11 (0.96 – 1.29)	1.03 (0.94 – 1.14)
SdrD ¶	0.89 (0.61 – 1.31)	0.99 (0.89 – 1.09)	0.66 (0.34 – 1.31)
SdrE	0.49 (0.17 – 1.40	0.49 (0.24 – 1.00)	0.42 (0.14 – 1.20)
SEA ¶	0.99 (0.78 – 1.26)	1.01 (0.86 – 1.19)	1.17 (0.99 – 1.39)
SEB	0.88 (0.70 – 1.12)	0.92 (0.77 – 1.10)	0.85 (0.66 – 1.10)
SEI	0.95 (0.54 – 1.76)	1.07 (0.70 – 1.65)	0.93 (0.49 - 1.76)
SEM ¶	0.97 (0.86 – 1.10)	1.01 (0.95 – 1.07)	0.97 (0.87 – 1.09)
SEO ¶	1.09 (0.83 – 1.45)	0.75 (0.51 – 1.11)	0.70 (0.36 – 1.33)
SEQ ¶	0.98 (0.89 – 1.08)	1.02 (0.99 – 1.05)	1.03 (0.99 – 1.07)
TSST-1	1.08 (0.90 – 1.31)	0.95 (0.84 – 1.08)	1.02 (0.87 – 1.19)
SCIN	0.92 (0.72 – 1.18)	0.93 (0.80 – 1.08)	0.89 (0.75 – 1.06)
Efb	0.93 (0.72 – 1.21)	0.78 (0.61 – 0.99) *	0.86 (0.66 – 1.11)
CHIPS	1.04 (0.79 – 1.36)	0.84 (0.69 – 1.02)	0.96 (0.77 – 1.21)

Using binary logistic regression analyses differences in colonisation prevalence were assessed per MFI unit. MFI values are divided by 1000 except for the ones marked with a ¶, they are divided by 100. * = p-value 0.05, ** = p-value < 0.025, which we consider statistically significant. Swabs are missing at 1.5 month (*n*=17), 6 months (*n*=8) and 14 months (*n*=7).

TABLE 2. Anti-staphylococcal antibodies and *S. pneumoniae* colonisation in infants

		S. pneumoniae colonisation	
Infant staphylococcal antibodies		6 months	14 months
ClfA	6 months	1.01 (0.71 – 1.44)	0.82 (0.54 – 1.25)
	14 months	N/A	0.43 (0.19 – 0.95) *
ClfB ¶	6 months	0.33 (0.07 – 1.59)	0.68 (0.41 – 1.13)
	14 months	N/A	0.64 (0.39 – 1.05)
SasG ¶	6 months	0.31 (0.06 – 1.75)	0.65 (0.24 – 1.75)
	14 months	N/A	0.94 (0.78 – 1.12)
IsdA	6 months	1.07 (0.46 – 2.52)	1.22 (0.51 – 2.96)
	14 months	N/A	0.63 (0.21 – 1.84)
IsdH	6 months	1.87 (0.68 – 5.19)	0.72 (0.37 – 1.41)
	14 months	N/A	0.53 (0.23 – 1.18)
FnbpA	6 months	17805.28 (0.01 - 3.71 10^10)	0.37 (0.04 – 3.25)
	14 months	N/A	0.66 (0.16 – 2.75)
FnbpB ¶	6 months	1.81 (0.16 – 20.87)	0.71 (0.33 – 1.53)
	14 months	N/A	0.48 (0.18 – 1.33)
SdrD ¶	6 months	0.91 (0.22 – 3.86)	0.36 (0.07 – 1.78)
	14 months	N/A	0.35 (0.09 – 1.41)
SdrE	6 months	0.24 (0.01 – 4.94)	0.07 (0.00 – 1.91)
	14 months	N/A	0.49 (0.19 – 1.26)
SEA ¶	6 months	0.84 (0.46 – 1.52)	0.05 (0.00 – 8.07)
	14 months	N/A	0.09 (0.00 – 6.15)
SEB	6 months	1.14 (0.54 – 2.34)	1.09 (0.72 – 1.66)
	14 months	N/A	0.96 (0.70 – 1.32)
SEI	6 months	0.48 (0.05 – 5.01)	0.02 (0.00 – 2259.67)
	14 months	N/A	0.14 (0.00 – 18.69)
SEM ¶	6 months	1.11 (0.68 – 1.83)	0.60 (0.14 – 2.64)
	14 months	N/A	0.87 (0.49 – 1.52)
SEO ¶	6 months	0.00 (0.00 – 16.35)	0.72 (0.23 – 2.28)
	14 months	N/A	0.72 (0.32 – 1.61)
SEQ ¶	6 months	0.96 (0.77 – 1.21)	0.76 (0.36 – 1.64)
	14 months	N/A	0.95 (0.81 – 1.11)
TSST-1	6 months	0.96 (0.80 – 1.15)	1.08 (0.95 – 1.23)
	14 months	N/A	0.95 (0.82 – 1.10)
SCIN	6 months	1.08 (0.89 – 1.31)	0.99 (0.86 – 1.15)
	14 months	N/A	0.90 (0.76 – 1.07)
Efb	6 months	1.03 (0.45 – 2.40)	0.87 (0.58 – 1.32)
	14 months	N/A	0.56 (0.26 – 1.19)
CHIPS	6 months	1.10 (0.93 – 1.30)	0.98 (0.87 – 1.11)
	14 months	N/A	0.90 (0.79 – 1.02)

All MFI values are divided by 1000 except for the ones marked with a ¶, they are divided by 100. * = p-value 0.05
** = p-value < 0.0125, which we consider statistically significant.

No significant association was seen for anti-staphylococcal antibodies at 14 months with pneumococcal colonisation at 14 months and anti-pneumococcal antibodies with *S. aureus* colonisation at the same age (data not shown).

Moreover, no significant association was observed for differences in IgA levels at 6 and 14 months for both anti-staphylococcal and anti-pneumococcal antibodies on subsequent colonisation. In addition, we analysed the correlation between *S. aureus* and pneumococcal colonisation in this particular study population. We did not observe a significant correlation between the two pathogens at 1.5, 6 and 14 months. However, the odds ratios at 1.5 and 14 months are directed to an inverse correlation (OR 0.26 95% CI 0.04 -1.55 and OR 0.18 95% CI 0.02 – 1.55, respectively), in contrast to the correlation at 6 months (OR 1.64 95% CI 0.33 – 8.02).

TABLE 3. Anti-pneumococcal antibodies and *S. aureus* colonisation in infants

		S. aureus colonisation	
Infant pneumococcal antibodies		6 months	14 months
BVH-3	6 months	0.03 (0.00 – 53.05)	1.77 (0.35 – 8.86)
	14 months	N/A	0.88 (0.57 – 1.36)
PspC	6 months	0.57 (0.20 – 1.61)	1.16 (0.88 – 1.51)
	14 months	N/A	1.02 (0.89 – 1.19)
PdBD	6 months	0.82 (0.27 – 2.54)	1.04 (0.74 – 1.46)
	14 months	N/A	1.05 (0.81 – 1.36)
Eno	6 months	0.72 (0.07 – 7.23)	0.09 (0.00 – 151.15)
	14 months	N/A	0.09 (0.00 – 117.08)
Hyl	6 months	0.06 (0.00 – 11.41)	0.93 (0.66 – 1.30)
	14 months	N/A	1.05 (0.86 – 1.29)
IgA-1 protease	6 months	-	0.05 (0.00 – 39.38)
	14 months	N/A	0.58 (0.08 – 4.12)
NanA ¶	6 months	0.68 (0.15 – 3.04)	0.83 (0.44 – 1.47)
	14 months	N/A	1.04 (0.92 – 1.18)
PLY	6 months	1.37 (0.41 – 4.54)	1.31 (0.64 – 2.70)
	14 months	N/A	1.09 (0.65 – 1.84)
PpmA	6 months	0.00 (0.00 – 16359. 82)	1.31 (0.48 – 3.59)
	14 months	N/A	0.95 (0.63 – 1.41)
PsaA	6 months	0.93 (0.66 – 1.32)	1.01 (0.84 – 1.21)
	14 months	N/A	1.01 (0.83 – 1.23)
PspA	6 months	0.16 (0.00 – 18.07)	0.72 (0.25 – 2.03)
	14 months	N/A	1.32 (0.85 – 2.04)
SlrA	6 months	0.00 (0.00 – 1.33 10^10)	0.26 (0.00 – 37.48)
	14 months	N/A	0.99 (0.23 – 4.28)
SP0189	6 months	0.00 (0.00 – 2.61 10^11)	0.08 (0.00 – 192820.15)
	14 months	N/A	0.41 (0.01 – 28.20)
SP0376 ¶	6 months	0.81 (0.29 – 2.28)	1.49 (0.86 – 2.59)
	14 months	N/A	1.16 (0.79 – 1.69)
SP1003	6 months	0.46 (0.08 – 2.62)	1.02 (0.79 – 1.30)
	14 months	N/A	1.05 (0.89 – 1.24)
SP1633 ¶	6 months	0.81 (0.29 – 2.27)	1.21 (0.83 – 1.78)
	14 months	N/A	0.96 (0.60 – 1.55)
SP1651 ¶	6 months	0.06 (0.00 – 18.46)	0.62 (0.20 – 1.94)
	14 months	N/A	1.00 (0.85 – 1.18)

All MFI values are divided by 1000 except for the ones marked with a ¶, they are divided by 100. * = p-value 0.05 ** = p-value < 0.0125, which we consider statistically significant.

DISCUSSION

We assessed the effect of the humoral immune response on the inverse correlation between *S. aureus* and *S. pneumoniae* which significantly adds to the discussion on determinants of the inverse correlation that has been described for these two species. A recently published study revealed that the anti-staphylococcal IgG, IgA and IgM levels show large inter-individual variability in healthy infants from the same cohort as the present study. The levels of anti-staphylococcal IgA and IgM increase from birth until the age of 2 years, whereas the levels of anti-staphylococcal maternal-IgG decrease. These placentally transferred maternal IgG antibodies do not protect against nasal staphylococcal colonisation. Several anti-staphylococcal antibodies (e.g. those directed to CHIPS, Efb, IsdA and IsdH) seemed to play a role in nasal colonisation of young children [18]. In the current study we investigated whether levels of systemically produced IgG and IgA against staphylococcal and pneumococcal antigens are correlated to later colonisation with the other pathogen. In infancy, no positive nor negative effect of anti-pneumococcal and anti-staphylococcal IgG and IgA was seen on *S. aureus* and pneumococcal colonisation, respectively. We hypothesized that an increased level of specific anti-pneumococcal antibodies (following clinical or subclinical infection) reflects prior pneumococcal colonisation, and thus decreases the risk for *S. aureus* colonisation. However, no such cross-protectiveness by anti-pneumococcal antibodies on *S. aureus* colonisation seems to exist and vice versa. On the other hand, one can hypothesize that increased levels of specific anti-pneumococcal antibodies protect children from pneumococcal colonisation and infection and therewith increase the risk of *S. aureus* colonisation. No such association was seen either. In this sample no significant negative association between *S. aureus* and pneumococcal colonisation can be observed. However, at 1.5 and 14 months a non-significant trend towards an inverse correlation was found.

In conclusion, our study aimed to explore the aetiology and immunological effects of the negative association between *S. aureus* and pneumococcal colonisation. We found no role for the early specific humoral immune response against *S. aureus* and pneumococcal protein-antigens.

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Chapter 4

Bacterial colonisation and disease outcome

Chapter 4.1

Role of *Staphylococcus aureus* nasal colonisation in atopic dermatitis in infants

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ABSTRACT

Staphylococcus aureus is an important pathogen associated with atopic dermatitis. Longitudinal data on nasal colonisation with *S. aureus* in infancy was recently described. However the risk of developing atopic dermatitis following nasal colonisation with *Staphylococcus aureus* in infants is unknown. Therefore, the objective was to study the association between *Staphylococcus aureus* nasal colonisation and atopic dermatitis in infancy.

This project was embedded in the Generation R Study in Rotterdam, the Netherlands, which is a population-based prospective cohort study of pregnant women and their children. Postnatal, 1,079 Dutch children participated in the Focus Cohort.

Nasal swabs for *Staphylococcus aureus* cultivation were taken at the age of 1.5, 6 and 14 months. Questionnaires on atopic dermatitis and confounders (parental eczema, birth weight, gestational age and gender) were obtained pre- and postnatal. The outcome was atopic dermatitis in the first and second year of life.

First positive culture of *Staphylococcus aureus* at 6 months was associated with atopic dermatitis prevalence in the first and second year of life (aOR 2.25 95% CI 1.74–4.30 and aOR 2.59 95%CI 1.60–5.19) and also with severity (aOR 3.30 95%CI 1.68–6.47). Moreover, frequent colonisation in the first year of life (≥2) held a 4.50 (95% CI 1.04–19.43) fold risk to develop moderate/severe atopic dermatitis in the second year of life.

Staphylococcus aureus colonisation at 6 months as well as frequent colonisation in the first year of life is associated with atopic dermatitis and its severity in young children.

INTRODUCTION

Staphylococcus aureus is a human commensal as well as a cause of a wide range of infections. Besides several invasive diseases, it plays an important role in cutaneous diseases including atopic dermatitis (AD) [1-3]. AD is an inflammatory skin disease which usually presents in the first years of life [4-5]. As reported in many studies, *S. aureus* is the most important pathogen associated with AD. Skin colonisation with *S. aureus* is known to be related with AD disease severity [6].

A significant fraction of the healthy population is colonised with *S. aureus* on epithelial surfaces, of which the anterior nares are the most frequent carriage sites [1, 7-8]. Longitudinal studies distinguish three types of carriage patterns among healthy adult individuals, non carriers, intermittent carriers and persistent carriers [9-12]. Persistent carriers have a well documented higher risk of acquiring *S. aureus* infection, but they barely exist in infancy [13-16]. The anterior nares may serve as an important endogenous reservoir for involvement in AD, reaching a colonisation prevalence of 39-82% in adult patients with AD [17-18]. *S. aureus* might play a role in the chronicity and severity of AD through its release of superantigenic exotoxins [19]. Specifically, colonisation with superantigen producing *S. aureus* is associated with increased severity of AD. *S. aureus* enterotoxins A through E and the toxic shock syndrome toxin (TSST)-1, acting as superantigens, have been shown to trigger atopic dermatitis occurrence and severity [20-21]. *S. aureus* enterotoxins [22] increase the inflammation in atopic dermatitis and provoke the generation of enterotoxins specific IgE which correlates with the severity of the disease [19, 23-24]. Longitudinal data on nasal colonisation with S. aureus in infancy was recently described [16]. Additionally we aim to assess the risk of developing atopic dermatitis following nasal colonisation with *S. aureus* in healthy infants in the first year of life.

METHODS

Study design and population

This project was embedded in the Generation R Study, a population-based prospective cohort study of pregnant women and their children. Detailed assessments were conducted in 1,232 Dutch pregnant women and their children. Three infants died perinatally. The remaining mothers gave birth to 1,244 infants, of whom 138 were excluded as the consent was withdrawn after birth. Twins (n = 27) were excluded for analyses since they are related, leaving 1,079 infants in the group of postnatal participants. All children were born between February 2003 and August 2005 [25-26]. The Medical Ethics Committee of the Erasmus Medical Center, Rotterdam, has approved the study. Written informed consent was obtained from all participants [26].

The infants visited the Generation R study center at age 1.5 months (n = 884), 6 months (n = 882) and 14 months (n = 863). At each visit, nasal samples for *S. aureus* isolation were taken,

627 got a swab taken at 1.5 months, 832 at 6 months and 757 at 14 months. 758 infants attended all visits of whom 353 provided us with three swabs to use for longitudinal analysis. The amount of infants with a swab at 1.5 months is considerably lower compared to the other visits due to a later start of swab sampling as part of the visit to the research center. None of the infants used antibiotics in the preceding 48 hours.

Staphylococcus aureus

Research nurses obtained a nasal swab for *S. aureus* isolation at each visit. Nasal samples were taken using a swab that was rubbed through the nostrils. The methods of sampling were described in more detail previously [16]. *S. aureus* colonisation was analysed age specifically at 1.5, 6 and 14 months of age. Additionally we obtained analyses to assess the importance of first positive culture. To assess the importance of frequent *S. aureus* colonisation in the first year of life on the development of atopic dermatitis, three groups were created; infants who were never positive, positive once and positive twice or more.

Atopic dermatitis

Information regarding AD was obtained using an age-adapted version of the validated questionnaire of the "International Study of Asthma and Allergies in Childhood" (ISAAC) at the age of 12 and 24 months [27-28]. Parents were asked questions regarding previous episodes of eczema, AD treatment or episodes of itchy rash. These categories were combined to define a dichotomous outcome: presence or absence of AD in the first and the second year of life. The severity score was based on questions regarding the level of suffering. Questions related to continuous or intermittent rash. This resulted in three groups: no AD, mild AD (episode of rash without additional symptoms) and moderate/severe AD (episode of rash with additional symptoms as mentioned above).

Statistical methods

Information about date of birth, birth weight and gender was obtained from midwives and hospital registries. Gestational age was based on pregnancy dating by early ultrasound. Information on parental history of eczema was obtained by prenatal questionnaires.

We compared the 353 infants with all three swabs available to the total cohort of 1,079 infants who were available for postnatal analysis. To study the association between AD in both the first and the second year of life and colonisation we performed univariate and multivariate binary logistic regression, adjusted for important confounders such as gender, gestational age, birth weight and parental history of eczema. To study the association between severity of AD in the second year of life and colonisation we conducted multinomial logistic regression analyses, both univariate and multivariate. Infants with missing data in the outcome variable were excluded from the analyses (11.2% of 1,079 infants), missing data in the confounders were analysed in the model as separate category and thus accounted for in the analyses. Measures

of association are presented by crude odds ratios (OR) and adjusted odds ratios (aOR) with their 95% confidence interval (CI). The statistical analyses were performed using the Statistical Package of Social Sciences version 11.0 for Windows (SPSS Inc, Chicago, IL, USA).

RESULTS

Table 1 presents parental and infant characteristics. Of the 1,079 infants, 48.3% are girls (*n* = 521), they have a mean birth weight of 3509 grams (SD 538), the median gestational age was 40.3 weeks (95%range 37.1–42.1). Eczema symptoms in the parents occurred in 3.4% of the mothers (*n* = 32) and 4.4% of the fathers (*n* = 39). In the period of 6-12 months of age, 259 of 1079 infants (26.8%) suffered from AD symptoms. A total of 273 of 1079 infants (28.5%) infants suffered from AD in the second year of life (Table 1).

Presented in the table 2 are only the multivariate analyses. Table 2 shows that *S. aureus* colonisation at 6 months was clearly associated with AD in both the first and the second year of life (aOR 1.77 95%CI 1.20 – 2.61 and aOR 1.84 95%CI 1.25 – 2.70 respectively). Infants with their first positive swab at 6 months (a negative swab at 1.5 months) had an even more increased risk to develop atopic dermatitis symptoms in the first (aOR 2.25 95% CI 1.74 – 4.30) and the second year of life (aOR 2.59 95%CI 1.60 – 5.19). Infants with a higher frequency of colonisation in the first year of life (twice or more) have an increased risk to suffer from AD in

TABLE 1. Population descriptive

	Total cohort	Atopic Dermatitis in the 1st year of life		Atopic Dermatitis in the 2nd year of life	
	n = 1,079	No (n=706)	Yes (n=259)	No (n=685)	Yes (n=273)
Birth weight (grams)	3509 (538)	3506 (546)	3537 (505)	3523 (532)	3507 (554)
Gestational age (wk)	40.3 (37.1-42.1)	40.3 (37.1-42.1)	40.4 (37.1-42.1)	40.3 (37.1-42.1)	40.3 (36.7-42.1)
Gender					
- Female (ref)	521 (48.3%)	350 (50.4%)	125 (51.7%)	332 (48.5%)	133 (48.7%)
- Male	558 (51.7%)	356 (49.6%)	134 (48.3%)	353 (51.5%)	140 (51.3%)
Eczema symptoms of the mother					
- No (ref)	897 (96.6%)	595 (97.5%)	210 (93.8%)	573 (97.1%)	221 (95.7%)
- Yes	32 (3.4%)	15 (2.5%)	14 (6.2%)	17 (2.9%)	10 (4.3%)
Eczema symptoms of the father					
- No (ref)	848 (95.6%)	562 (96.2%)	203 (92.7%)	552 (96.7%)	211 (91.7%)
- Yes	39 (4.4%)	22 (3.8%)	16 (7.3%)	19 (3.3%)	19 (8.3%)

Values are presented as means with a standard deviation (birth weight), medians with a 5-9% range for variables with a skewed distribution (gestational age) and absolute numbers with percentages.
In the total cohort data was missing in mother's history of eczema symptoms (*n*=150), father's history of eczema symptoms (*n*=192), atopic dermatitis between 6-12 months (*n*=114), atopic dermatitis in the second year of life (*n*=121).

