Bacterial carriage in infancy
Risk factors and consequences
The Generation R study

Joost A.M Labout

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Risicofactoren en consequenties
Het Generation R Onderzoek

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Contents

Chapter 1

Introduction

Background

In this thesis various studies on the prevalence, risk factors and consequences of carriage of *Streptococcus pneumoniae* (pneumococcus), *Haemophilus influenzae*, *Moraxella catarrhalis* and *Staphylococcus aureus* in young children are presented. These bacteria are considered important (airway) pathogens in this age group. The definition of *pathogen* needs further explanation, since the boundary between commensal organisms and pathogenic organisms is not always obvious. [1-5] According to Casadevall and Pirofski, defining a pathogen as an organism that causes disease in a host is inadequate because some pathogens do not cause disease in all human hosts. [3] The adjective *opportunistic* is used for pathogens that cause disease only in hosts that are immunocompromised or whose pathogenesis is facilitated by traumatic breaching of an epithelial barrier. [4] For example, about 50% of children two years of age are colonized by *S. pneumoniae*. [6-11] This does not lead to disease in most cases, and thus, the pneumococcus can be considered a commensal organism. Weiser et al. have shown that colonization is the natural state of the pneumococcus, and invasive disease, also from the perspective of the pneumococcus, is not favourable. [12] However, *S. pneumoniae* is the main cause of bacterial otitis media and bacterial pneumonia in young children worldwide. It is also an important cause of life threatening sepsis and meningitis. Moreover, pneumonia is "the leading killer in children", and since *S. pneumoniae* is considered to cause more than half the cases of bacterial pneumonia worldwide, it is without any doubt a fearful pathogen. [13]

Bacterial carriage

After rupture of the amniotic membranes the mucosal surfaces of the body are exposed to tremendous numbers of organisms. During a lifetime, humans encounter a vast number of bacterial species that have the potential to colonize the upper respiratory tract, but only few do so. Of these, many are cleared from the mucosal surfaces rapidly. Never the less, some may persist for months or years forming a complex ecosystem on the upper respiratory mucosa. [14] Asymptomatic colonization of the nasopharynx by bacteria is considered to be the first step in the pathogenesis of infections caused by these organisms. [15] To this respect, the most important bacteria that are able to cause disease after colonizing the upper respiratory tract are *S. pneumoniae, H. influenzae* and *M. catarrhalis*. In addition, healthy individuals carrying these bacteria are thought to be primarily responsible for the person-to-person

bacterial transmission of these pathogens. [15] Therefore, it is of great importance to study the prevalence of bacterial carriage, especially in the young age group.

Risk factors for bacterial carriage

Numerous factors influence the upper respiratory tract flora. Firstly, the host and in particular the immune system of mucosal surfaces. The host immune system has evolved an extensive defence system to protect against pathogens whereas also maintaining "coexistence" with commensal organisms. Secondly, the bacteria, which have evolved ways to live in the human nasopharynx and are able to evade the host immune response, play an important role in the upper respiratory tract flora. Different species of bacteria interact in the human nasopharynx. Some species prevent subsequent colonization by other species, and, conversely, there is cooperation between different species to maintain colonization. For example, a negative association between carriage of pneumococci and *S. aureus*, and a positive association between carriage of pneumococci and *H. influenzae* as well as *M. catarrhalis* carriage has also been described. [7, 9, 16-22] The interactions between bacteria in the nasopharynx are considered to be caused by microbe-micobe as well as microbe-host-microbe interactions. Thirdly, environmental factors play an important role in the establishment of the mucosal bacterial flora. Several risk factors for bacterial carriage have been identified. Crowding is a well-established risk factor for bacterial carriage, but other risk factors like gender, socioeconomic status, smoking and breast-feeding are also associated with bacterial carriage. [9, 10, 23-31] Never the less, it is likely that other, yet unknown environmental factors play a role in the establishment of the nasopharyngeal flora.

Consequences of bacterial carriage

Asymptomatic colonization of the nasopharynx by bacteria is considered to be the first step in the pathogenesis of infections. However, details of the association between bacterial carriage and infections are not clearly established. For example, acute otitis media is the most frequently diagnosed respiratory disease in children and mostly caused by *S. pneumoniae, H. influenzae* and *M. catarrhalis*, but the association between carriage of these bacteria and the risk for otitis is not completely elucidated. Early age of colonization considered to increase the risk of otitis media. [32] Syrjänen et al. have suggested that carriage of newly acquired

bacteria is associated with otitis media. [33] Likewise, carriers of *S. aureus* are at an increased risk to develop *S. aureus* infection. It was even shown that the number of surgical-site *S. aureus* infections can be reduced by decolonizing of nasal carriers of *S. aureus* on hospital admission. [34] However, studies have shown that non-carriers who acquire exogenous *S. aureus* bacteraemia have a fourfold increased mortality rate compared with *S. aureus* nasal carriers. [35]

Bacterial carriage with *S. pneumoniae, H. influenzae* and *M. catarrhalis* in young infants is also associated with the development of asthma at the age of five years. [36] Bronchoalveolar lavage in young children with severe recurrent wheeze contains increased numbers of macrophages and neutrophils but not of eosinophils and mast cells suggesting an association of bacterial colonization with the initiating events of early asthma. [36] Furthermore, carriage with *S. aureus* is thought to be related to the development of atopic dermatitis. Semic-Jusufagic et al. have shown a positive association between specific IgE staphylococcal enterotoxin-mix and atopic dermatitis in children. [37] Other studies reported on increased levels of antistaphylococcal IgE and staphylococcal toxins A-E in the serum of atopic dermatitis patients. [37-39] Like the details of the association between bacterial carriage and infections, the mechanisms behind the association between bacterial carriage and atopic disease are far from elucidated.