TABLE 2. The association between *S. aureus* colonisation and atopic dermatitis in infancy

	Atopic dermatitis in the 1st year of life			Atopic dermatitis in the 2nd year of life		
	No (n=706)	Yes (n=259)	aOR (95%CI)	No (n=685)	Yes (n=273)	aOR (95%CI)
S. aureus 1.5 month						
- no (ref)	195 (47.2%)	63 (42.6%)	1.00	201 (47.6%)	61 (43.3%)	1.00
- yes	218 (52.8%)	84 (57.4%)	1.21 (0.82–1.78)	221 (52.4%)	80 (56.7%)	1.24 (0.83–1.83)
S. aureus 6 months						
- no (ref)	417 (81.8%)	140 (71.4%)	1.00	413 (81.9%)	142 (71.0%)	1.00
- yes	93 (18.2%)	56 (28.6%)	1.77 (1.20–2.61)*	91 (18.1%)	58 (29.0%)	1.84 (1.25–2.70)*
S. aureus 14 months						
- no (ref)	413 (85.3%)	154 (84.6%)		406 (85.8%)	151 (82.5%)	1.00
- yes	71 (14.7%)	28 (15.4%)	N.A. ¶¶	67 (14.2%)	32 (17.5%)	1.23 (0.77–1.97)
First positive culture						
- never (ref)	87 (22.4%)	23 (15.2%)	1.00	90 (23.0%)	25 (16.4%)	1.00
- 1.5 month	212 (54.5%)	84 (55.6%)	1.46 (0.86–2.47)	216 (55.2%)	79 (52.0%)	1.30 (0.77–2.20)
- 6 months	54 (13.9%)	31 (20.5%)	2.25 (1.17–4.30)*	50 (12.8%)	34 (22.4%)	2.59 (1.34–4.91)*
- 14 months	36 (9.3%)	13 (8.6%)	N.A. ¶¶	35 (9.0%)	14 (9.2%)	1.39 (0.64–3.02)
S. aureus ¶						
- never	87 (37.0%)	23 (24.2%)		90 (35.6%)	25 (29.4%)	1.00
- once	102 (43.4%)	44 (46.3%)	N.A. ¶¶	115 (45.5%)	32 (37.6%)	1.02 (0.56–1.87)
- ≥2	46 (19.6%)	28 (29.5%)		48 (19.0%)	28 (32.9%)	2.04 (1.04–3.98)*

Of the total group (*n*=1,079), 631 infants had a swab at 1.5 month, 787 infants at 6 months and 717 infants had a swab at 14 months.
Results are presented as adjusted odds ratios (aOR) corrected for gender, birth weight, gestational age and eczema history of the parents.
¶ Only 353 infants with 3 swabs available were analysed. ¶¶ Not applicable as determinant should occur before outcome. * p-value<0.05

TABLE 3. *S. aureus* colonisation and atopic dermatitis severity in the 2nd year of life

	Atopic dermatitis in the 2nd year of life			
	Mild (n=218)		Moderate/Severe (n=55)	
	OR	AOR	OR	aOR
S. aureus 1.5 month				
- no (ref)	1.00	1.00	1.00	1.00
- yes	1.04 (0.69–1.58)	1.10 (0.72 – 1.67)	2.34 (0.96–5.72)	2.21 (0.89 – 5.48)
S. aureus 6 months				
- no (ref)	1.00	1.00	1.00	1.00
- yes	1.56 (1.03–2.38)*	1.55 (1.01 – 2.37)*	3.36 (1.72–6.53)*	3.30 (1.68 – 6.47)*
S. aureus 14 months				
- no (ref)	1.00	1.00	1.00	1.00
- yes	1.52 (0.94–2.45)	1.45 (0.89 – 2.37)	0.52 (0.16–1.74)	0.50 (0.15 – 1.70)
S. aureus ¶				
- never (ref)	1.00	1.00	1.00	1.00
- once	0.93 (0.49–1.74)	0.94 (0.49 – 1.80)	1.57 (0.38–6.43)	1.57 (0.37 – 6.69)
- twice or more	1.79 (0.90–3.58)	1.69 (0.81 – 3.49)	4.38(1.08–17.69)*	4.50 (1.04 – 19.43)*

Results are presented as crude odds ratios (OR) and adjusted odds ratios (aOR) corrected for gender, birth weight, gestational age and eczema history of the parents.

¶ Only 353 infants with 3 swabs available were analysed. * p-value<0.05

the second year of life, this effect remains significantly associated after adjustment for confounders (aOR 2.04 95%CI 1.04 – 3.98).

Of the 273 children who suffered from AD in the second year of life, 55 (20.1%) suffered from moderate to severe AD and 218 from a mild phenotype (79.9%). (Table 3) *S. aureus* colonisation was also correlated with severity of AD. Infants colonised at 6 months had an increased risk to suffer from both mild and moderate/severe atopic dermatitis in the second year of life. However, a higher risk was found for the children with moderate/severe AD (aOR 3.30 95%CI 1.68 – 6.47) as compared to mild AD (aOR 1.55 95% CI 1.01 - 2.37). Frequent *S. aureus* colonisation in the first year of life (twice or more) was associated with moderate/severe AD symptoms in the second year of life, but not with mild AD (aOR 4.50 95%CI 1.04 - 19.43 and aOR 1.69 95% CI 0.81 – 3.49, respectively).

An additional analysis was done to assess if children with AD in the first year of life have an increased risk to become colonised as a result. We did not find a significant association between AD in the first year of life and *S. aureus* colonisation at 14 months.

Since we selected 353 out of 1,079 infants for a part of the analyses, selection bias may have occurred. Overall, looking at the differences between the selected 353 infants with three swabs available and the total cohort of 1,079 infants, the selected infants had less missing data from the questionnaires. The infants without three swabs available were more likely to have not filled out the questionnaire or return the questionnaire incompletely.

28.8% of the 353 infants suffered from AD in the first year of life, and 25.1% in the second year of life. We missed data on AD symptoms in 6.5% and 4.2% in this selected group. Of the remaining

infants in the total cohort (*n* = 726), 25.8% and 30.3% of the children suffered from AD in the first and second year of life, and data was missing in 12.5% and 14.6% in the first and second year of life respectively. Eczema history of the mother was similar in the group of 353 infants as compared to the total cohort; 82.7% of the mothers from the 353 infants versus 83.3% in the total cohort had no positive history of eczema symptoms. The numbers of missing data were fairly equal in these groups (15.0% versus 13.4%), contrary to the eczema history of the fathers which was missing more often in the total cohort as compared to the selected group (21.3% versus 10.5%).

DISCUSSION

Bacterial colonisation is considered an important factor in the pathophysiology involved in AD [29]. We found a clear association, after adjustment for important confounders, between the prevalence of *S. aureus* nasal colonisation at 6 months and the occurrence of AD in both the first and the second year of life in a healthy infant cohort. Moreover, frequent nasal colonisation with *S. aureus* in the first year of life held an increased risk to develop atopic dermatitis in the second year of life, this risk was especially increased to moderate/severe AD. This is in line with previous studies, showing a relation between *S. aureus* and AD in several ways. Semic-Jusufagic et al showed, in a similar cohort study, a positive association between specific IgE staphylococcal enterotoxin-mix and AD in children [24]. Other studies reported on increased levels of anti-staphylococcal IgE and staphylococcal toxins A-E in the serum of AD patients [21, 30]. No other studies ever reported on nasal colonisation of *S. aureus* as risk factor preceding AD in infants.

Colonisation at 6 months may be a critical event in the development of the immune system of infants. Barely any immune globulins from the mother are left at this age and the infant's own immune system is still developing. It therefore is important to take this moment in the first year of life into account when studying bacterial colonisation and the development of AD in childhood.

We found severity of AD to be associated with *S. aureus* nasal colonisation. Infants positive at 6 months not only have an increased risk on AD in the first place, they also have a significant increased risk to suffer from moderate to severe AD compared to non-carriers. Moreover, a more than fourfold risk was found in our cohort for infants with at least two moments of *S. aureus* colonisation in the first year of life. This is additional to data on severity presented by others [24]. Several studies describe an association between colonisation and a higher eczema severity score [24, 31-32].

Our study provides data on nasal colonisation of *S. aureus* preceding AD adjusted for several confounders, supporting a direct link between colonisation and AD in one of the largest birth-cohorts. With the smallest number of 353 in infants with all three swabs available, it is still large as compared to other studies. A larger sample was studied for the individual swab

results. Selection bias may have occurred by choosing to analyse 353 infants out of the total cohort of 1,079 infants. These 353 infants were selected based on their attendance to the research centre and willingness to provide a nasal swab. One can speculate whether these are the children with less or more physical problems. Either, the parents may be more willing to participate when their child is ill or the child is too ill to participate or seeks medical care in different ways that participation is not wanted by the parents. However, since we do not see great difference in AD prevalence between the selected 353 children and the total cohort a selection by atopic dermatitis is not likely to have happened. It could be the case for other illnesses and infections and also selection bias by socio-economic status may have occurred.
Aside from the analyses of bacterial colonisation preceding episodes of AD, we analysed bacterial colonisation following an episode of AD in the first year of life as well which showed no significance. This allows us to draw conclusions of AD following bacterial colonisation with *S. aureus*, rather than the other way around, bacterial colonisation following AD.
AD symptoms are not confirmed by a clinician in our study. However the questionnaire used was validated and age-adapted [27-28]. Information bias due to knowledge on the main determinant is unlikely to have occurred since the parents, the ones who filled out the questionnaire on AD and confounders were not notified of the infant's colonisation status.
We did not study mythically-resistant *Staphylococcus aureus* (MRSA) colonisation in this study. Not only was this out of scope of this study, the prevalence of MRSA in the Netherlands is among the lowest in the world [33]. Studying MRSA in a Dutch population cohort is thus not very important.
Nasal colonisation and colonisation of the affected skin in patients with AD are strongly associated, which may explain a pathophysiological role for *S. aureus* nasal colonisation and AD [34]. One can speculate a systemic release of enterotoxins specific IgE against super antigens of *S. aureus* to lead to atopic dermatitis.
Our results are in line with, and in addition to, literature suggesting a potentially pathophysiological role for *S. aureus* in AD [24, 29]. Further studies are required to clarify the exact pathophysiological role of *S. aureus* colonisation in relation to AD.

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Chapter 4.2

Glucocorticoid receptor variants are associated with *Staphylococcus aureus* colonisation and atopic dermatitis

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ABSTRACT

Nasal *Staphylococcus aureus* colonisation, which is associated with atopic dermatitis (AD), is common in children. In adults, glucocorticoid receptor (GR) single nucleotide polymorphisms (SNPs) were associated with *S. aureus* colonisation. The aim was to assess whether GR SNPs are associated with *S. aureus* colonisation until age 3 and whether the association between *S. aureus* colonisation and AD depends on GR variants.

This study was embedded in a birth cohort. DNA was obtained from cord blood samples and assessed for five GR polymorphisms: *Bcl*I, *TthIII*I, GR-9β, N363S and ER22/23EK. Nasal swabs for *S. aureus* isolation were obtained in 1,079 children at 1.5, 6, 14, 24 and 36 months. AD symptoms were questionnaire-derived at age 4.

Six haplotypes were distinguished. GR haplotypes were not associated with *S. aureus* colonisation during infancy. At age 3, however, four haplotypes were significantly associated with reduced nasal colonisation; haplotype 1 (OR 0.46 95%CI 0.25–0.85 as compared to wildtype), 2 (*Bcl*I, OR 0.46 95%CI 0.25–0.85), 3 (*Bcl*I+*TthIII*I, OR 0.40 95%CI 0.20–0.79) and 4 (GR-9β+*TthIII*I, OR 0.47 95%CI 0.24–0.93). However, the odds of AD were highest for those children with frequent *S. aureus* colonisation holding 2 of the 5 minor GR haplotypes compared to children holding two copies of wildtype (aOR 2.89 95%CI 1.03–8.07).

Several GR-haplotypes were associated with a reduced risk of *S. aureus* colonisation at age 3, but not in infancy. Yet, the children with non-wildtype GR-haplotypes were prone to develop AD symptoms given prior *S. aureus* colonisation.

INTRODUCTION

Bacterial colonisation is an important cause of morbidity in the Western world as it increases the chance of infection. Nasal *Staphylococcus aureus* colonisation, associated with for instance postoperative wound infections and cutaneous diseases, is very common among the general population [1-2]. Recently we reported on the association between frequent early staphylococcal colonisation in the first year of life and the development of atopic dermatitis (AD) in toddlers [3]. The next step would be to study mechanisms behind this association. In adults three types of *S. aureus* colonisation patterns can be distinguished; non-carriage, intermittent carriage and persistent carriage [4-7], although recently the intermittent and non-carriers were suggested to be indistinguishable [8]. Persistent colonisation barely exists in infancy and it is not known when or how the match between host and pathogen occurs which will then lead to persistent colonisation of subjects [9]. In other words, we do not know when the apparently random staphylococcal colonisation patterns of children mature into an adult pattern. The variations in colonisation frequency and pattern may be explained by host or other characteristics. In adults, an association between glucocorticoid receptor (GR) polymorphisms and *S. aureus* carriage was described [10]. Homozygotes of the GR-9β polymorphism stood a reduced risk of persistent *S. aureus* nasal carriage, whereas carriers of ER22/23EK were at increased risk [10]. The GR is expressed in almost every cell in the body and it is the main receptor for glucocorticoids, such as cortisol. Glucocorticoids are involved in regulation of inflammation by inhibiting the immune response [11-15]. They play an important role in the pharmacology of immune suppression and are potent and frequently used immunosuppressive drugs. Glucocorticoids are frequently applied in treatment of AD, but patients differ in their therapy response and GR variants are thought to play a role in ineffective responses [16]. Patients with atopic diseases such as AD and asthma were found to express a GR with lowered binding affinity for glucocorticoids [17]. Moreover, varying cortisol levels during infancy and early childhood might play a critical role in the development of immune responses at a time when the atopic phenotype is developing [18]. No study has as yet investigated the effect of GR polymorphisms on *S. aureus* colonisation in young children, or the potential role of these polymorphisms in the association between *S. aureus* colonisation and AD. We hypothesize that *S. aureus* may be acquired from the environment thereby triggering genetically susceptible children to develop AD. Therefore, our objective was to assess whether GR SNPs are associated with *S. aureus* colonisation in early childhood and whether the association between *S. aureus* colonisation and AD in childhood may depend on GR haplotype.

METHODS

Study design and population

This project was embedded in a population-based birth cohort; the Generation R Study. Detailed assessments were conducted in 1,232 Dutch pregnant women and their children. The Medical Ethics Committee of the Erasmus Medical Center, Rotterdam, has approved the study. Written informed consent was obtained from all participants [19-20].

All children were born between February 2003 and August 2005. Three infants died perinatally, overall the 1,232 mothers gave birth to 1,244 infants, of whom 138 were not included in the cohort of analysis as the consent was withdrawn after birth. Twins (n = 27) were excluded as well leaving 1,079 infants for postnatal analyses. The infants visited the Generation R research center at 1.5 months (n = 900), 6 months (n = 901), 14 months (n = 882), 24 months (n = 856) and 36 months (n = 862). During these visits, nasal swabs were obtained in 631 children at 1.5 month (70%), 787 at 6 months (87%), 717 (81%) at 14 months 618 (72%) at 24 months and 624 (72%) at 36 months. This subgroup study started in April 2003, data collection on bacterial colonisation started in November 2003, and so data on bacterial colonisation in the first 224 participants at 1.5 months of age are missing. Information about date of birth, birth weight and gender was obtained from midwives and hospital registries. Gestational age was based on pregnancy dating by early ultrasound.

Staphylococcus aureus

At the research center nasal swabs were obtained for *S. aureus* isolation. Methods of sampling were described previously [9]. To assess colonisation frequency, we combined the results of 5 swabs into a single dichotomous determinant: zero or one positive swab versus at least 2 swabs positive for *S. aureus* [8] in order to distinguish frequent colonisers from non-frequent colonisers at an age where persistent colonisation may not exist as yet.

Atopic dermatitis

Information on AD symptoms was obtained using an adapted parentally reported questionnaire from the validated "International Study of Asthma and Allergies in Childhood" (ISAAC) questionnaire [21], at the child's age of 2 months and at 4 years. Parents were asked questions regarding physician attendance for AD symptoms and/or previous episodes of itchy rash. These categories were combined to define a dichotomous outcome: presence or absence of AD in the fourth year of life.

Glucocorticoid Receptor polymorphisms

DNA was collected from cord blood samples at birth. All participants were genotyped for five GR gene SNPs: *Bcl*I (rs41423247), *Tth*III*I* (rs10052957), GR-9β (rs6198), N363S (rs6195) and ER22/23EK (rs6189 and 6190). Genotyping of the five GR gene polymorphisms was performed using Taqman allelic discrimination assays (Applied Biosystems, Foster City, CA) and Abgene

QPCR ROX mix (Abgene, Hamburg Germany), as described previously ([22]). We used the genotype data for each of the 5 polymorphisms to infer the haplotypes present in the population using the program PHASE, which implements a Bayesian statistical method for reconstructing haplotypes from population genotype data. Instead of individual SNPs, we studied the haplotype structure of the GR gene. Rare SNPs were excluded as these may explain only a small fraction of variation in response to glucocorticoids seen between individuals. For each haplotype, 3 genotype combinations were distinguished as carrying 0, 1 or 2 copies of the haplotype alleles. Haplotype 1 (wildtype) carries the major alleles of the SNPs; therefore the reference allele is defined as presence of 2 copies of wildtype. Genotype and allele frequencies were in Hardy Weinberg equilibrium ($p>0.01$).

Statistical methods

To study the association between GR receptor haplotypes and *S. aureus* colonisation univariate binary logistic regression analyses were performed. Children with two copies of the wildtype haplotypes were used as reference category for each haplotype. Because of the low number of homozygous subjects for the haplotypes, the haplotypes were analysed as carriers (1 or 2 copies) and non-carriers (no copies) of the polymorphism. To assess the association between colonisation and AD in the fourth year of life we performed univariate and multivariate binary logistic regression, adjusted for potential confounders such as gender, gestational age, birth weight and episode of AD symptoms early in life (< 2 months), prior to colonisation. Additionally we repeated this analysis stratified by GR haplotypes (2 copies of wildtype versus one copy and no copy of wildtype). Measures of association are presented by crude odds ratios (OR) and adjusted odds ratios aOR with their 95% confidence interval (CI). A p-value less than 0.05 was considered statistically significant. The statistical analyses were performed using the Predictive Analytics SoftWare (PASW) version 17.0 for Windows (SPSS Inc, Chicago, IL, USA).

RESULTS

Table 1 presents child characteristics. Of the 1,079 infants, 48.3% are girls ($n = 521$), they have a mean birth weight of 3,509 grams (SD 538) and their mean gestational age was 40.0 weeks (SD 1.71). The *S. aureus* colonisation rate decreases during the first year of life (52.3% - 14.5%) and remains stable thereafter until the age of three (14.9%). Of the 428 children providing us data on frequent colonisation, nearly 40% of the children are colonised with *S. aureus* at least twice in this period ($n = 169$). Atopic dermatitis symptoms occurred in 205 of 1,079 children (22.8%) at the age of 4, data was missing for 108 subjects.

Six haplotypes were distinguished in our study population. Table 2 describes the distribution of these haplotypes. Haplotype 1, 2, 3 and 4 were most frequent with haplotype frequencies

TABLE 1. Child characteristics

Parameter	Value for children
	n = 1,079
Gender - female	521 (48.3%)
Gestational age	40.0 (SD 1.7)
Birth weight	3,509 (SD 538)
S. aureus colonisation	
- 1.5 month	330 (52.3%)
- 6 months	258 (20.1%)
- 14 months	104 (14.5%)
- 24 months	84 (13.6%)
- 36 months	93 (14.9%)
Frequency	
- 0-1x	259 (60.5%)
- ≥2x	169 (39.5%)
Atopic dermatitis before the age of 2 months	303 (35.8%)
Atopic dermatitis in the 4th year	205 (22.8%)

of 41.2%, 38.0%, 32.5% and 31.0%, respectively. Haplotype 5 and 6 were less frequent (17.4% and 14.1%). Data on GR polymorphisms was missing for 164 children.

No association was observed between GR haplotypes and *S. aureus* colonisation in the first two years of life (infancy). At the age of 3, however, several haplotypes were found to be associated with decreased nasal colonisation rates; haplotype 1 (wildtype, OR 0.46 95%CI 0.25 – 0.85), 2 (*Bcl*I, OR 0.46 95%CI 0.25 – 0.85), 3 (*Bcl*I + *TthIII*I, OR 0.40 95%CI 0.20 – 0.79) and 4 (GR-9β + *TthIII*I, OR 0.47 95%CI 0.24 – 0.93). There was a non significant trend towards less frequent colonisation rates in the first three years of life in children with GR polymorphisms (Table 3).

Children colonised with *S. aureus* at least twice until the age of 3 are at increased risk to develop AD complaints in the fourth year of life (aOR 1.66 95%CI 1.04 – 2.65, after adjusting for AD symptoms before the age of 2 months aOR 1.57 95%CI 0.99 – 2.53, data not shown). Although the interaction terms were not significant, the odds ratios showed a trend similar to our hypothesis (Wildtype/non-Wildtype interaction with frequent *S. aureus* colonisation OR 1.06, p-value 0.94, non-Wildtype/non-Wildtype interaction with frequent *S. aureus* colonisation OR 1.86, p-value 0.46). Increasing odds ratio's for AD symptoms at age 4 given GR variants and *S. aureus* colonisation. Hence, we repeated this analysis in a model stratified for glucocorticoid wildtype haplotype. Due to small numbers we were not able to show results stratified by each individual haplotype. Figure 1 shows the odds for developing atopic dermatitis symptoms following frequent colonisation in the first three years of life stratified by 2 copies of wildtype versus 1 copy of wildtype and no copy of wildtype. Children with at least two positive swabs for *S. aureus* whom carry two non-wildtype haplotypes on the GR, as compared to two copies of wildtype, have a nearly 3-fold increased risk of developing AD symptoms in the fourth year (aOR 2.89 95%CI 1.03 – 8.07). In other words, a recessive effect

TABLE 2. Glucocorticoid receptor haplotypes

Glucocorticoid receptor haplotype (copies)		Variant	N (%)
Haplotype 1	Wildtype	G GG A C A	
- 0			322 (35.2%)
- 1			432 (47.2%)
- 2			161 (17.6%)
Haplotype 2	*Bcl*I	G GG A **G** A	
- 0			161 (31.2%)
- 1			318 (61.6%)
- 2			37 (7.2%)
Haplotype 3	*Bcl*I + *TthIII*I	**A** GG A **G** A	
- 0			161 (38.6%)
- 1			241 (57.8%)
- 2			15 (3.6%)
Haplotype 4	GR-9β + *TthIII*I	**A** GG A C **G**	
- 0			161 (41.0%)
- 1			220 (56.0%)
- 2			12 (3.0%)
Haplotype 5	N363S	G GG **G** C A	
- 0			161 (67.4%)
- 1			73 (30.5%)
- 2			5 (2.1%)
Haplotype 6	GR-9β + *TthIII*I + ER22/23EK	**A AA** A C **G**	
- 0			161 (71.9%)
- 1			63 (28.1%)
- 2			-

was observed for two copies of non-wildtype haplotypes on the GR. Although out of scope of the aim of this study, GR receptor variants were not significantly associated with AD at age 4. In total 428 children provided longitudinal data on *S. aureus* colonisation until age 3. Non-response analyses revealed that children with missing data in the longitudinal swab collection until age 3 reported less atopic dermatitis complaints in the fourth year (20.4% versus 25.9%). Moreover, significantly fewer children received breastfeeding at 6 months in the group of children with missing data in the swab results (24.7% versus 31.8%, respectively). In addition, their mothers were more likely not to provide data on postnatal smoking habits and they had significantly more missing data on daycare attendance and presence of siblings. There was no significant difference in gender, educational level of the mother, prenatal smoking and parental history of AD. Data on AD was missing in 180 children. These children were more likely to be boys (58.9% versus 50.3%) and were born to lower educated mothers (55.0% versus 31.5%). The prevalence of maternal pre- and postnatal smoking was higher in this group of non-responders (17.2% versus 6.7% and 9.4% versus 8.1%, respectively). Moreover, they were less likely to receive breastfeeding at 6 months (13.9% versus 30.3%). No association was present with daycare attendance. The group of children with missing data on AD more often had missing data on daycare attendance and presence of siblings as well. There was no significant difference in parental history of AD.

TABLE 3. Glucocorticoid receptor haplotypes and Staphylococcus aureus colonisation

	1.5 month	6 months	14 months	24 months	36 months	At least twice
	OR (95%CI)	OR (95%CI)	OR (95%CI)	OR (95%CI)	OR (95%CI)	OR (95%CI)
Haplotype 1						
- non carrier	1.00	1.00	1.00	1.00	1.00	1.00
- carrier of 1 copy	1.23 (0.77 – 1.96)	1.69 (0.96 – 2.96)	0.97 (0.51 – 1.83)	1.03 (0.54 – 1.97)	0.51 (0.28 – 0.92)*	0.87 (0.48 – 1.58)
	p-value 0.39	*p-value 0.07*	*p-value 0.92*	*p-value 0.94*	*p-value 0.03*	*p-value 0.64*
- carrier of 2 copies	0.97 (0.60 – 1.58)	1.14 (0.62 – 2.09)	0.80 (0.41 – 1.56)	0.53 (0.25 – 1.12)	0.46 (0.25 – 0.85)*	0.52 (0.28 – 0.99)*
	p-value 0.97	*p-value 0.68*	*p-value 0.51*	*p-value 0.10*	*p-value 0.01*	*p-value 0.05*
Haplotype 2						
- non carrier	1.00	1.00	1.00	1.00	1.00	1.00
- carrier	1.03 (0.64 – 1.65)	1.42 (0.79 – 2.55)	0.95 (0.49 – 1.83)	0.81 (0.41 – 1.60)	0.46 (0.25 – 0.85)*	0.63 (0.34 – 1.15)
	p-value 0.92	*p-value 0.24*	*p-value 0.88*	*p-value 0.55*	*p-value 0.01*	*p-value 0.13*
Haplotype 3						
- non carrier	1.00	1.00	1.00	1.00	1.00	1.00
- carrier	1.05 (0.63 – 1.76)	1.51 (0.82 – 2.78)	0.93 (0.46 – 1.86)	0.89 (0.43 – 1.85)	0.40 (0.20 – 0.79)*	0.75 (0.39 – 1.44)
	p-value 0.84	*p-value 0.18*	*p-value 0.83*	*p-value 0.76*	*p-value <0.01*	*p-value 0.38*
Haplotype 4						
- non carrier	1.00	1.00	1.00	1.00	1.00	1.00
- carrier	1.19 (0.71 – 2.00)	1.43 (0.76 – 2.69)	0.82 (0.40 – 1.66)	0.55 (0.24 – 1.24)	0.47 (0.24 – 0.93)*	0.66 (0.33 – 1.29)
	p-value 0.51	*p-value 0.27*	*p-value 0.58*	*p-value 0.15*	*p-value 0.03*	*p-value 0.22*
Haplotype 5						
- non carrier	1.00	1.00	1.00	1.00	1.00	1.00
- carrier	1.03 (0.51 – 2.07)	1.43 (0.65 – 3.15)	0.38 (0.12 – 1.22)	0.64 (0.22 – 1.89)	0.70 (0.29 – 1.66)	0.60 (0.26 – 1.39)
	p-value 0.95	*p-value 0.38*	*p-value 0.10*	*p-value 0.42*	*p-value 0.42*	*p-value 0.23*
Haplotype 6						
- non carrier	1.00	1.00	1.00	1.00	1.00	1.00
- carrier	1.27 (0.60 – 2.67)	1.16 (0.47 – 2.89)	1.25 (0.50 – 3.11)	0.24 (0.03 – 1.87)	0.46 (0.17 – 1.24)	0.64 (0.25 – 1.62)
	p-value 0.53	*p-value 0.75*	*p-value 0.64*	*p-value 0.17*	*p-value 0.13*	*p-value 0.34*

Results are presented as crude odds ratios (OR) with a 95 % Confidence Interval (CI) and p-values.

P-values <0.05 are considered statistically significant and are indicated with a * .

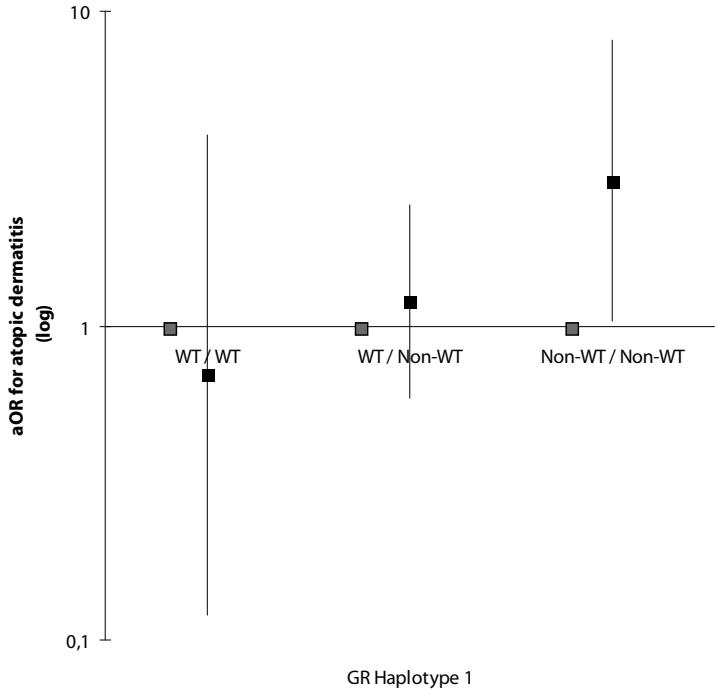

Figure 1. This figure shows the association between frequent *Staphylococcus aureus* colonisation (at least twice) and atopic dermatitis symptoms, stratified by GR haplotype 1. Results are presented as odds ratios, adjusted for gender, gestational age, birth weight and episode of AD prior to colonisation (AD in the first 2 months of life) with a 95% confidence interval on a logarithmic scale. The odds of developing atopic dermatitis in the fourth year of life following frequent *S. aureus* colonisation (black square) as compared to the children never or once colonised (grey square) was highest for children holding two copies of non-WT (aOR 2.89 95%CI 1.03 – 8.07, p-value 0.04). * = statistically significant, p-value 0.04.

DISCUSSION

In a large population-based birth cohort, we discovered that GR SNPs are associated with decreased prevalence of *S. aureus* colonisation at age 3, but not in infancy. At the age of 2 we see a trend towards reduced rate of S. aureus colonisation as well, though not significant. Earlier we described absence of persistent colonisation in infancy, which is contrary to colonisation patterns in the adult population [9]. *S. aureus* colonisation rates among young infants were found to be clearly different from that among adults. It is still unknown at what age a so called host-pathogen match occurs that will lead to persistent colonisation. An association between GR SNPs and persistent *S. aureus* nasal colonisation was earlier described in healthy Dutch adults. The fact that we find an association between the GR SNPs and colonisation at age 3 may be a first clue relating to the establishment of an adult staphylococcal colonisation pattern in childhood. Van den Akker et al found other haplotypes in Dutch adults compared

to the haplotypes found in our children, hence we can not completely compare the results. However, in their study the haplotype containing GR-9β resulted in reduced risk of *S. aureus* colonisation which is comparable to what we find in our haplotypes number 1 to 4 at the age of 3 [10]. The activity of the GR is regulated by the balance of two isoforms GR-α and GR-β. GR-α positively mediates the receptor activity and GR-β is a dominant negative inhibitor of GR-α [23]. The mechanism for the protective effect of GR polymorphism on *S. aureus* colonisation is not known. However, van den Akker speculated on a role for stabilization of the GR-β mRNA by the presence of the G allele [24-25]. Through a negative influence on GR-α action, this may lead to an immune enhancement predisposing to chronic autoimmune disease [26], while reducing the risk of *S. aureus* colonisation. An explanation may also be found in the negative association of the reduced glucocorticoid induced immune suppression [10].

In addition, we hypothesized that the association between frequent *S. aureus* colonisation and AD may depend on GR SNPs. In the current study we show that children colonised with *S. aureus* at least twice until the age of 3, have a significantly increased risk to develop AD at age 4. This is in line with our earlier study conducted in infants [3]. In the current study, we searched for mechanistic aspects accounting for the association by assessing the effect of GR polymorphisms. The association between frequent *S. aureus* colonisation and development of AD seems to depend on a recessive effect of GR polymorphisms. In a stratified model, children with no copy of wildtype have a nearly 3-fold increased risk to develop AD symptoms following frequent *S. aureus* colonisation. Unfortunately, possibly due to a small sample size, the interaction term was not significant.

The glucocorticoid receptor is involved in immune suppression and alterations in its gene may lead to persistent inflammatory responses [15, 27]. As explained above it is possible that through a negative influence on GR-α action, variants of the GR gene may lead to autoimmune diseases. This is in line with our findings: a reduced risk of *S. aureus* colonisation, but an increased risk of AD following *S. aureus* colonisation. In other words, we hypothesize that genetic variants of GR indeed reduce the risk of *S. aureus* colonisation as result of a reduced immune suppression, but if a child is unlucky enough to get colonised with *S. aureus* while having one of the GR variants, the chances are high to develop atopic dermatitis through a possible mechanism of reduced immune suppression resulting in enhanced immune activity [16].

Large, prospective, population based birth cohorts facilitate research as described here and provide an opportunity to study the association between GR polymorphisms, *S. aureus* colonisation longitudinally and AD in young children. Moreover, this study presents data in a group of healthy subjects, rather than patients. The limitation is that our study is quite descriptive. AD was, although by means of a validated questionnaire, parentally reported rather than diagnosed by a physician. Moreover, even though the Generation R cohort is a multi-ethnical cohort, this particular study was conducted in a Dutch subgroup. Hence, the results might not apply to groups of different genetic or environmental backgrounds. On the other hand, by selecting children from the same ethnical background we reduce genetic heterogeneity in

our analyses. Additionally, since this was conducted in a subgroup, we did not have enough subjects to conduct analyses for every separate haplotype to explain the association between *S. aureus* colonisation and development of AD in childhood. Non-response analysis revealed some selection. Since these determinants were no biological confounders of the studied association, nor did they alter the risk estimate of the association between frequent *S. aureus* colonisation with AD substantially, we did not add these determinants to the presented model.

In conclusion, our study observes that children with variants of the GR have a decreased risk of *S. aureus* colonisation at the age of 3 which is similar to what was observed in adults. Moreover, our study suggests that if these children with variants of the glucocorticoid receptor get frequently colonised with *S. aureus* in the first three years of life, they may be more prone to develop AD symptoms in the fourth year of life. Hence, *S. aureus* seems to be an environmentally acquired factor, triggering genetically susceptible children to develop AD. This provides yet another example of human genotypes being the main etiological factors in the establishment of *S. aureus* colonisation and its clinical consequences. This study generates a new hypothesis on the mechanisms behind the association between *S. aureus* colonisation and AD in which a role for gene-environment interactions can be reserved. Since our study is the first showing these associations, replication studies are needed.

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Chapter 4.3

Nasopharyngeal bacterial colonisation and respiratory symptoms in childhood

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ABSTRACT

Bacterial colonisation may result in respiratory tract infections and is also associated to asthma. We aim to study whether nasopharyngeal bacterial colonisation is associated with respiratory symptoms in pre-school children, prior to the pneumococcal conjugate vaccine-era.
This project is part of the Generation R study, a population-based prospective birth cohort which is conducted in Rotterdam, the Netherlands. We studied 1,079 Dutch children enrolled in the Generation R Focus Cohort.
Nasal and nasopharyngeal swabs were cultured for *Streptococcus pneumoniae, Moraxella catarrhalis, Haemophilus influenzae* and *Staphylococcus aureus* at 1.5, 6 and 14 months. Respiratory tract symptoms were questionnaire-derived at the age of 12, 24, 36 and 48 months using the ISAAC questionnaire to assess wheezing symptoms and doctor attendance for respiratory tract infections. Data on confounders was obtained: birth weight, gestational age, gender, maternal smoking, socio-economic status, breastfeeding, day-care and siblings.
A trend towards protection against respiratory tract infection, but not against wheezing, was observed following colonisation in the first year of life. Especially frequent airway pathogen colonisation, rather than specific co-colonisation combinations, was associated with a decreased risk of respiratory tract infection until the fourth year (aOR 0.17 95%CI 0.04–0.78) and an increased risk of wheezing symptoms in the second year (aOR 8.62 95%CI 2.06–36.11).
Bacterial colonisation in early life was associated with less respiratory tract infections at long term, but with increased rates of wheezing symptoms in the second year of life.

INTRODUCTION

The airway pathogen colonisation rate increase in early childhood with peak rates around one year of age (around 30% of the children is colonised with *Moraxella catarrhalis* or *Haemophilus influenzae*, over 40% is colonised with the pneumococcus) [1-2]. Several determinants of nasopharyngeal bacterial colonisation have been described [1-3]. The nasopharynx is a major ecologic niche for several pathogens such as airway pathogens *Streptococcus pneumoniae, Haemophilus influenzae* and *Moraxella catarrhalis,* whereas skin pathogen *Staphylococcus aureus* rather nestles in the anterior nares. Even though these pathogens act commensally most of the time, they can be a great burden by increasing the risk of infection and disease [4-6]. The spread of the airway pathogens from the nasopharynx often results in respiratory tract infections [4, 7]. A recent study suggested that bacterial colonisation with the airway pathogens may not only results in respiratory tract infections, but also contributes to the development of asthma-like symptoms at young age [8].

Pneumococcal infection has to be preceded by nasopharyngeal colonisation with the homologous strain; hence pneumococcal colonisation is the first step in infection caused by this pathogen [4]. However since pneumococcal colonisation duration is less than a month [9], the risk of infection is short-lived as well. For *Moraxella catarrhalis* this is slightly different. While colonisation with this pathogen increases risk of infection, this is often not caused by the homologous strain of colonisation [5]. Children tend to acquire and eliminate different strains in time. Again an increased risk of infection may be expected at time of colonisation. However, it is unknown what the effect is of nasopharyngeal bacterial colonisation early in life on respiratory tract infections at long term.

Therefore the aim of this study was to investigate whether nasopharyngeal colonisation with bacterial pathogens was associated with respiratory tract symptoms, both respiratory tract infections with fever and wheezing symptoms in early childhood from the first to the fourth year of life, in a healthy population-based cohort prior to the pneumococcal conjugate vaccine-era.

METHODS

Study design and population

The Generation R study is a population-based prospective cohort study following pregnant women and their children until young adulthood. The Generation R Study has been described in detail previously [10-11]. Detailed assessments were performed in the Focus cohort, consisting of 1,232 Dutch pregnant women and their children.

All children were born between March 2003 and August 2005. Of the 1,232 pregnant women, 1,079 infants remained in the postnatal cohort of analyses. Three infants died perinatally, consent was withdrawn in 138 infants and 13 twin-pairs were excluded (Figure 1). The children

visited the research center at age 1.5 months (n = 884), 6 months (n = 882) and 14 months (n = 863); during these visits swabs for microbiological cultures were obtained in 629 children at 1.5 month (71%), 785 at 6 months (89%) and 718 (83%) at 14 months. The Generation R Focus Study started in April 2003, data collection on bacterial colonisation started in November 2003, and so data on bacterial colonisation in the first 224 participants at 1.5 months of age are missing. A total of 368 children provided three samples. None of the children used antibiotics in the 48 hours preceding nasopharyngeal sampling. The Medical Ethics Committee of the Erasmus Medical Center, Rotterdam, has approved the study and written informed consent was obtained [12].

Bacterial cultures

At the research center a nasopharyngeal swab was obtained for isolation of *Streptococcus pneumoniae, Moraxella catarrhalis* and *Haemophilus influenzae* and a nasal swab for *S. aureus* isolation. Methods of sampling were described in detail previously [3, 13]. For the final analyses we grouped the airway pathogens *S. pneumoniae, M. catarrhalis* and *H. influenzae* [8] and assessed the total number of positive cultures with the airway pathogens during the first year of life. This generated a score between zero (never colonised) and nine (colonised with all three airway pathogens at all three measurements). Thus, children with more than one positive culture were co-colonised with more airway pathogens at the same moment, and/or colonised at more than one point in time.

Information about date of birth, birth weight and gender was obtained from midwives and hospital registries. Gestational age was based on pregnancy dating by early ultrasound. Information about pre- and postpartum maternal smoking, socio-economic status, breastfeeding at 6 months, day care attendance in the first year of life and the presence of siblings was assessed by questionnaires at the age of 6, 12 and 24 months and analysed in a dichotomous way.

Respiratory tract symptoms

Respiratory tract symptoms were assessed by using an age-adapted version of the asthma-core questionnaire of the "International Study of Asthma and Allergies in Childhood" (ISAAC) [14] at the age of 12, 24, 36 and 48 months. Parents were asked questions regarding wheezing in the preceding year. For respiratory tract infections we defined three subgroups: child has not been to a doctor with fever and cough/runny or blocked nose/ear ache in the preceding year, child has been to a doctor with fever and cough/runny or blocked nose/ear ache once or twice in the preceding year and child has been to a doctor with fever and cough/runny or blocked nose/ear ache more three times ore more in the preceding year. For the analyses we assessed the risk of colonisation on frequent respiratory tract infection (three times or more) compared to no doctor visit for respiratory tract infection.

Statistical methods

We compared the selected children with serial swab data (n = 368) versus the children who were available for postnatal analysis but did not have all three swabs available for analyses. We assessed the differences using chi-square tests for categorical variables and Mann-Whitney U tests for continuous variables. To reduce potential bias associated with missing data, for all determinants the missing values were put in the model as separate category and thus adjusted for in the analyses. Univariate logistic regression analysis was performed to report on the association of bacterial colonisation with wheezing and respiratory tract infection. Subsequently, we used gender, birth weight, gestational age, socio-economic status, pre- and postpartum smoking of the mother, breastfeeding, day care attendance in the first year of life and presence of siblings as determinants of bacterial colonisation and respiratory symptoms by adjusting for these variables with multivariate logistic regression analysis. The confounders were chosen based on literature and/or significant effect in our own model. Measures of association are presented by adjusted odds ratios with their 95% confidence interval (CI). A p-value less than 0.05 was considered statistically significant, a p-value of <0.0125 (p-value 0.05 divided by the four pathogens that are tested) was used to adjust for multiple testing. The statistical analyses were performed using the Statistical Package of Social Sciences version 17.0 for Windows (SPSS Inc, Chicago, IL, USA).

RESULTS

The cohort of analysis consists of 1,079 children participating in the Focus cohort who provided colonisation data in the first year of life (figure 1).

The median maternal age was 31.7 years (SD 4.1) and most mothers attended higher education (n = 683, 63.8%). During pregnancy, 8.9% of the mothers (n = 91) smoked, which increased to 12.9% (n = 90) postpartum. The median birth weight of the children was 3509 grams (SD 538) and the median gestational age was 40.0 weeks (SD 1.7). Of the 368 children, 48.3% (n = 512) were girls and 38.9% (n = 371) had at least one sibling. Of all mothers, 279 (29.4%) gave breastfeeding up until the infant's age of at least six months, and 79.4% of the children (n = 721) attended day care in the first year of life. Table 1 shows characteristics of the mothers and children.

Wheezing symptoms decreased in the first four years of life from 28.9% of the children in the first year to 11.1% in the fourth year. A peak incidence of respiratory tract infections was observed in the second year of life (23.8%), followed by a decrease until the fourth year of life (13.4% to 8.1%). Figure 2.

S. pneumoniae, M. catarrhalis and *H. influenzae* colonisation rates increased in the first 14 months of life from 8.9%, 11.6% and 7.0% at 1.5 month, respectively, to 43.5%, 29.6% and

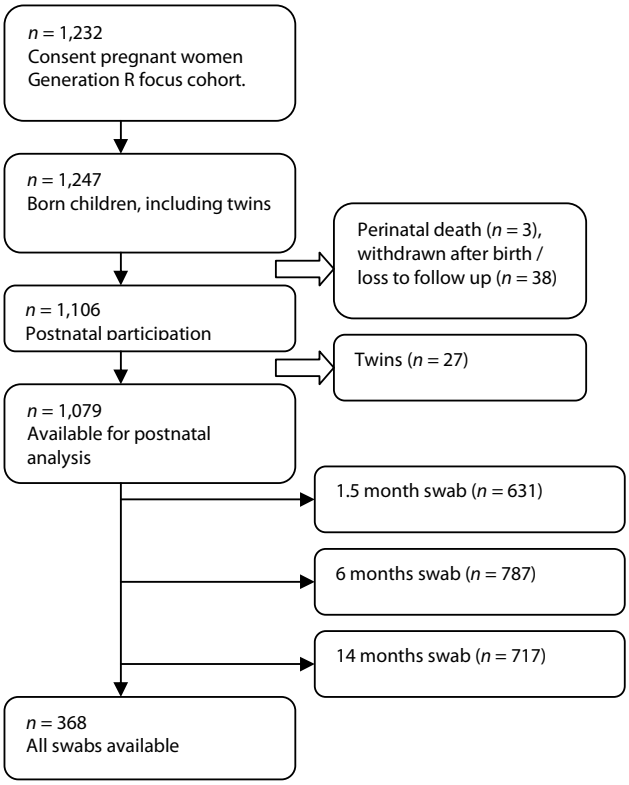

Figure 1 Cohort of Analysis: 1,232 Dutch mothers were invited for the Generation R Focus Cohort. 368 of their children provided three nasopharyngeal swabs in the first 14 months of life.

31.7% at 14 months, respectively [36]. Early in the first year, most infants are colonised with *S. aureus* only (*n* = 267), the most common co-colonisation combinations at
1.5 months of age are combinations with *S. aureus* as well. This turns around at 6 months, when the airway pathogen colonisation rates increase. The most common co-colonisation combinations at 6 and 14 months are the combinations with the airway pathogens, without *S. aureus*. 2.9% and 5.6% of the children at 6 and 14 respectively are colonised with all three airway pathogens at the same moment. Colonisation with all four pathogens at the same moment barely occurs in the first year of life. A fifth of the children (19.6%) never had a positive nasopharyngeal swab in the first 14 months of life. None of the children had three swabs positive for all three pathogens (a score of 9); however 19.3% of the children did have at least four positive samples.

Table 2 shows the association between the 4 pathogens separately at the three moments in the first year of life and respiratory symptoms up until the 4th year. A trend can be observed towards protection against respiratory tract infections later in childhood following colonisation early in life. None of the associations was significant after adjustment for multiple testing.

TABLE 1. Maternal and child characteristics

Parameter	Value for mothers
	n = 1,079
Maternal age (years)	31.7 (4.1)
Mother educational level	
- low/intermediate	382 (36.2%)
- high	683 (63.8%)
Parent atopy	132 (12.6%)
Mother smoking	
- prepartum	91 (8.9%)
- postpartum	90 (12.9%)
Parameter	Value for children
	n = 1,079
Gestational age (weeks)	40.0 (1.7)
Birth weight (grams)	3509 (538)
Gender female	512 (48.3%)
One or more siblings	371 (38.9%)
Day care attendance	721 (79.4%)
Breast feeding at 6 months	279 (29.4%)
Wheezing	
- 1st year	276 (28.9%)
- 2nd year	166 (17.5%)
- 3rd year	104 (11.7%)
- 4th year	99 (11.1%)
Respiratory tract infection ≥3x	
- 1st year	188 (20.7%)
- 2nd year	217 (23.8%)
- 3rd year	117 (13.4%)
- 4th year	70 (8.1%)

Values of maternal age, gestational age and birth weight are means (SDS). All other values are the numbers with percentages. Data were missing on mother educational level (*n*=14), maternal smoking prenatal (*n*=67), maternal smoking postnatal (*n*=383), siblings (*n*=125), day care attendance (*n*=261), breastfeeding (*n*=68), wheezing in the 1st year (*n*=124), in the 2nd year (*n*=132), in the 3rd year (*n*=193), in the 4th year (*n*=191), RTI in 1st year (*n*=170), in the 2nd year (*n*=168), in the 3rd year (*n*=207), in the 4th year (*n*=214).

The associations between airway pathogen co-colonisation combinations and respiratory tract symptoms are shown in table 3. A trend was observed towards protection against frequent respiratory tract infections in the following years for several airway pathogen co-colonisation combinations at 14 months.

Frequent colonisation in the first year of life was associated with increased risk of wheezing symptoms in the following year, but not in the years after. Children colonised at least 4 times in the first year of life held an 8.62 increased risk to develop wheezing complaints in the second year of life (95%CI 2.06 – 36.11), table 4. More frequent colonisation moments in the first year of life, give higher the risk to develop these complaints.

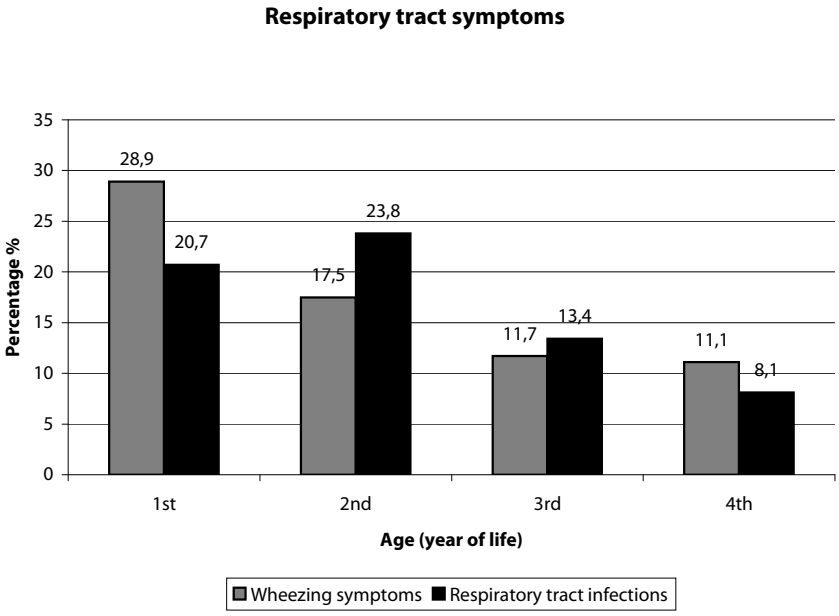

Figure 2 Parentally reported wheezing complaints an respiratory tract infections, presented by year. A decrease of respiratory symptoms was observed from the second to the fourth year (17.5% to 11.1 % for wheezing and 23.8% to 8.1% for respiratory tract infections), following an increase of respiratory tract infections from the first (20.7%) to the second year of life (23.8%).

In contrast, frequent colonisation in the first year of life seems to protect children from respiratory tract infections in the following years up until the 4th year of life. An airway pathogen score of three held the following adjusted odds ratios: aOR 0.19 (95%CI 0.06 – 0.66), aOR 0.18 (95%CI 0.04 – 0.86) and aOR 0.17 (0.03 – 1.08) for the second, third and fourth year of life respectively (Table 4.).

Comparisons between 368 children included in the final analyses and children in the total cohort of analyses revealed some selection bias, but since these determinants are also confounders and thus adjusted for in the analyses this will not affect the results significantly. The children with serial swab data available (n = 368) were less likely to be born to a mother who continued smoking prepartum (5.4% compared to 10.9%), no differences were seen in relation to postpartum smoking of the mother. These children were also less likely to attend day care (74.7% compared to 83.2%).

DISCUSSION

Frequent colonisation with any of the airway pathogens in the nasopharynx during the first 14 months was associated with an increased risk of wheezing and a decreased risk of respiratory tract infections. Our findings suggest that the cumulative number of airway pathogen-

TABLE 2. Nasopharyngeal bacterial colonisation and respiratory tract symptoms

	Wheezing 2nd year	Wheezing 3rd year	Wheezing 4th year
	aOR (95% CI)	aOR (95% CI)	aOR (95% CI)
1.5 month			
- *S. aur*	1.30 (0.82–2.08)	0.55 (0.29–1.05)	0.82 (0.44–1.51)
- *S. pneu*	1.08 (0.48–2.43)	1.24 (0.45–3.42)	0.59 (0.20–1.75)
- *H. inf*	0.42 (0.13–1.33)	0.99 (0.27–3.65)	1.04 (0.32–3.37)
- *M. cat*	1.14 (0.55–2.37)	1.03 (0.37–2.85)	0.86 (0.31–2.37)
6 months			
- *S. aur*	1.23 (0.76–1.98)	1.05 (0.57–1.92)	0.82 (0.42–1.58)
- *S. pneu*	0.93 (0.60–1.45)	0.91 (0.51–1.62)	0.90 (0.50–1.62)
- *H. inf*	1.44 (0.91–2.28)	1.50 (0.84–2.67)	1.11 (0.60–2.04)
- *M. cat*	1.43 (0.93–2.19)	1.82 (1.08–3.09)*	1.11 (0.62–1.97)
14 months			
- *S. aur*	1.31 (0.75–2.30)	1.73 (0.91–3.30)	2.08 (1.10–3.93)*
- *S. pneu*	0.95 (0.62–1.47)	1.16 (0.68–1.96)	0.97 (0.57–1.66)
- *H. inf*	1.35 (0.86–2.14)	0.64 (0.35–1.17)	0.59 (0.31–1.11)
- *M. cat*	1.22 (0.77–1.93)	0.73 (0.40–1.32)	1.20 (0.67–2.15)
	RTI 2nd year	RTI 3rd year	RTI 4th year
	aOR (95% CI)	aOR (95% CI)	aOR (95% CI)
1.5 month			
- *S. aur*	0.64 (0.40–1.01)	0.62 (0.34–1.12)	0.51 (0.26–0.98)*
- *S. pneu*	1.10 (0.48–2.52)	0.37 (0.09–1.44)	0.97 (0.31–3.06)
- *H. inf*	0.92 (0.33–2.55)	0.85 (0.21–3.41)	2.32 (0.75–7.21)
- *M. cat*	1.04 (0.49–2.21)	0.59 (0.17–2.05)	1.59 (0.63–4.04)
6 months			
- *S. aur*	1.04 (0.65–1.67)	0.89 (0.48–1.63)	1.31 (0.62–2.74)
	0.60 (0.38–0.95)*	0.76 (0.42–1.34)	0.89 (0.42–1.87)
- *S. pneu*	1.25 (0.78–2.01)	0.69 (0.37–1.31)	0.53 (0.21–1.35)
- *H. inf*	1.35 (0.88–2.06)	1.18 (0.69–2.04)	1.15 (0.57–2.33)
- *M. cat*			
14 months			
- *S. aur*	1.42 (0.80–2.49)	1.07 (0.52–2.23)	0.90 (0.34–2.34)
- *S. pneu*	0.82 (0.53–1.26)	0.89 (0.52–1.53)	0.78 (0.40–1.50)
- *H. infl*	0.69 (0.44–1.09)	0.76 (0.43–1.37)	0.46 (0.20–1.05)
- *M. cat*	0.79 (0.50–1.25)	0.51 (0.27–0.96)*	0.50 (0.22–1.12)

RTI = Respiratory tract infections. Results are presented as adjusted odds ratios (24) and a 95% confidence interval (CI) * *p-value <0.05, ** p-value <0.0125*

positive cultures in the first 14 months of life is an important risk factor for the development of wheezing symptoms a year after, but seems to protect children from respiratory tract infection at the long run, at least until age four.

Our findings support the theory that children attending day-care in the first year of life, which is an important determinant of airway pathogen colonisation, will go through several clinical and subclinical infections [15-18], resulting in an immune response that seems to protect the children from respiratory tract infections in the following years. However, colonisation

TABLE 3. Nasopharyngeal bacterial co-colonisation and respiratory tract symptoms

	n	Wheezing 2nd year	Wheezing 3rd year	Wheezing 4th year
		aOR (95% CI)	aOR (95% CI)	aOR (95% CI)
Co-colonisation 6 months				
S. pneu and *H. inf*	42 (5.3%)	1.76 (0.72–4.29)	1.17 (0.31–4.36)	1.57 (0.47–5.23)
S. pneu and *M. cat*	41 (5.2%)	0.89 (0.33–2.37)	1.33 (0.41–4.33)	0.54 (0.12–2.53)
H. inf and *M. cat*	34 (4.3%)	1.51 (0.57–4.01)	3.16 (1.07–9.28)*	0.79 (0.20–3.06)
S. pneu and H. inf and M. cat	23 (2.9%)	1.11 (0.29–4.29)	0.81 (0.10–6.76)	1.66 (0.40–6.90)
Co-colonisation 14 months				
S. pneu and *H. inf*	75 (10.5%)	1.93 (0.95–3.93)	1.10 (0.46–2.62)	0.79 (0.27–2.31)
S. pneu and *M. cat*	54 (7.5%)	2.11 (1.01–4.43)*	1.21 (0.48–3.09)	1.69 (0.67–4.27)
H. infl and *M. cat*	31 (4.3%)	2.17 (0.83–5.72)	1.07 (0.29–3.92)	n/a
S. pneu and H. inf and M. cat	40 (5.6%)	0.38 (0.08–1.70)	n/a	0.66 (0.14–3.10)
		RTI 2nd year	RTI 3rd year	RTI 4th year
		aOR (95% CI)	aOR (95% CI)	aOR (95% CI)
Co-colonisation 6 months				
S. pneu and *H. inf*	42 (5.3%)	0.52 (0.18–1.52)	0.16 (0.02–1.24)	0.35 (0.04–2.96)
S. pneu and *M. cat*	41 (5.2%)	0.87 (0.34–2.19)	1.69 (0.60–4.75)	2.47 (0.71–8.58)
H. inf and *M. cat*	34 (4.3%)	2.65 (1.03–6.81)*	2.04 (0.73–5.70)	0.37 (0.04–3.26)
S. pneu and H. inf and M. cat	23 (2.9%)	0.38 (0.08–1.82)	n/a	0.85 (0.10–7.40)
Co-colonisation 14 months				
S. pneu and *H. inf*	75 (10.5%)	0.54 (0.26–1.11)	0.93 (0.40 – 2.18)	0.41 (0.11–1.50)
S. pneu and *M. cat*	54 (7.5%)	0.54 (0.25–1.15)	0.53 (0.19 – 1.48)	0.40 (0.11–1.42)
H. infl and *M. cat*	31 (4.3%)	0.64 (0.23–1.77)	0.78 (0.21 – 2.91)	0.58 (0.12–2.70)
S. pneu and H. inf and M. cat	40 (5.6%)	0.45 (0.18–1.14)	0.51 (0.14 – 1.86)	0.53 (0.11–2.43)

RTI = Respiratory tract infections. Results are presented as adjusted odds ratios and a 95% confidence interval (CI)
** p-value <0.05, ** p-value <0.0125*

seems to increase the chance of wheezing symptoms in the second year of life. Several studies observe an increased risk of wheezing complaints in early childhood following day-care attendance [19-20], this may reflect our results showing an association between colonisation and an increased risk of wheezing. Wheezing in these first few years of life are often thought to be caused by viral infections. However, some of these wheezing symptoms are not transient and develop into childhood asthma.

Bisgaard et al presented the importance of bacterial colonisation with AWP in the development of asthma in childhood [8]. In a high-risk cohort of infants born to asthmatic mothers, they discovered that early hypopharyngeal colonisation with AWP *S. pneumoniae, H. influenzae and M. catarrhalis* increases the risk of wheezing episodes in pre-school children and asthma in 5-years old. However, we can not yet confirm Bisgaard's finding of an important role on early bacterial colonisation on the development of wheezing at older ages and later asthma. The effect of colonisation on wheezing symptoms was not documented in our cohort in the third and fourth year of life. These conflicting results may be due to their cohort

TABLE 4. Frequent bacterial colonisation and respiratory tract symptoms.

	N	Wheezing 2nd year	Wheezing 3rd year	Wheezing 4th year
		aOR (95% CI)	aOR (95% CI)	aOR (95% CI)
Frequency of airway pathogen colonisation				
- never	72 (19.6%)	1.00	1.00	1.00
- once	80 (21.7%)	2.48 (0.65–9.44)	1.18 (0.29 – 4.76)	1.59 (0.44 – 5.78)
- twice	87 (23.6%)	5.26 (1.47–18.88)**	0.47 (0.09 – 2.41)	0.45 (0.10 – 2.11)
- three times	58 (15.8%)	5.65 (1.31–24.31)*	0.88 (0.16 – 4.93)	3.49 (0.80 – 15.28)
- 4 times or more	71 (19.3%)	8.62 (2.06–36.11)**	2.99 (0.66 – 13.62)	0.99 (0.19 – 5.19)
	N	RTI 2nd year	RTI 3rd year	RTI 4th year
		aOR (95% CI)	aOR (95% CI)	aOR (95% CI)
Frequency of airway pathogen colonisation				
- never	72 (19.6%)	1.00	1.00	1.00
- once	80 (21.7%)	0.57 (0.23 – 1.42)	0.59 (0.21 – 1.67)	0.30 (0.08 – 1.14)
- twice	87 (23.6%)	0.28 (0.10 – 0.77)*	0.19 (0.05 – 0.71)*	0.17 (0.04 – 0.78)*
- three times	58 (15.8%)	0.19 (0.06 – 0.66)**	0.18 (0.04 – 0.86)*	0.17 (0.03 – 1.08)
- 4 times or more	71 (19.3%)	0.41 (0.14 – 1.20)	0.22 (0.05 – 0.98)*	0.26 (0.05 – 1.34)

For this analyses *n*=368 children were included who provided all three swabs in the first year of life.
RTI = Respiratory tract infections. Results are presented as adjusted odds ratios (aOR) and a 95% confidence interval (CI) ** p-value <0.05, ** p-value <0.0125*

with children high at risk for atopy compared to our healthy birth cohort. More over, small number of cases in our analyses may also play a role.

Whether there is a direct causal relationship between colonisation with bacterial pathogens early in life and the development of childhood asthma or whether this colonisation is an epiphenomenon secondary to an alternative asthma predisposition remains unclear [21]. Bacterial colonisation in infants may act as an environmental trigger in genetically susceptible individuals. However, and most likely, the mechanisms linking microorganisms to wheeze will be primed or caused by a variety of heterogeneous mechanisms [22].

In the current study we observe a protective effect of colonisation on respiratory tract infection in the long run. Apparently, colonisation may result in a clinical or sub-clinical infection during the days to weeks when a subject is colonised. The induced immune response during that episode may result in protection against respiratory tract infections later on. There is such a strong protective effect of frequent airway pathogen colonisation on respiratory tract infections that we may expect that not only bacterial infections but also viral infections are prevented. We hypothesize that bacterial colonisation may actually be the first step towards a viral infection. Due to the presence of a bacterial pathogen a novel port d'entrée may allow viruses to enter the mucosa. Even though it is most commonly thought that viral infection is followed by a bacterial super-infection rather than the other way around, this data suggests

that bacterial colonisation actually protects against viral infection in the long run. We may hypothesize that bacterial colonisation may increase the risk of viral infection at the moment of colonisation, resulting in an immune response not only against the bacterial coloniser but also against the viral microbes.

Our study is conducted in a population-based cohort which makes our findings generalisable to the general population. Another strength of our study is the availability of a broad range of data on potential confounders. Our outcome measurement was based on parental report. This has a lower diagnostic specificity as compared to a genuine clinical work-up. However, the questionnaires were validated in school-aged children and age adapted for the younger ones and have successfully been used before [23]. Some limitations of our studies need to be discussed. We found a significant and strong association between frequent airway pathogen colonisation in the first year of life and wheezing symptoms early in life. The 95% confidence intervals, however, were wide, as can be expected with relatively small numbers of individuals. Furthermore, there might be underreporting of smoking, which is an important confounder in our study that was put in our model. . However the children with missing data on maternal smoking, as compared to the children with no missing data did not significantly differ in the outcomes.

Missing data in confounding factors were analysed as separate category and thus accounted for. The 368 children with all three swabs available were slightly less likely to attend day care. Day care increases the risk on both nasopharyngeal carriage as well as infections. Again, the children with missing data on day care attendance did not significantly differ in the outcomes as compared to the children with no missing data.

In conclusion, we show in an unselected birth cohort that the nasopharyngeal colonisation frequency with airway pathogens in the first year of life is associated with increased wheezing symptoms in the second year of life, but protects children from frequent respiratory tract infections from the second to the fourth year of life. This latter protection may be due to humoral immune response following a clinical or subclinical infection at the time of colonisation. Further studies are required to clarify the pathophysiologal mechanisms of bacterial colonisation and co-colonisation with other bacterial and viral pathogens on the immune system and the development of wheezing illness in early life and asthma later in childhood and respiratory tract infections. Moreover, the role of the immune response in colonisation and the path from colonisation to disease should be further clarified.

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Chapter 5

From colonisation to disease: a helicopter view

What did we know about nasal and nasopharyngeal bacterial colonisation and where were the gaps?
What did we add to the existing knowledge on nasal and nasopharyngeal bacterial colonisation?
Future perspectives: What issues became or remained gaps? What should the next steps be?

WHAT DID WE KNOW ABOUT NASAL AND NASOPHARYNGEAL BACTERIAL COLONISATION AND WHERE WERE THE GAPS?

Bacterial colonisation has long been an established risk factor for horizontal spread of pathogens and disease development even in healthy subjects [1].

Colonisation with the airwaypathogens (AWP) *S. pneumoniae, H. influenzae* and *M. catarrhalis* increases the risk of both upper and lower respiratory tract infections in children at the time of colonisation [2-7]. Respiratory infections are still a primary cause of hospitalization in young children and cause a great burden to the child, the care-givers and society. The nasopharynx, the main location for colonisation with these pathogens, is in direct connection with other parts of the respiratory tract including the ears and lower respiratory tract. Migration from the nasopharynx to these other parts may result in respiratory infections such as otitis media, sinusitis and pneumonia [1, 7-9].

S. aureus is also considered a common bacterial cause of pneumonia, though most frequently *S. aureus* infections are located in the skin. *S. aureus* is a common inhabitant of the anterior nares in both adults and children which has been identified as a risk factor for *S. aureus* infection [10].

Due to the great burden of pneumococcal disease worldwide in children below the age of 5 [11], prevention policies have led to the implementation of pneumococcal conjugate vaccine (PCV) in national vaccine programmes in most developed countries. In The Netherlands, the PCV-7 was implemented in our vaccine programme in 2006, which successfully resulted in less vaccine-type pneumococcal infections [12]. However, following the implementation of PCV, which eradicated the most common pneumococcal serotype species from the nasopharynx, a shift has occurred towards more frequent colonisation with non-vaccine pneumococcal serotypes and other pathogens such as *S. aureus, H. influenzae* and *M. catarrhalis* [13-15]. New interaction between species may occur. This all may lead to new problems related to these species. In order to develop alternative strategies, understanding of the dynamics and determinants of colonisation in healthy children and the impact of colonisation, the consequences, in youngsters is essential. This thesis aimed to study colonisation with *S. aureus, S. pneumoniae, H. influenzae* and *M. catarrhalis* in healthy children in the first years of their life.

WHAT DID WE ADD TO THE EXISTING KNOWLEDGE ON NASAL AND NASOPHARYNGEAL BACTERIAL COLONISATION?

Bacterial colonisation dynamics and determinants

We described, in a large group of children born in Rotterdam, the Netherlands, colonisation dynamics of four bacterial species in the first years of childhood.

We discovered that newborns show similar colonisation rates with *S. aureus* as compared to adults, which was independently influenced by their gender (boys are at higher risk). More

than 50% of the 6-week old infants were found culture-positive for nasal colonisation with this pathogen [16]. This is a similar number to what one would expect from a random healthy adult sample [10, 17]. As previously described, in an adult population we can distinguish persistent carriers, subjects whom are permanently colonised with *S. aureus* in their anterior nares, from intermittent and non-carriers [18-19]. These persistent carriers are not only identified by the fact that they are culture positive for every single swab, also they were found to be inhabited by genotypically the same strain over time. This suggests some type of match between host and pathogen that leads to a comfortable situation for both. In our study we tried to identify the initiation of this match by studying the phenomenon of persistent colonisation in early childhood. In the first year of life, no such persistent carriers were identified among our Dutch infants [16]. A few of these children (10 of 353) were culture-positive for all three swab moments in the first year of life, however only 3 of them carried a genetical identical strain. This may suggest persistent carriage in these 3 individuals, however, it may also reflect persistent carriage in their care-givers. Re-colonisation form a close-contact person seems more likely to occur in these rare cases. Consequently we may conclude that this match between host and pathogen that leads to permanent colonisation does not seem to have its origins in the first year of life. Hence, we continued to study these children until age 3. A quick glance at the colonisation data of our children at age two and three gives us no indication to believe persistent colonisation starts at age 2 (unpublished data). Many children colonised at age two were not at age three and vice versa. The next step was to study an adult risk factor for persistent colonisation in children to see if that may give us a clue for a maturing match. Glucocorticoid receptor single nucleotide polymorphisms (SNPs) were reported to be associated with persistent *S. aureus* colonisation in healthy Dutch adults [20]. Interestingly, these SNPs were not associated with colonisation in infancy until age 2, which we would have expected given the fact that we concluded persistent carriage does not exist at this age. But in addition to the lack of association until age two, we showed similar associations as shown in Dutch adults at the child's age of 3. This may be a first hint to suggest that a match between host and pathogen may occur at or around this age, as before age three the apparent random colonisation dynamics were not associated with the glucocorticoid receptor gene SNPs.

Persistent colonisation with S. aureus does not exist in infancy. A perfect match between host and pathogens may establish at age 3 when similar associations are found with glucocorticoid receptor gene polymorphisms as earlier described in adults. This may be a first clue towards a maturing staphylococcal colonisation pattern.

In infancy, a search for a perfect host-pathogen match may be the reason for the lack of persistent colonisation with *S. aureus*. However, several studies reported interactions between common habitants of the airways, which may also play a role. We may hypothesize that inter-

actions with species such as *S. pneumoniae, H. influenzae and M. catarrhalis* [1, 21-22], most emerging in early childhood, result in the lack of persistent colonisation of *S. aureus* at a young age. Whereas *S. aureus* colonisation occurs very frequently in newborns with rates above 50%, common childhood pathogens such as *S. pneumoniae, H. influenzae and M. catarrhalis* are much less frequently found in newborns with rates up to 10% in healthy infants [23-24]. In the first year of life, the number of infants colonised with *S. aureus* rapidly decreases, and with the same speed the airway pathogen colonisation rates increase. These AWP remain more frequently found in the nasopharynx up until at least the age of three years. Since we know that these pathogens rarely colonise healthy adults [25] in contrast to *S. aureus*, we expect a decrease in colonisation rate with the AWP and a catch up in *S. aureus* colonisation rate. It is unknown, though, at what age this occurs. *Preliminary data* from the Generation R Study of the 5 year old children suggests this happens in young school-aged children, as presented in Figure 1.

Moreover, a proper look at the graphs makes one realize that the *S. aureus* curve is a mirror image-like from the pneumococcal colonisation prevalence curve. This supports our previously mentioned hypothesis of bacterial interference as a potential reason for the lack of *S. aureus* persistent colonisation. The AWP are often studied individually in their risk of causing infectious problems. However and obviously, the nasopharyngeal environment should not be sketched in such simplicity. Interaction between species take place, both bacteria and viruses and co-infections are often present. These organisms are in a complex yet balanced relationship with each other and thereby manipulation of one may trigger effects on other components of the flora. This also became obvious upon the implementation of the pneumococcal PCV's, where pneumococcal infections were replaced by staphylococcal infections [12]. Earlier studies suggested interference between *S. pneumoniae* and *H. influenzae*. However suggestions on the direction of this interaction are contradicting in different studies. Moreover, *S. aureus* and *S. pneumoniae* colonisation patterns were described to be inversely correlated which is supported by the mirror-image graphs in figure 1 [21-22]. The aetiology of this interaction is, as yet, unknown. It was found to be genotype independent, the pneumococcal pilus was proposed to play a role as well as the immune response and hydrogen peroxide production by the pneumococcus [26-29].

We tried to help explain this interaction on the basis of differential natural IgG and IgA responses. We did not observe an effect from maternal pathogen specific IgG against colonisation with the other pathogen. Nor did we find an effect from the IgG and IgA-response of the child him/herself on colonisation with the other pathogen. So even though we know that there are significant interactions between pathogens in the airways of children, we could not explain the interaction between *S. pneumoniae* and *S. aureus* on the basis of certain specific antibodies.

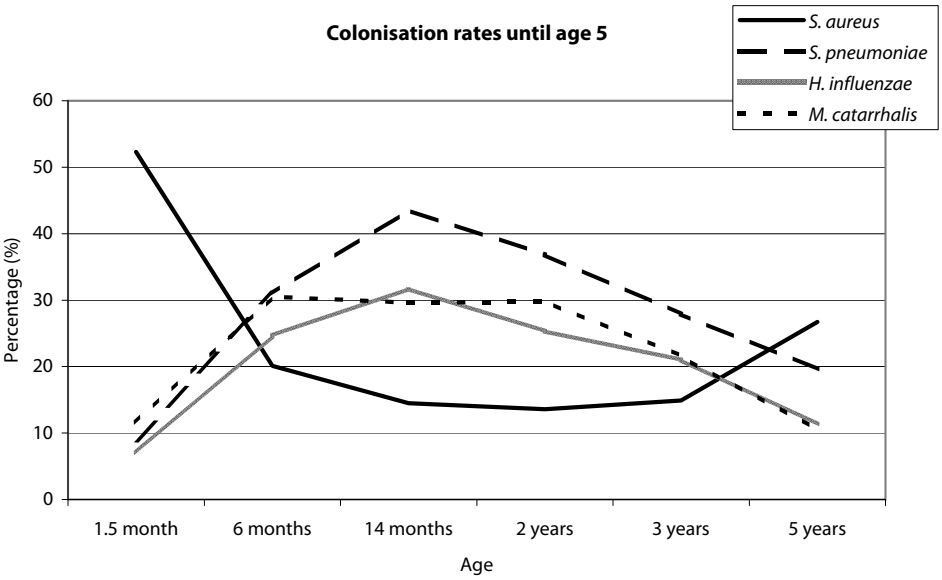

Figure 1. Colonisation rates until age 5

Mirror-image graphs represent opposing bacterial colonisation curves in the first years of life. The interaction between S. pneumoniae and S. aureus can not be explained by certain specific antibodies.

Bacterial colonisation and immune response

It is still debatable where the line is between colonisation and infection. Nasal and nasopharyngeal colonisation may be considered as normal representative flora of humans. At the moment an individual presents signs and symptoms of local or systemic infection it is clear that one speaks of infection. However there is a grey area with so-called subclinical infections. May infection be identified by multiplication of the organism, by migration to other sites or by immune response of the host? Colonisation may be just colonisation, but it may also be the start of an infection, an end of an infection, an infection by itself or an innocent bystander.

We studied in a subsample of 57 children the humoral immune response following colonisation and discovered several specific anti-pneumococcal antibodies to be significantly raised following colonisation. Moreover, we studied the anti-staphylococcal humoral immune response following colonisation. At both 14 and 24 months, previously colonised children had significantly higher levels of IgG directed against several staphylococcal proteins. Their levels of IgA directed against certain proteins were elevated as well, whereas the levels of antigen-specific IgM did not differ significantly between colonised and non-colonised children.

These results suggest that colonisation induces a specific humoral immune response. At the very least this indicates that colonisation does something to the human body other than just

inhabiting the anterior nares and nasopharynx, whether it is only inducing an immune response or even a (sub) clinical infection remains to be elucidated.

These specific antibodies may help the body to get rid of the microorganism within days to weeks, although this does not seem to be true for *S. aureus* colonisation in permanent carriers. We have also studied whether these anti-pneumococcal specific antibodies act protective against future colonisation which turned out not to be the case. Specific anti-pneumococcal antibodies were not associated with colonisation later in time. Summarizing, humans systemically produce IgG, IgM and IgA following colonisation, but this does not protect us against future colonisation. This systemical humoral immune response may reflect a (sub) clinical infection prior to, during or following colonisation. Colonisation itself may be perceived as a physically restricted problem where locally produced IgA in saliva and other secretes may play a role. Nasopharyngeal colonisation with the pneumococcus can lead to the development of antibodies against specific capsular polysaccharides [30] and surface proteins [30-31]. Moreover, a study conducted in children attending day-care provided evidence for serotype-specific acquired immunity to pneumococcal carriage for some, but not all serotypes [32]. Hence, we assume that the development of antibodies to capsular and non-capsular components of pneumococcus may contribute to the gradual resistance to colonisation that is observed as children grow older. This is also supported by the elimination of colonisation with vaccine-type pneumococcus following the PCV implementation [13, 33]. This protective effect of PCV against colonisation may be due to transmigration of the antibodies that are produced upon the vaccine from the serum to mucosa. In our study, however the anti-pneumococcal protein antibodies are induced upon colonisation but are no markers of protection against later colonisation. This is in line with results from other observational studies in children where no clear association between antibodies to certain pneumococcal proteins and nasopharyngeal colonisation could be demonstrated [34]. Thus, the role of antibodies, whether capsular or non-capsular, in the natural development of resistance of pneumococcal colonisation, remains unclear.

Recently it has been proposed that the development of antibody independent but pneumococcus-specific CD4+ Th17 cells play an important role in the reduction of duration of colonisation and mucosal disease [35]. Experiments in genetically modified mice confirmed that protection did not depend on antibody [36-38]; instead, protection was critically dependent on CD4+ T-cells. Further studies implicated CD4+ T cells of the IL-17A lineage, which was subsequently shown by others to contribute to the monocyte/ macrophage-derived clearance of primary pneumococcal infection in mice as well [39]. It was documented that significant CD4+ T-cell-dependent reduction in colonisation could be achieved with a mix of pneumococcal proteins [40]. Based on these data we hypothesize that, in humans, protection against pneumococcal colonisation may partly derive from the development of CD4+ IL-17A producing T-cells. These CD4+ IL-17A producing T-cells recognize pneumococcal antigens that are expressed during the course of colonisation. Subsequently, secretion of IL-17A from these cells may recruit phagocytes includ-

ing neutrophils or macrophages to the site of colonisation and help reduce the duration of carriage, rather than complete eradication.

The systemical humoral immune response following colonisation may reflect a (sub)clinical infection prior to, during or following colonisation and is not associated with future colonisation.

Bacterial colonisation and atopic diseases

Early 2010 two publications following the 99th Dahlem Conference on Infection, Inflammation and Chronic Inflammatory Disorders reported an update on the current ideas about the so-called 'hygiene hypothesis' . One article was pro- and one article was clearly questioning this hypothesis that dates from 1958 following a study by Strachan who observed an inverse correlation between hay fever and the number of older siblings in British children [41]. This illustrates that the role of microbes in the development of atopic diseases is still unravelled and leads to discussion amongst specialists in this field. The hygiene hypothesis proposes that the epidemic of allergic and auto-immune diseases is due to a lack of interactions between humans and microbes. This may be due to a changed lifestyle in developed countries during last century. Basically it precipitates into the idea that the children in society grow up in and environment too sterile to adequately trigger the immunesystem with pathogens and therewith increase our risk of atopic diseases. Okada et al feel that the hygiene hypothesis is strengthened by epidemiological data showing that subjects migrating from a place with a low-incidence of infection to a high-incidence country acquire the immune disorders with a high incidence at the first generation [42]. This and other data showing a correlation between high atopic disease incidence and high socio-economic status reckons them to favour the hygiene hypothesis, even though these data do not provide a causal link between infections and immune disorders. These data, however, have been challenged by more recent epidemiological literature on the high incidence of allergic asthma in unhygienic American inner cities [43-44], the lack of a preventive effect of probiotics on allergic disease [45] and the involvement of bacterial colonisation in the development of atopic diseases such as asthma and atopic dermatitis [46-47].

In this thesis we describe the risk of developing atopic dermatitis following frequent nasal colonisation with *S. aureus*. Infants with frequent colonisation in the first year of life have double the odds to develop AD complaints in the second year of life [47]. Moreover, this effect is still present when we study the children at older ages. Frequent *S. aureus* colonisation in the first three years of life significantly increases the odds of developing atopic dermatitis in the fourth year of life. We also suggest a role for gene-environment interactions in this association. Firstly, noticed by the fact that the association between frequent *S. aureus* colonisation and atopic dermatitis is strongest in children born to parents with a history of asthma or eczema (unpublished data from the Generation R Study). More specifically we identified that the

effect of *S. aureus* colonisation on the development of atopic dermatitis does not count for children with two copies of wildtype of the glucocorticoid receptor gene. In other words, we may hypothesize that one has to be genetically susceptible to develop AD following *S. aureus* colonisation. In this case there seems to be an effect-modifying role for glucocorticoid receptor SNPs and there may be more SNPs on other genes involved as well [48-49]. At first thought these findings are in conflict with the hygiene hypothesis giving a role of importance to *S. aureus* in the development of atopic dermatitis.

The association between S. aureus colonisation and development of atopic dermatitis is dependent on glucocorticoid receptor polymorphisms. A so-called gene-environment interaction seems to take place.

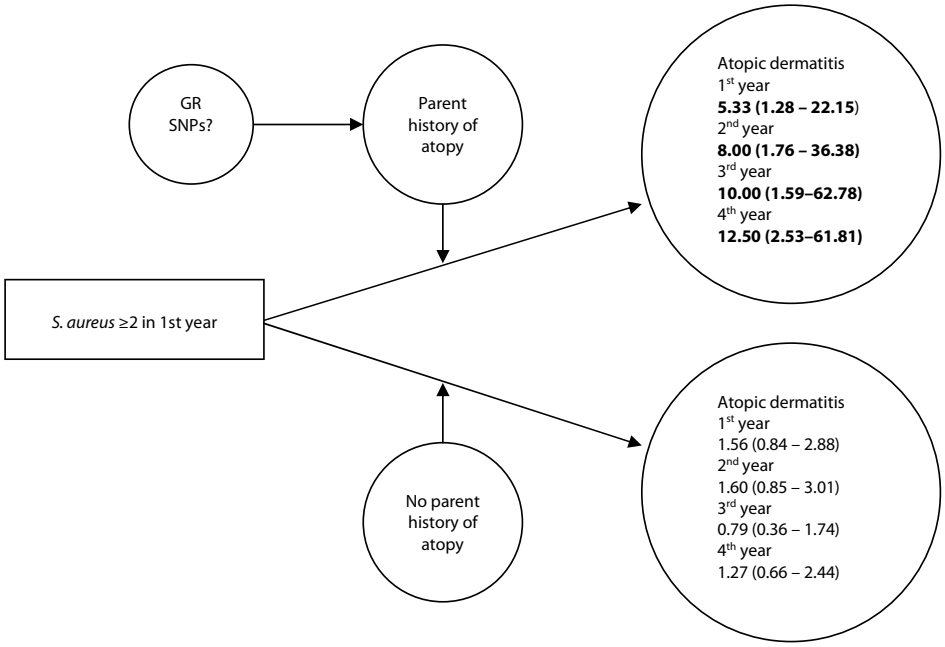

Figure 2. The association between *S. aureus* colonisation in the first year of life seems to be dependent on genetic predisposition towards atopic diseases

Results are presented as crude odds ratios (95% confidence interval) of the association of frequent *S. aureus* colonisation in the first year of life and atopic dermatitis in the 1st to 4th year of life, stratified by parental history of asthma or eczema (atopy).

Bisgaard et al presented the importance of bacterial colonisation with AWP in the development of asthma in childhood [46, 50]. In a high-risk cohort of infants born to asthmatic mothers, they discovered that early hypopharyngeal colonisation with AWP *S. pneumoniae, H. influenzae and M. catarrhalis* increases the risk of wheezing episodes in pre-school children and

asthma in 5-years old. This is yet another example that seems to undermine the hygiene hypothesis.

In our cohort, we find that frequent nasopharyngeal colonisation with the AWP in the first year of life is associated with an increased risk of wheezing symptoms in the following year. Once again, these findings do not plead in favour of Strachan's hypothesis. However, we can not, as yet, confirm Bisgaard's finding of an important role on early bacterial colonisation on the development of wheezing at older ages and later asthma. The effect of colonisation on wheezing symptoms was not documented in our cohort in the third and fourth year of life. Conflicting results are found for frequent airway pathogen colonisation and wheezing at age 3 and 4. Another contrast with their cohort is the lack of association between *S. aureus* colonisation and AD which we observed in our healthy cohort. It seems as if other aetiological mechanisms may play a role in this high-risk cohort of children with a positive atopic disease family history compared to our children from a general population-based birth cohort.

A Finnish intervention trial on probiotic treatment in healthy infants showed that this treatment prevented eczema and atopic eczema significantly [45], but the data could not be replicated by Australian colleagues in a high-risk infant-cohort [51]. Apparently there may be differences in aetiological mechanisms among children who are genetically at a high risk for atopic diseases as compared to low-risk children.

The results of our study, in anyway, challenge the hygiene hypothesis saying that a lack of interaction between pathogens and humans is at the base of the development of atopic diseases. The original proposed mechanism behind the hygiene hypothesis suggests that a deviation in Th1 and Th2-cells explains the protective influence of pathogens from immunological disorders [42]. Th1 T-cells produce inflammatory cytokines such as IL-2, IFN-γ, TNF-α which are involved in cell-mediated immunity. Contrary to Th1-cells, Th2-cells produce IL-4, IL-5, IL-6 and IL-13 that contribute to IgE production and allergic responses. A lack of stimuli of the Th1 cell response may redirect the immune response towards a Th2 response, and therewith predisposes the host to allergic disease [42]. Some recent clinical studies demonstrated that the concept that atopic diseases reflect a Th2/Th1 imbalance is probably too simplistic.

Even though our results are partly in conflict with the hygiene hypothesis, there possibly is some truth in the hypothesis. However, it does not seem to be a matter of black and white. Our and also other recent studies propose that specific bacterial pathogens seem to be associated with later development of atopic diseases. However the lack of presence of certain other pathogens and infections may be an underlying reason. Most likely a shift in microbial environment has taken place over the last decades, which has led to different exposure. This difference in exposure may explain the increased risk of atopic diseases in developed countries. This latter hypothesis saying that microbiota changes could contribute to the modulation of immune disorders was earlier suggested but evidence is still limited [52].

Nasopharyngeal colonisation with AWP is associated with wheezing symptoms at short term, but not at long term. This seems to be in conflict with the results from a Danish cohort with children high-at risk for asthma. This may be explained by our healthy, low-at risk population.

It is difficult to put these slightly conflicting results in one model (figure 3). Even though we provided new insights into the mechanisms of atopic diseases following bacterial colonisation, new questions were raised as well. We hypothesized that different mechanisms play a role in different asthma phenotypes, but our results on atopic dermatitis following *S. aureus* colonisation became even stronger after stratification for parental history of atopy. Still, different mechanisms may explain the conflicting results between high-risk and low-risk co-

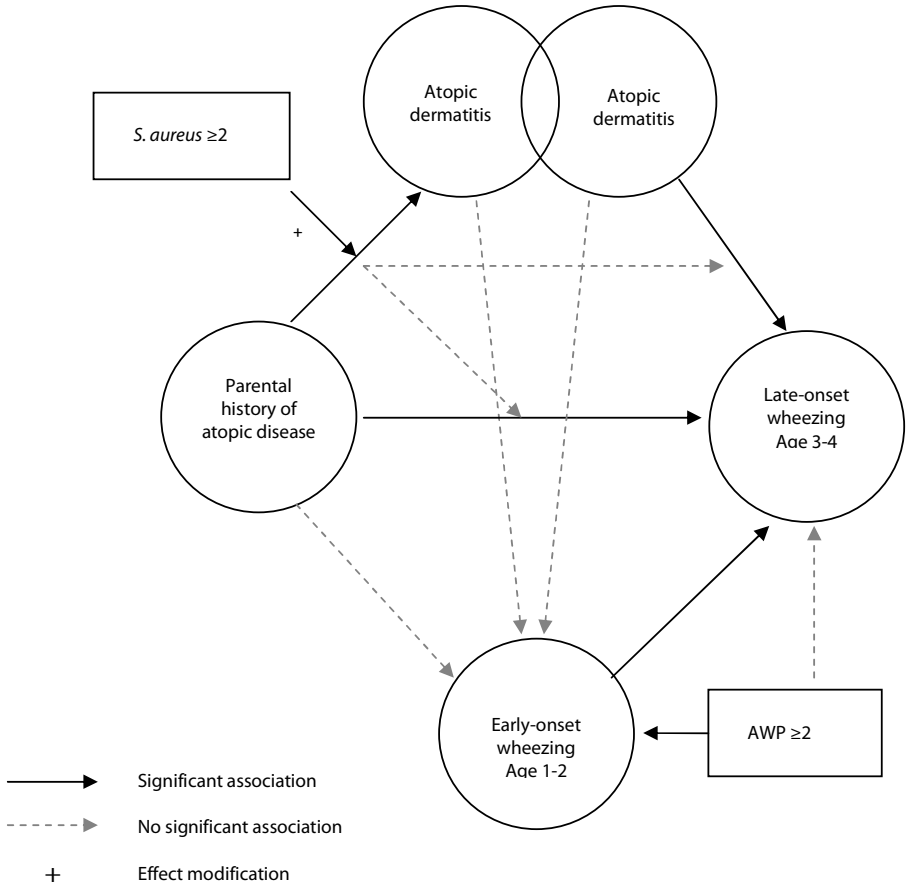

Figure 3. A global overview of the associations between bacterial colonisation and atopic diseases (in parents defined as asthma or eczema)

horts. Moreover these different mechanisms may also explain the discrepancy in our own cohort. *S. aureus* colonisation is associated with AD. AD is associated with late-onset wheezing (third and fourth year of life), but *S. aureus* colonisation is not associated with late-onset wheezing. This suggests perhaps two types of AD. One type of AD that is associated with development of asthma and a type of AD that is triggered by *S. aureus*.

Bacterial colonisation and respiratory tract infections

Colonisation with AWP is undoubtedly known as a direct risk factor for respiratory tract infection [2-4, 6, 8-9, 53]. During colonisation, usually no longer than days to weeks in case of many of the AWP, the microbes get the chance to migrate from the nasopharynx to the ears (via the Eustachian tube), the sinuses, the larynx and the lower airways [1, 7, 54] (figure 4). This may result in both upper and lower respiratory tract infections including otitis media, sinusitis, laryngitis, bronchitis or pneumonia.

In most developed countries, respiratory tract infections, otitis media in particular, are the most common reasons for children to visit a doctor. *S. pneumoniae, H. influenzae and M. catarrhalis* are predominant causes of otitis media as well as members of the commensal flora in the nasopharynx of healthy children [3-5, 8, 54-57]. This has important implications for vaccine strategies for preventing respiratory tract infections including otitis media.

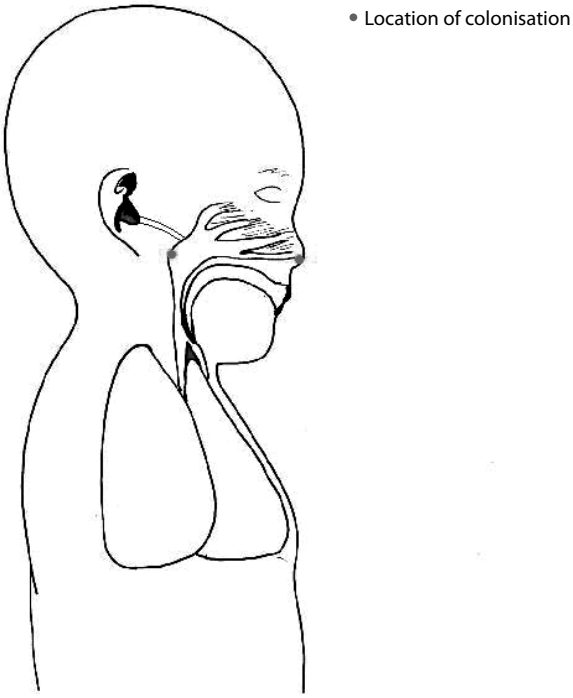

Figure 4. Migratory routes leading from the location of colonisation to focal areas of the upper and lower respiratory tract.

Since there is extensive interaction between microbes in the airways, it is likely that a combination of microbes and their interactions are at the base of respiratory tract infections. Most respiratory tract infections are considered to have a viral origin. They either remain as such, are complicated by a bacterial super-infection, or they may be preceded by bacterial colonisation [58].

Kukavica et al. developed a murine model that showed that co-infection with hMPV and pneumococcus, similarly to co-infection with the influenza virus and pneumococcus, predispose the host to severe pneumococcal pneumonia through different mechanisms [59]. Their study underlines the importance of the PCV in reducing the severity of hMPV and influenza virus infection by preventing pneumococcal super-infection. This is in line with an earlier report from South Africa concluding that the pathogenesis of hMPV-associated respiratory tract infections that result in hospitalization involves bacterial co-infection with pneumococcus and thus will be prevented by pneumococcal vaccination [60].

In our study we observe an association between bacterial colonisation and less respiratory tract infection in the long run. Apparently, based on our results on induction of antibodies following colonisation, colonisation may result in a clinical or sub-clinical infection during the days to weeks when a subject is colonised. The induced immune response during that episode may result in protection against respiratory tract infections later on. In the long run we see that children frequently colonised with AWP in the first year of life are at a significantly lower risk to suffer from respiratory tract infections in the second, third and fourth year of life. Moreover, in our subsample study of 57 children, we noticed that several anti-pneumococcal specific antibodies that were produced upon colonisation were markers of protection against respiratory tract infections. These antibodies, however, did not protect against future colonisation.

Low odds were presented for the association between frequent airway pathogen colonisation and respiratory tract infections that we may expect that not only bacterial infections but also viral infections are prevented. Even though it is most commonly thought that viral infection is followed by a bacterial super-infection rather than the other way around, this data suggests that bacterial colonisation actually may protect against viral infection.

Frequent nasopharyngeal colonisation with AWP in the first year of life is associated with reduced odds to suffer from respiratory tract infections until age 4. This preventive effect may be due to a humoral immune response following a (sub) clinical infection prior to, during or following colonisation.

We hypothesize that bacterial colonisation may actually be the first step towards a viral infection. Due to the presence of a bacterial pathogen a novel port d'entrée may allow viruses to enter the mucosa. This is supported by a recent study that reported on increased levels of hMPV antibodies following pneumococcal colonisation (yet unpublished). Moreover, this is in line with the increased risk on wheezing symptoms at short term following frequent

colonisation. Apparently children colonised in the first year of life are more prone during colonisation to suffer from respiratory tract infections such as virally-induced wheezing. In the long run, it prevents them from respiratory tract infections. The fact that we do not see such a protective effect of wheezing at older ages may be due to difference in wheezing phenotype at age 3 and 4 compared to age 2. At these elevated ages we may be looking at atopic wheezing or the onset of asthma rather than respiratory tract infections.

Vaccines

Pneumoccoccal vaccine

The existing pneumococcal vaccines are based on mixtures of capsular polysaccharides. These polysaccharides enable the distinction of over 90 different pneumococal serotypes. Currently two types of pneumococal vaccines are available: unconjugated and conjugated pneumococcal vaccines. Both of these two are based on capsular polysaccharides. Unconjugated polysaccharide vaccines are not efficacious in children less than 2 years old and, therefore, fail to protect those at highest risk. The conjugated polysaccharide vaccine protects infants as well [61] but is expensive, needs refrigeration, requires multiple injections, and does not include all capsular serotypes that cause pneumococal disease in the developing world [62]. In The Netherlands children are vaccinated with the 7-valent PCV which will soon be replaced by a 23-valent vaccine, covering more serotypes. The pneumococcal serotypes included in the vaccine were chosen on the basis of the relative distribution of the individual serotypes that cause approximately 80-90% of invasive pneumococcal infections [63]. Even though the conjugate vaccine is very successful in reducing overall pneumococal diseases and colonisation with vaccine-type serotypes, there is a co-incidental increase of non-vaccine type disease and colonisation serotype replacement whereby pneumococcal serotypes not included in the conjugate vaccine become more prevalent in colonisation and disease following implementation of conjugate vaccine in national programs [12-13, 33, 64-65]. Additionally, there is also evidence for shifts towards more frequent colonisation with other pathogens inhabiting the same colonisation sites. This may increase the overall burden of disease caused by these pathogens [15].

These are important early signs to suggest that the impressive efficacy of the conjugate vaccine is reduced by this side effect. Therefore, despite the success of the conjugate vaccine, alternative strategies are urgently needed. Hence, the suggestion was raised to develop protein-based vaccines that may work protective against pneumococcal disease regardless of serotype. Adding virulence proteins to the current polysaccharide vaccine may combine the best of both worlds which will hopefully lead to a reduction and maybe even eradication of pneumococcal disease. Several capsular virulence proteins have been identified as putative vaccine candidates such as Ply, CbpA, PspA, PsaA, PiaA, PhtB, PhtE (BVH3) and NanA [66-69]. There is evidence that immunization with certain combinations of virulence proteins provides ad-

ditive or even synergistic protection. Especially the combination of Ply, PspA and CbpA was discovered to work successfully protective in a mouse model of pulmonary infection [67].

In this thesis we describe an innovative tool to measure immunoglobulines against 17 pneumococcal proteins in cord blood and serum samples in children. As discussed above, no protective effect of these specific anti-pneumococcal antibodies on colonisation prevalence was observed. In contrast, we did observe that children with higher levels of certain anti-pneumococcal antibodies were less likely to visit a doctor for respiratory tract infections. In other words, when we compare children who have not visited a doctor for respiratory tract infections as compared to children whom visited a doctor for this reason at least 3 times in a year, this latter group has significantly lower levels of specific antibodies. This counts only for antibodies produced by the child him- or herself at the age of 14 and 24 months. Maternal antibodies from cord blood were not observed to be significantly different among these two groups.

Figure 5 shows associations studied in this thesis with regard to future vaccine research. In our study we found several anti-pneumococcal antibodies to act protective against respiratory tract infections but not against colonisation. If we can create a similar humoral immune response by means of a protein-based vaccine, we may be able to prevent children from suffering from respiratory tract infections although this does not seem to prevent nasopharyngeal colonisation.

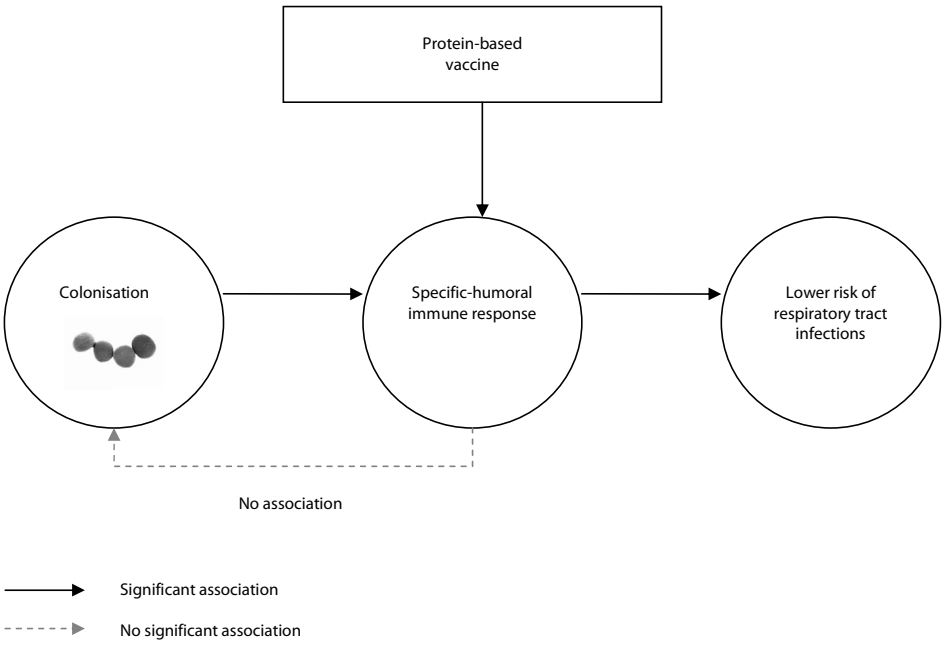

Figure 5. Associations studied in this thesis on anti-pneumococcal antibodies, colonisation and respiratory tract infection.

Combined immunization against Ply, PspA and CbpA works protective in mouse models, which is in line with our results. Levels of antibodies against pneumococcal virulence proteins were significantly higher in the group of children that suffered from less respiratory tract infections.

Since colonisation is the first step towards infection this may sound odd. Should we not aim for prevention of colonisation? There are two considerations

1. If the risk of respiratory tract infections remains decreased even though there is no effect on decreased nasopharyngeal colonisation we should not mind. It may actually be beneficial to keep the pneumococcus in the nasopharynx as this may prevent increased rates of colonisation with other pathogens and the problems associated with those species. It may be the case that the specific humoral immune response is enough to prevent viruses from invade and causing infections following pneumococcal colonisation. It may also be enough to prevent the colonising pneumococcus strain to become invasive and cause infection, however, it may not be enough to prevent the pneumococcus from colonising in the first place. As mentioned before, perhaps we should not worry about this and consider the putatively beneficial part of it, as long as it prevents children from respiratory tract infections!
2. We suggest that antibodies against specific pneumococcal proteins generate protection against disease, whereas the antibodies induced by the current polysaccharide vaccine protect against both infection as well as colonisation, against certain serotypes. As discussed before, colonisation, regardless of serotype, may be shortened in duration by CD4+ IL-17A producing T-cells. It may be worthwhile, considering pneumococcal colonisation as main risk factor for pneumococcal disease, to create an optimal strategy for prevention of pneumococcal disease and colonisation. Perhaps a vaccine addressing both immune responses is most promising [62].

Other vaccines

Besides a multitude of viruses and the pneumococcus, *H. influenzae* and *M. catarrhalis* account for a significant proportion of respiratory tract infections as well. Especially otitis media is a disease for which these pathogens are commonly held responsible. Vaccine-protection against non-typeable *H. influenzae (NTHi)* and *M. catarrhalis* is expected to significantly reduce the burden of otitis media [5].

An 11-valent pneumococcal vaccine conjugate to the *H. influenzae*-derived protein D was demonstrated to provide vaccine-induced protection against acute otitis media. However this has only been demonstrated in one study hence the evidence is limited [70]. Currently NTHi vaccine candidates are being identified [71-72]. Vaccination against *M. catarrhalis* is in the same stage, to date most studies are focussing on identification of potential vaccine candidates [73-74].

FUTURE PERSPECTIVES: WHAT ISSUES BECAME OR REMAINED GAPS? WHAT SHOULD THE NEXT STEPS BE?

Research often provides at least as many new questions as answers and this thesis did so accordingly. The results in this thesis will add to the discussion on colonisation by it self, colonisation as early determinant of atopic diseases and perhaps most clinically relevant these days: it will add to the discussion on the development of improved pneumococcal vaccines. In this section we will address some of the newly raised questions, partly due to some limitations of the current study, and an idea will be provided on how to move on in this field of research.

Bacterial colonisation dynamics and determinants

1. We identified polymorphisms in the glucocorticoid receptor gene as a genetic determinant of *S. aureus* colonisation. We expect other genetic variants to play an additional role. In adults, host polymorphisms in IL-4, complement factor H and C-Reactive Protein (CRP) were associated with *S. aureus* nasal carriage. Future studies should analyse additional genetic aspects, preferably by means of Genome Wide Association Studies (GWAS), of the host-pathogen match. Large consortia with equal longitudinal colonisation data are therefore urgently needed. Until then, candidate gene approaches may help to identify genes involved in colonisation.
2. We studied the potential role of IgG and IgA on the interaction between *S. pneumoniae* and *S. aureus* and found no role for this specific humoral immune response. Within the same cohort, interactions between the four described species were studied and clear associations between *S. pneumoniae* and *S. aureus* and between *S. pneumoniae* and *H. influenzae* were identified. Especially in the light of the current vaccination policy it is important to know the natural interactions that take place in healthy children from the pre-pneumococcal vaccine era in order to properly monitor this in a pneumococcal vaccine era. Moreover, these interactions may lead to a shift in burden of disease from one species to the other following the pneumococcal vaccine implementation. A better understanding of why and how these interactions take place and their consequences may help in the discussion on vaccine policies.
3. This thesis described several determinants of bacterial colonisation. Male gender was an independent risk factor for *S. aureus* colonisation in infancy and crowding for *M. catarrhalis* colonisation. Moreover, in case of *S. aureus* and *H. influenzae* maternal colonisation statuses were associated with colonisation status of the child. Even though we addressed some determinants, there are many more known and unknown determinants that are considered to play a role. Again genetic make-up of the host may predispose children towards (more frequent) colonisation. GWAS may provide clues for identifying those colonisation-prone children. There are also reasons to believe air pollution may play an important role in acquisition of bacterial colonisation, this, however, needs to be further elucidated.

Bacterial colonisation and immune response

1. We described in this thesis that colonised children induce a systemic IgG, IgA and IgM response. We hypothesize that this may be due to (sub) clinical infections. This hypothesis should be explored in future studies in which IgG, IgA and IgM should be monitored more closely around colonisation, but also taking precisely defined respiratory tract infection into account, while the children age.
2. In this thesis we limited our results to systemic IgG, IgA and IgM responses, though many believe colonisation is due to local rather than a systemic condition. In order to assess the difference between a systemic and a local response, it is informative to study IgG, IgA and IgM levels in saliva and nasopharyngeal swab samples.
3. CD4+ IL-17A producing T-cells are considered to be important in reduction of colonisation duration, rather than elimination. No protection by specific anti-pneumococcal antibodies against elimination was discovered in the present study. Future studies should clarify the role of both the specific cellular and specific humoral response in colonisation acquisition, duration and elimination.
4. Since immune responses, both cellular and humoral, seem important in bacterial colonisation it would be interesting to identify host factors associated with such different responses. By means of GWAS we may be available in the future to identify SNPs involved in the immune response-associated genes with bacterial colonisation.

Bacterial colonisation and atopic diseases: relevance of the hygiene hypothesis?

1. In the current presented study, we find early bacterial colonisation to be associated with early wheezing complaints. Another study provided evidence for a role of early colonisation on the development of childhood asthma. The Generation R Study provides a large sample of healthy children in which this question should be addressed once the children further age and may get the diagnosis asthma by age 5 and older.
2. Our study shows that *S. aureus* colonisation is associated with atopic dermatitis, especially in children prone to atopic disease due to genetic susceptibility. Future studies should put this in the light of the association between atopic dermatitis and asthma. What is the mechanism behind the increased risk to develop AD following *S. aureus* colonisation and what is the mechanism behind the increased risk to develop asthma following airway pathogen colonisation while this AD is also associated with development of asthma?
3. Genetic components seem to play an important role in the development of atopic diseases that is why children born to atopic parents are especially prone to develop it themselves. GWAS should identify children genetically at risk to develop atopic diseases. Moreover, in relation to these genetic components environmental factors triggering genetically susceptible infants should be identified.
4. Viruses are commonly considered able to trigger the development of wheezing episodes and childhood asthma. Further studies should explore the role of viruses in bacterial

colonised and bacterial non-colonised children to understand the true (prophylactic or predisposing) role(s) of these pathogens.

5. Earlier in this chapter we questioned the hygiene hypothesis. Further studies should clarify the exact role of microbes and a potential shift in microbial environment in the development of atopic diseases to confirm or undermine the hygiene hypothesis.

Bacterial colonisation and respiratory tract infections: clues for vaccine development?

1. We hypothesized that the association between frequent colonisation and reduced respiratory tract infections at long term may be due to (sub) clinical infections. This hypothesis should be explored in future studies. Children should be closely monitored for respiratory tract infections and signs of infections in serum, prior, during and following the moment of sampling. Sampling should be done highly frequent including quantitative measurements of the cultures. This will give us more insight in the relevance of colonisation in respiratory tract infections.
2. Frequent colonisation with AWP early in life results in protection against respiratory tract infection at later ages. This may be due to increased susceptibility to infections during colonisation resulting in an immune response which later on protects against infections. We additionally hypothesized that a potential protective effect of early frequent bacterial colonisation on later viral respiratory tract infections occurs as well. Future studies should address this hypothesis and describe in more detail the interactions between bacterial colonisation and viruses and, subsequently, the consequences for respiratory tract infections.
3. The emergence of serotypes not included in the first generation 7-valent conjugate vaccine and the demonstration that these strains are important causes of disease, morbidity, and mortality are worrisome. Future studies should definitely prioritize the development of a pneumococcal vaccine with broader serotype coverage. We provide a suggestion on associations between antibodies directed against certain specific virulence proteins and respiratory tract infections. Immunogenicity in humans, its combination with polysaccharides, its safety and efficacy should be further explored. While the proposal to use such protein-based pneumococcal vaccines is not new no such vaccine has made it beyond Phase I clinical trials to date.
4. As proposed earlier in this chapter, it may be worthwhile, in order to create an optimal strategy for prevention of pneumococcal disease, to develop a vaccine that triggers multiple immune responses. Aside from anti-polysaccharide antibodies and anti-pneumococcal protein antibodies, we may be interested in the requirements for the development of IL-17A responses in humans to shorten duration of colonisation and thus shorten the duration of the period at-risk.

5. The anti-pneumococcal antibodies as presented in this thesis may act protective after transmigration to the mucosa. They may also be antibodies produced locally during colonisation that are measurable in serum as well without being directly effective on the local action. This latter suggest the most important role for a local immune response, hence mucosal vaccines, focussing on CD4+ IL-17A producing T-cells, may be interesting. However, this thesis also describes that colonisation is associated with reduced number of future respiratory tract infections. This may suggest that colonisation may actually not just be commensal colonisation but rather a (sub) clinical infection resulting in an immune response. Therefore, it is more likely a systemical response takes place. It should be further explored what the role is for local versus systemic response in the light of vaccine development.

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Chapter 6

Conclusions: Q&A based on the specific aims of this thesis

Question # 1.

What is the prevalence of colonisation with Staphylococcus aureus and Moraxella catarrhalis? Is colonisation in the first year of life a dynamic process?

The prevalence of *S. aureus* colonisation decreases in the first year of life from 53.8% at the age of 1.5 month, to 22.9% at 6 months and 11.9% at 14 months of age. This colonisation rate pattern of *S. aureus* was opposite from the figures obtained for the airway pathogens such as *M. catarrhalis*, which increased in the first year of life up to ~30%. Figure 1 in chapter 5 shows the prevalence of colonisation with *S. aureus, M. catarrhalis, S. pneumoniae* and *H. influenzae* until the age of five. The line that represents *S. aureus* colonisation in young children is a mirror-image from the three lines representing the airway pathogens.

Persistent colonisation, defined as continuous colonisation by the same *S. aureus* strain in time, was rarely detected in infancy whereas this is a common phenomenon in adults. Using PFGE, a mixture of different genotypes was observed in the children from our study, with only three children carrying the same genotype over time. Hence, *S. aureus* colonisation in infancy is a dynamic process of relatively frequent elimination and acquisition with an apparent lack of persistent carriage.

Question # 2.

What are determinants of Staphylococcus aureus and Moraxella catarrhalis colonisation? Are the colonisation statuses of mothers and children linked?

Several determinants had been suggested in the literature to influence staphylococcal colonisation rate in healthy children: number of older siblings, family size, breast-feeding, passive smoking and colonisation with other pathogens.

In our study, only male gender incurred a doubled risk to be colonised in the first year of life. No other determinants were detected for *S. aureus* colonisation. *M. catarrhalis* colonisation, however, was strongly determined by crowding factors such as siblings and day-care attendance.

Moreover, we determined whether colonisation statuses of mothers and children were correlated. Such a correlation was observed for *S. aureus* and *H. influenzae.* Genotyping strains from mothers and children revealed high rates of genotypically indiscriminate strains for *S. aureus* colonisation but not for *H. influenzae*. Direct transmission seems to occur in case of *S. aureus*, whereas other factors such as genetic susceptibility may explain the correlation between maternal and infant *H. influenzae* colonisation rate.

Question # 3.

Are glucocorticoid receptor polymorphisms associated with Staphylococcus aureus colonisation?

Several glucocorticoid receptor (GR) haplotypes were associated with a reduced risk of *S. aureus* colonisation at age three, but not earlier in infancy. In adults, glucocorticoid receptor SNPs were also found to be associated with *S. aureus* colonisation. As described in question 1, persistent colonisation does not exist in infancy. The apparent random colonisation patterns of infants may still have to mature into an adult state. The association between GR SNPs at age three but not in infancy may explain a more mature colonisation state in 3-year-old children as compared to the younger ones.

Moreover, the association between frequent *S. aureus* colonisation and atopic dermatitis in children seems to be dependent on glucocorticoid receptor polymorphisms. An effect-modifying role for the glucocorticoid receptor in the association between *S. aureus* colonisation and atopic dermatitis may exists.

Question #4.

Do pathogen-specific antibodies protect against colonisation and disease? Does colonisation result in a humoral immune response?

For both *S. pneumoniae* and *S. aureus*, the antibody responses showed extensive inter-individual variability. On average, IgG levels were high in cord blood, decreased in the first 6 months and increased again thereafter, contrary to the course of IgA and IgM levels which increased from birth until the age of 2.

Placentally transferred specific maternal IgG did not protect against nasal colonisation with *S. aureus*, against nasopharyngeal colonisation with *S. pneumoniae*, nor against disease outcome.

In staphylococcus-colonised children, IgG and IgA levels for a number of proteins were higher than in the non-colonised children. Therefore, CHIPS, Efb, IsdA and IsdH seem to play a role in *S. aureus* nasal colonisation of young children.

Increased levels of IgG against BVH-3, NanA and SP1003 at 6 months, NanA, PpmA, PsaA, SlrA, SP0189 and SP1003 at 14 months and SlrA at 24 months were associated with decreased RTI in the 3rd year of life. Some of these are induced upon colonisation, however no protection against later colonisation was observed. Nor were maternal antibodies were associated with decreased colonisation and infection.

Question #5.

Is bacterial colonisation associated with an increased risk of respiratory tract infections and atopic diseases?

Staphylococcus aureus colonisation at 6 months as well as frequent colonisation in the first year of life is associated with atopic dermatitis in young children. This association was also documented at an older age: frequent colonisation with *S. aureus* in the first three years of life is associated with an increased risk to suffer from atopic dermatitis complaints in the fourth year of life. As described in the answer to question 3, this effect was dependent on glucocorticoid receptor haplotype.

Frequent nasopharyngeal colonisation with the airway pathogens; *S. pneumoniae, H. influenzae* and *M. catarrhalis* in the first year of life was associated with increased wheezing symptoms in the second year of life, but with lower number of doctor visits for respiratory tract infections from the second to the fourth year of life. This latter association may be due to humoral immune response following a clinical or subclinical infection at the time of colonisation.

Chapter 7

English summary &
Nederlandse samenvatting

SUMMARY

The background and scope of the studies presented in this thesis are given in chapter 1. Colonisation with *Staphylococcus aureus, Streptococcus pneumoniae, Haemophilus influenzae* and *Moraxella catarrhalis* is harmless most of the time, but it increases the risk of endogenous infections. A large fraction of the healthy human population is colonised with *S. aureus*. The most common place where *S. aureus* can be detected is the anterior nares and is permanently cultured in nearly 30% of the adult population. Several determinants have been suggested to influence carriage rates in healthy children. The number of older siblings, family size, breast-feeding and passive smoking were found to be positively associated with *S. aureus* nasal carriage in cross-sectionally observed groups of children. Also, genetic make-up of both host and pathogen may be important in colonisation status. *S. pneumoniae*, also reffered to as pneumococcs, is a cause of superficial respiratory as well as invasive infections. Morbidity and mortality are high; worldwide it causes around 11% of all deaths in children below the age of 5 years. Similar to *S. aureus* colonisation, asymptomatic nasopharyngeal carriage is the primary source for pneumococcal (auto-) infection. Over 90 different pneumococcal serotypes have been identified on the basis of variability in the capsular polysaccharides. The current vaccines cover only some of these serotypes by inducing antibodies against certain capsular polysaccharides. Recent studies report on vaccination-associated shifts in pneumococcal serotypes causing nasopharyngeal colonisation and infection. Moreover, an increase in colonisation rates with other pathogens was observed following the pneumococcal conjugate vaccine implementation in national projects. Protein-based vaccines may provide a future perspective as these vaccines may broadly prevent pneumococcal colonisation and infections regardless of serotype. *H. influenzae* and *M. catarrhalis* colonise the same area as the pneumococcus does, namely the nasopharyngeal cavities and have the same risk factor, namely crowding. Both pathogens are involved in respiratory tract infections as well, most commonly otitis media. Microbial interactions among these four pathogens may also contribute to both colonisation rates as well as the burden of diseases. Although these respiratory tract infections have a low mortality in the developed world and are often self-limiting, morbidity and burden is high to patients, caregivers and the healthcare system. A peak incidence in respiratory tract infections occurs in the children's first two years of life. Asymptomatic colonisation with respiratory pathogens shows its peak incidence around that same age. Recently a Danish study reported the risk of developing asthma following colonisation with the airway pathogens *S. pneumoniae, H. influenzae* and *M. catarrhalis*. Nasal colonisation with *S. aureus* is though to play a role in atopic dermatitis disease severity. Atopic diseases such as asthma and eczema are an increasing clinical problem worldwide. Asthma and AD are supposed to be multifactorial diseases; multiple factors such as genetic and environmental determinants may lead to the development of symptoms. In order to stimulate the development of new preventive measures against the burden caused by certain bacterial species,

it is important to increase knowledge on the human antibody response. A new, innovative, high-throughput immunological assay has been developed recently and is known as so-called xMAP® Technology (Luminex Corporation). This allows for simultaneous quantification of antibodies in the same sample directed to different proteins. *S. aureus* and *S. pneumoniae* multiplex assays were developed by using this flow cytometry-based technique. This technique may help us to gain additional insight in the humoral immune response involved in colonisation and disease in childhood, which may enhance development of new preventive measures (i.e. vaccines) against respiratory tract infections and atopic diseases. The general aim of this thesis was to study the dynamics and kinetics of bacterial nasal and nasopharyngeal colonisation and its consequences in early childhood.

In chapter 2 we describe the dynamics and determinants of bacterial colonisation in infancy. *S. aureus* carriage among young infants is clearly different from that among adults as described in chapter 2.1. Long-term persistent carriage rarely occurs among infants and the incidence of carriage drops enormously in the first year of life. Moreover, boys have a higher risk to be colonised as compared to girls. Chapter 2.2 describes how colonisation rates of *M. catarrhalis* are completely the opposite of *S. aureus* colonisation rates in the first year of life, with low rates at birth and increasing during the first year of life. Crowding was found to be an independent risk factor for colonisation with this pathogen. Colonisation status of mothers and children are correlated as described in chapter 2.3. For both *S. aureus* and *H. influenzae*, the odds for one to be colonised is doubled if the other is colonised as well. Direct transmission seems to occur in the case of *S. aureus* whereas other factors such as genetic susceptibility seem to play a role in the correlation of *H. influenzae* colonisation between mothers and children.

The natural specific antibody response to *S. pneumoniae* and *S. aureus* is discussed in chapter 3. For both anti-pneumococcal and anti-staphylococcal antibodies we described high levels of IgG in cord blood, but these levels significantly decreased in the first 6 months of life. A significant increase was observed after these first six months. Low values were obtained for IgA and IgM in the cord blood samples, which increased significantly in the first two years of life. Anti-pneumococcal and anti-staphylococcal maternal IgG did not protect children against colonisation with the pneumococcus and *S. aureus*, respectively, nor were maternal anti-pneumococcal IgG levels correlated to respiratory tract infections in early childhood.
Several antibodies against pneumococcal virulence proteins are associated with reduced numbers of respiratory tract infections. Some of these antibody responses are associated with colonisation, hence, most likely elicited during natural colonisation. However no protection against colonisation was observed, suggesting that the selected specific antibodies do not protect against asymptomatic carriage. In chapter 3.1 we suggest that potential vaccine candidates should not only be selected on level of protection against colonisation but also

on level of protection against infections, regardless of the association with colonisation. This is because some anti-pneumococcal antibodies appear not to be associated with colonisation but are strongly associated with a decreased risk of respiratory tract infections. Future studies should put effort in developing protein-based vaccines, in particular the effect of combination of several pneumococcal proteins and their correlation of protection in humans should be studied.
Chapter 3.2 reveals how specific antibodies directed against staphylococcal antigens such as CHIPS, Efb, IsdA and IsdH are expressed *in vivo*, and therefore, seem to play a role in nasal colonisation of young children.
S. aureus and the pneumococcus are known to be inversely correlated. In chapter 3.3 we studied whether specific antibodies play a role in this inverse correlation. We found no role for the early specific maternal IgG levels, nor for the child his or her own produced IgG and IgA response against *S. aureus* and pneumococcal protein-antigens.

The consequences of bacterial colonisation on the development of infections and atopic diseases are reported in chapter 4. In chapter 4.1 we describe an association between *S. aureus* colonisation in the first year of life and atopic dermatitis in the first and second year of life. Especially colonisation at 6 months, as well as frequent colonisation in the first year of life, was strongly associated with atopic dermatitis and its severity. In the following chapter, chapter 4.2, we describe that this association is dependent on the glucocorticoid receptor gene. Infants with two copies of wildtype of the glucocorticoid receptor gene were not prone to develop atopic dermatitis following *S. aureus* colonisation. Moreover, the glucocorticoid receptor gene polymorphisms were found to be associated with decreased *S. aureus* colonisation rates at three years of age, but not in infancy. This was earlier described in adults, which may be the reason why we do not observe this association in infancy where, apparently, random colonisation dynamics occur. In chapter 4.3 we report the association between frequent colonisation with the airway pathogens *S. pneumoniae, H. influenzae* and *M. catarrhalis* and respiratory tract symptoms such as wheezing and respiratory tract infections. Frequent colonisation with these pathogens is associated with an increased risk of wheezing complaints in the second year of life, but not at older ages of the child. In contrast, frequent colonisation is associated with reduced rates of respiratory tract infections in the second third and fourth year of life. This latter protection may be due to humoral immune response following a clinical or subclinical infection at the time of colonisation.
Chapter 5 gives an overview of this thesis and discusses the aforementioned studies of this thesis opposite of each other and the literature. In conclusion, bacterial colonisation is a dynamic process in early childhood. Interference between species, environmental and genetic determinants play important roles in colonisation acquisition, dynamics and the development of infections and atopic diseases. Finally, in chapter 6 the main research questions as proposed in chapter 1 are briefly answered.

SAMENVATTING

In hoofdstuk 1 wordt de achtergrond en het doel van de in dit proefschrift gepresenteerde studies belicht.

Kolonisatie met *Staphylococcus aureus, Streptococcus pneumoniae, Haemophilus influenzae* en *Moraxella catarrhalis* is vaak onschuldig, maar het verhoogt wel het risico op infecties veroorzaakt door de koloniserende stammen. Een groot deel van de volwassen bevolking is gekoloniseerd met *S. aureus*. Hoewel dit microorganisme vaak op de huid te vinden is, is de meest bekende kolonisatie plek van *S. aureus* voorin de neusgaten. Bijna 30% van de volwassen populatie draagt dit microorganisme permanent met zich mee op deze locatie. In de literatuur zijn verschillende risicofactoren beschreven voor het dragen van *S. aureus* bij gezonde kinderen. Het aantal broertjes en zusjes, de gezinsgrootte, het al dan niet gevoed zijn met borstvoeding en passief roken zijn onafhankelijke determinanten van dragerschap. Daarnaast lijken genetische aspecten van zowel het individu als het pathogeen ook een belangrijke rol te spelen in de kolonisatie status. *S. pneumoniae*, ook wel de pneumokok genoemd, is een bekende veroorzaker van milde luchtweginfecties tot invasieve infecties. De morbiditeit en mortaliteit zijn hoog, wereldwijd veroorzaakt het rond de 11% van alle mortaliteit bij kinderen onder de 5 jaar. Net als bij *S. aureus* kolonisatie is asymptomatisch nasofaryngeaal dragerschap de hoofdbron voor pneumokokken infectie. Meer dan 90 verschillende pneumokokken serotypes zijn geïdentificeerd op basis van verschil in de kapsel polysacchariden. Recente studies rapporteren een verschuiving in voorkomende serotypen als gevolg van het in 2006 in Nederland geïntroduceerde pneumokokken conjugaat vaccine (PCV). Daar waar het vaccin zich succesvol richt op de voorheen meest voorkomende serotypen in kolonisatie en infectie, komen nu andere serotypen op die koloniseren en infectie veroorzaken. Bovendien zien we ook een toename van nasale en nasofaryngeale kolonisatie met andere pathogenen. Vaccines gebaseerd op virulentie proteinen die aanwezig zijn op alle serotypen bieden hierin mogelijk een uitkomst. Dit aangezien deze vaccins pneumokokken kolonisatie en infectie zou kunnen voorkomen ongeacht welk serotype. *H. influenzae* en *M. catarrhalis* koloniseren ook de nasofarynx, hetzelfde gebied als de pneumokok, en zijn beiden eveneens betrokken bij luchtweginfecties. Otitis media in het bijzonder. Deze drie luchtwegpathogenen hebben ook een gezamenlijke risicofactor. Crowding, oftewel het hebben van broertjes en zusjes en/of het bezoeken van een crèche, predisponeert voor kolonisatie met alle drie de luchtweg pathogenen. Verder dragen de microbiële interacties tussen alle vier de hierboven beschreven pathogenen ook bij aan de kolonisatie frequenties en de mate van ziekte uitkomst.

Luchtweginfecties bij kinderen hebben doorgaans een lage mortaliteit in de Westerse wereld en zijn vaak zelflimiterend. Doch is de morbiditeit en de last voor zowel de patiënten, hun verzorgers als de maatschappij hoog. In het tweede levensjaar zien we een piek incidentie

van luchtweg infecties. Asymptomatische kolonisatie met de luchtwegpathogenen heeft ook zijn piek incidentie rond die leeftijd.
Recentelijk is er een studie gepubliceerd van een Deense groep die rapporteert over het risico op de ontwikkeling van astma na vroege kolonisatie met de luchtweg pathogenen *S. pneumoniae, H. influenzae* en *M. catarrhalis.* Nasale kolonisatie met *S. aureus* wordt doorgaans een rol in de ernst atopische dermatitis toegewezen. Atopische ziekten zoals astma en atopische dermatitis zijn een toenemend klinisch probleem in voornamelijk de Westerse wereld. Zowel astma als atopische dermatitis zijn multifactoriële aandoeningen. Meerdere factoren, zoals genetische en omgevingsdeterminanten en hun interacties, kunnen leiden tot de ontwikkeling van symptomen passend bij atopische dermatitis en/of astma. Om de ontwikkeling van nieuwe preventieve maatregelen te stimuleren die helpen tegen morbiditeit en last veroorzaakt door de pathogenen, is het belangrijk om de kennis te verbreden over de humane antistof respons. Een nieuwe, innovatieve, immunologische essay werd recent ontwikkeld. Deze xMAP® Technology (Luminex Corporation) kan antistof niveaus tegen meerdere antigenen kwantificeren in één hoeveelheid serum. *S. aureus* en *S. pneumoniae* essays werden ontwikkeld met behulp van deze techniek gebaseerd op flow cytometry. Deze techniek zal helpen meer inzicht te krijgen in de humorale immuun respons die betrokken is bij kolonisatie en ziekten in de kinderleeftijd, wat mogelijk de ontwikkeling van nieuwe preventieve maatregelen (zoals vaccins) tegen luchtweg infecties en atopische ziekten zal faciliteren. Het hoofddoel van dit proefschrift is het bestuderen van de dynamiek en kinetiek van bacterieel nasale en nasofaryngeale kolonisatie en de consequenties hiervan op de jonge leeftijd.

In het tweede hoofdstuk worden de dynamiek en de determinanten beschreven van kolonisatie tot de peuterleeftijd. *S. aureus* dragerschap in het eerste levensjaar is beduidend anders dan bij volwassenen, wat wordt beschreven in hoofdstuk 2.1. Het permanent dragen van dezelfde *S. aureus* stam komt nauwelijks voor op deze jonge leeftijd. Verder zien we een hoge prevalentie van dragerschap vlak na de geboorte, welke flink daalt in het eerste levensjaar. Daarnaast is met dit onderzoek aangetoond dat jongens een groter risico lijken te hebben om gekoloniseerd te zijn met *S. aureus* dan meisjes. In hoofdstuk 2.2 worden de kolonisatie prevalentie en de determinanten van kolonisatie met *M. catarrhalis* beschreven. De prevalentie van kolonisatie met dit pathogeen is het tegenovergestelde van *S. aureus*. Rond de geboorte zijn maar weinig kinderen gekoloniseerd in de nasofarynx met dit pathogeen, maar dit getal neemt flink toe in het eerste levensjaar. Crowding wordt in dit hoofdstuk beschreven als onafhankelijke risicofactor voor kolonisatie. Verder blijkt uit hoofdstuk 2 dat kolonisatie status van moeders en kinderen gecorreleerd zijn aan elkaar. In hoofdstuk 2.3 wordt aangetoond dat dit het geval is voor zowel *S. aureus* als *H. influenzae*. Er lijkt sprake te zijn van directe transmissie in het geval van *S. aureus*. Moeders en kinderen dragen vaak genotypisch dezelfde stam in de neus. Dit in tegendeel voor *H. influenzae* waar

andere factoren zoals genetische vatbaarheid mogelijk een rol speelt in de correlatie van *H. influenzae* kolonisatie tussen moeders en kinderen.

De natuurlijke specifieke antistof respons tegen *S. pneumoniae* en *S. aureus* wordt in hoofdstuk 3 vermeld.
Zowel anti-pneumokokken als antistafylokokken IgG niveaus waren hoog in het navelstrengbloed, maar deze niveaus daalde flink in de eerste 6 maanden conform verwachting in verband met de halfwaarde tijd van maternale antistoffen. Na deze daling in de eerste 6 maanden werd een significante stijging van de IgG niveaus waargenomen. Lage waarden voor IgA en IgM werden gedetecteerd in het navelstreng bloed, welke overigens wel direct na de geboorte stegen in het eerste levensjaar. Maternale antistoffen tegen pneumokokken en stafylokokken proteïnen beschermde de kinderen niet tegen kolonisatie met respectievelijk de pneumokok en de *S. aureus*. Verder waren maternale anti-pneumokokken IgG niveaus ook niet gecorreleerd aan luchtweg infecties op de jonge kinderleeftijd. Verscheidene antistoffen tegen pneumokokken virulentie proteïnen, namelijk IgG gericht tegen PspC, Eno en PspA en IgA gericht tegen Eno, PsaA en PspA, lijken een indicatie te zijn voor een verlaagd risico op luchtweg infecties bij kinderen. Een aantal van deze specifieke antistoffen zijn eveneens gecorreleerd met kolonisatie status, waarschijnlijk zijn zij geproduceerd in reactie op kolonisatie en een eventueel daarbij horende klinische of subklinische infectie. Deze specifieke antistoffen lijken echter geen bescherming te geven tegen toekomstige asymptomatische kolonisatie. In hoofdstuk 3.1 wordt aangegeven dat verder onderzoek naar op proteïnen gebaseerde vaccins nuttig en noodzakelijk is. In dit hoofdstuk wordt een aantal potentiële vaccin kandidaten aangewezen, die niet alleen op basis van bescherming tegen kolonisatie maar vooral op basis van bescherming tegen luchtweg infecties geselecteerd zouden moeten worden. Er zijn aantal anti-pneumokokken antistoffen die niet geassocieerd zijn met kolonisatie maar wel het verminderd voorkomen van luchtweginfecties blijken. Toekomstig onderzoek moet leiden tot het ontwikkelen van op proteïnen gebaseerde vaccins, en dan in het bijzonder de combinatie van bepaalde virulentie proteïnen.
Hoofdstuk 3.2 onthult hoe bepaalde specifieke antistoffen gericht tegen stafylokokken antigenen, zoals CHIPS, Efb, IsdA en IsdH *in vivo* tot expressie komen, en zodoende een rol lijken te spelen in nasale kolonisatie van jonge kinderen. In de literatuur is een negatieve correlatie beschreven tussen *S. aureus* en de pneumokok. In hoofdstuk 3.3 wordt beschreven of specifieke antistoffen een rol spelen in deze negatieve correlatie. De conclusie is dat er geen rol lijkt te zijn weggelegd voor specifieke maternale IgG, en ook niet voor IgG en IgA respons tegen *S. aureus* en pneumokokken proteïn-antigenen geproduceerd door het kind zelf.

De consequenties van bacteriële kolonisatie op de ontwikkeling van infecties en atopische ziekten worden aangehaald in hoofdstuk 4. In hoofdstuk 4.1 wordt een associatie beschreven tussen *S. aureus* kolonisatie in het eerste levensjaar en atopische dermatitis in het eerste en

tweede levensjaar. In het bijzonder kolonisatie op 6 maanden en frequente kolonisatie in het eerste levensjaar waren geassocieerd met atopische dermatitis en de ernst van symptomen. In het volgende hoofdstuk, hoofdstuk 4.2, wordt aangetoond dat deze associatie tussen *S. aureus* kolonisatie en atopische dermatitis afhankelijk is van het glucocorticoid receptor gen. De associatie was niet aanwezig bij jonge kinderen met twee kopieën van wildtype op het glucocorticoid receptor gen. Verder werd geconstateerd dat kinderen met verschillende glucocorticoid receptor gen polymorfismen lagere kans hadden op *S. aureus* kolonisatie in het derde jaar. Deze associatie was er niet bij jonge kinderen. Bij volwassenen was deze associatie eerder ook al aangetoond. Het feit dat deze associatie niet op de jonge leeftijd bestaat past bij de eerdere bevindingen besproken in hoofdstuk 2 waar geen permanent dragerschap werd geobserveerd op de jonge leeftijd. Het feit dat de glucocorticoid receptor polymorfismen op de leeftijd van 3 jaar net als bij volwassenen geassocieerd is met *S. aureus* kolonisatie geeft aan dat deze laatste groep kinderen mogelijk een volwassener kolonisatie patroon beginnen te ontwikkelen. In hoofdstuk 4.3 wordt de associatie gerapporteerd tussen frequente kolonisatie met de luchtweg pathogenen *S. pneumoniae, H. influenzae* en *M. catarrhalis* en luchtweg symptomen zoals wheezing en luchtweginfecties. Frequente kolonisatie met deze pathogenen in het eerste levensjaar is geassocieerd met een verhoogd risico op klachten van piepende ademhaling in het daaropvolgende jaar. De kans op luchtweg infecties echter, lijkt verlaagd te zijn na frequente kolonisatie in het levensjaar. Deze laatste associatie houdt stand tot en met het vierde levensjaar. Dit beschermende effect van frequente kolonisatie op luchtweginfectie ontstaat wellicht door de productie van een humorale immune respons na een klinische of subklinische infectie rond het moment van kolonisatie.

In hoofdstuk 5 wordt een overzicht gegeven van dit proefschrift en bediscussiëren we de eerdergenoemde studies in dit proefschrift ten opzichte van elkaar en de literatuur. Samenvattend kan geconcludeerd worden dat bacteriële kolonisatie op de jonge kinderleeftijd een dynamisch proces is. Interacties tussen pathogenen, omgevings- en genetische factoren spelen een belangrijke rol in kolonisatie prevalentie, dynamiek, en de ontwikkeling van infecties en atopische ziekten.

Tot slot wordt in hoofdstuk 6 kort en bondig antwoord gegeven op de specifieke onderzoeksvragen die in het eerste hoofdstuk beschreven staan.

APPENDICES

I ABBREVIATIONS

AD	Atopic Dermatitis
aOR	Adjusted Odds Ratio
AWP	Airway Pathogens
CbpA	Choline binding protein A
CHIPS	Chemotaxis Inhibitory protein of *S. aureus*
CI	Confidence Interval
Clfa/b	Clumpingfactor A/B
DNA	Desoxyribonucleidacid
Efb	Extracellular fibrinogen-binding protein
Eno	Alpha-Enolase
Fnbp	Fibronectin-binding protein
GEE	Generalized Estimation Equation
GR	Glucocorticoid Receptor
H. influenzae	*Haemophilus influenzae*
Hyl	Hyaluronidase
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IsdA/H	Iron-regulated surface determinants A/H
M. catarrhalis	*Moraxella catarrhalis*
MFI	Median Fluorescence Intensity
MLST	Multi Locus Sequence Typing
MSCRAMMs	Microbial surface components recognizing adhesive matrix molecules
N	Natural number (sample size)
NanA	Neuraminidase A
OR	Odds Ratio
PCV	Pneumococcal Conjugate Vaccin
PFGE	Pulsed Field Gel Electrophoresis
PdbD	Pneumolysoid
Pht	Pneumococcal histidine triad
PLY	Pneumolysin
PpmA	Protease maturation protein A
PsaA	Pneumococcal surface adhesion A
PspA	Pneumococcal surface protein A
PspC	Pneumococcal surface protein C
RTI	Respiratory Tract Infections
S. aureus	*Staphylococcus aureus*

SasG	*Staphylococcus aureus* surface protein G
SCIN	Staphylococcal complement inhibitor
SD	Standard deviation
Sdr	Serine-aspartate repeat protein
SE	Staphylococcal enterotoxins
SlrA	Streptococcal lipoprotein rotamase A
SNP	Single Nucleotide Polymorphism
S. pneumoniae	*Streptococcus pneumoniae*
SPSS	Statistical Package for the Social Sciences
TSST	Toxic shock syndrome toxine

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15. Tummers, C., Lebon, A. Jaddoe, V.W.V., Hofman, A., de Jongste, J.C., Moll, H.A. Caesarean section increases the risk of atopic disease: the Generation R Study. Submitted

IV ABOUT THE AUTHOR

Ankie Lebon was born on February 28th 1984 in Amsterdam, The Netherlands. In 2002 she completed secondary school at the 'Amstelveen College' in Amstelveen. After working for a general practitioner and the medical lab of the Amstelveen Hospital for a year from 2002 to 2003 she started studying medicine at the Erasmus University in September 2003. In the second year of her study she got enrolled in the one-year Erasmus Honours Programme. From the start of the third year she worked on a Master of Science degree in Clinical Research at The Netherlands Institute for Health Sciences, which she obtained in February 2008. Her MSc thesis topic was on *Staphylococcus aureus* colonisation in infancy. This study was embedded in the Generation R Study and was retrospectively the start of her PhD project at the Department of Pediatrics (head Prof. A.J. van der Heijden, promotor Prof. H.A. Moll) and Department of Medical Microbiology and Infectious Diseases (head Prof. H.A. Verbrugh, promotor Prof. A. van Belkum).

During her medicine study she spent a month at the Child Health department of the Africa Centre in Mtubatuba, South Africa (head Prof. M.L. Newell). She also spent a month at Johns Hopkins School of Medicine in Baltimore, United States of America, to attend a summer programme at the Johns Hopkins Bloomberg School of Public Health on Epidemiology. From 2009 to 2010 she conducted the first year of her internships while she continued working on her thesis. During the internships she obtained her clinical training in Psychiatry at the Tribhuvan Teaching Hospital in Kathmandu, Nepal. In 2010 she returned back to the PhD project to complete this while focussing fulltime on the project. Early 2011 she will continue her internships which she will then complete at the end of September 2011

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General courses		
- Training solliciteren naar een opleidingsplaats, KNMG, Leids Universitair Medisch Centrum	2010	1.4
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- Master of Science Program in Clinical Research at the Netherlands Institute for Health Sciences (NIHES, Rotterdam)	2005-2008	
- Genome Wide Association Studies, NIHES	2008	1.4
Seminars and workshops		
- MOLMED 2nd Symposium & Workshops on Molecular Microbiology of Infectious diseases	2008	0.4
- Generation R Research Meetings	2007-2010	2.6
- Seminars at the department of Epidemiology	2007-2010	1.0
National presentations		
- SSWO (Sophia Scientific Research Organisation)	2007	1.4
- General Paediatrics research meeting	2007-2008	0.8
- Generation R research meeting	2008	0.4
- Medical Microbiology and Infectious Diseases Research Day	2008	0.8
- Sophia Children's Hospital Research Day	2008	0.8
- PAOS (Post Graduate Education Sophia Children's Hospital)	2010	0.4
- SSWO (Sophia Scientific Research Organisation)	2010	1.4
- Sophia Children's Hospital Research Day	2010	0.8
(Inter) national presentations at conferences and congresses		
- European Academy of Paediatrics (EAP) - oral presentation	2008	1.4
- Dutch Society for Paediatrics - Young Investigators day (NVK-JOD)	2008	0.4
- European Society Paediatric Infectious Diseases (ESPID) - poster presentation	2009	1.4
- European Society Paediatric Infectious Diseases (ESPID) - oral presentation	2010	1.4
- Dutch Society for Paediatrics - Young Investigators day (NVK-JOD)	2010	0.4
Other		
- Participation in the organizing committee planning the opening of the new research centre with HRH Princess Maxima.	2008	1.4

2. Teaching		
	Year	**Workload (ECTS)**
Supervising Master's theses		
- Determinants of *Moraxella catarrhalis* colonisation in infancy Mw. Jolanda van Zwol – Saarloos, MSc	2008	2.0
- Smoking during pregnancy and respiratory tract infections in children Mw. Drs. Bonnie Huang Fan, MSc	2009	2.0

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