Our research

Although it is clear that bacterial carriage is an important state towards the development of a variety of diseases, large longitudinal studies on dynamics and risk factors for bacterial carriage in infancy, and consequences of bacterial carriage for (respiratory) diseases in infancy are lacking. Therefore, we studied in the Generation R cohort the prevalence, dynamics and risk factors for bacterial carriage and the consequences of bacterial carriage in a cohort of healthy of young children. The Generation R Study is a prospective population-based cohort study conducted in Rotterdam, the Netherlands, which was designed to identify early environmental and genetic causes of normal and abnormal growth, development and health from fetal life until young adulthood. [40-42] The cohort includes 9778 mothers and their children living in Rotterdam, the Netherlands. Detailed assessments of fetal and postnatal growth and development were conducted in 1,232 Dutch pregnant women and their children in the Generation R Focus Study.

Aims and outline of this thesis

The overall aim of this research was to determine prevalence, risk factors and consequences of bacterial carriage in infancy and to study risk factors of upper respiratory tract infections in young children in a population-based prospective cohort study. The following issues were investigated:

Part 1.

The prevalence, dynamics and risk factors for carriage of *S. pneumoniae*, *H. influenzae*, *M. catarrhalis* and *S. aureus* in infancy. (Chapter 2, 3, 4 and 5)

The prevalence of pneumococcal carriage and factors associated with pneumococcal carriage in infants are presented in **chapter 2**. In **chapter 3**, the association between pneumococcal carriage and air pollution (PM_{10}) is presented. The dynamics and determinants of *S. aureus* carriage in infancy are discussed in **chapter 8**. In **chapter 5**, the microbial associations between S. *pneumoniae, H. influenzae, M. catarrhalis* and *S. aureus*, during colonization in infancy are studied.

Part 2.

The consequences of carriage of *S. pneumoniae*, *H. influenzae* and *M. catarrhalis* and otitis media, and the consequences of carriage of *S. aureus* and atopic dermatitis in young children. (Chapter 6 and 7)

In **chapter 6** risk factors for otitis media, especially, the association between bacterial carriage in the first year of life and otitis media in the second year of life are discussed. The association between colonization of *Staphylococcus aureus* and atopic dermatitis is presented in **chapter 7**.

Part 3.

The risk factors associated with upper respiratory tract infections in young children, in particular the socioeconomic status of the parents. (Chapter 8)

In **chapter 8** the role of socioeconomic status in the development of upper respiratory tract infections (URTI) is studied.

In **chapter 9**, the main findings of the studies are summarized and future perspectives are discussed.

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Part 1

The prevalence, dynamics and risk factors for carriage of *S. pneumoniae*, *H. influenzae*, *M. catarrhalis* and *S. aureus* in infancy.

Chapter 2

Factors associated with pneumococcal carriage in healthy Dutch infants: the Generation R study.

Labout JA, Duijts L, Arends LR, Jaddoe VW, Hofman A, de Groot R, Verbrugh HA, Hermans PW, Moll HA

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Abstract

Objective. To study the prevalence, risk factors and dynamics of pneumococcal carriage in infancy.

Study design. In a population-based prospective cohort study in Rotterdam, the Netherlands (June 2003 - November 2006), nasopharyngeal swabs were obtained at the age of 1.5, 6 and 14 months. Data on risk factors were obtained by midwives, hospital registries and by postal questionnaires.

Results. Prevalence of pneumococcal carriage increased from 8.3% to 31.3% to 44.5% at the ages of 1.5 (n=627), 6 (n=832) and 14 months (n=757), respectively. The prevalence of serotypes covered by the 7-valent conjugate increased from 3.0% to 16.2% and 27.7%, at the different ages. Having siblings (aOR 4.33, CI 1.22-15.35) and day care attendance (aOR 3.05, CI 1.88-4.95 at 6 months and aOR 2.78, CI 1.70-4.55 at 14 months) were associated with pneumococcal carriage. Pneumococcal carriage at 6 months was associated with pneumococcal carriage at 14 months (aOR 2.43, CI 1.50-3.94). Pneumococcal carriage was not associated with gender, maternal smoking, educational level mother and breast-feeding.

Conclusions. The prevalence of serotypes covered by the 7-valent conjugate vaccine increases in the first year of life. Siblings, day care attendance and previous pneumococcal carriage are independent risk factors for pneumococcal carriage.

Introduction

Streptococcus pneumoniae (pneumococcus) is one of the major causes of bacterial infections worldwide. Pneumococcal infections range from otitis media to life threatening invasive infections like sepsis, meningitis and pneumonia.[1-3] Young children, elderly people, and patients with immunodeficiencies have an increased risk for pneumococcal infections.[4] Pneumococci are frequently carried in the nasopharynx, especially in children. Although usually asymptomatic, pneumococcal colonization is the first step in the pathogenic route towards an invasive disease. Besides, carriage is the source for horizontal spread in the community.[5]

Numerous studies have shown that acquisition of pneumococci in the nasopharynx occurs early in life.[6] Prevalence of pneumococcal carriage increases in the first years of life. Crowding, defined by family size and day care attendance, is a well-established risk factor for pneumococcal carriage.[7,8] For other risk factors like gender, socioeconomic status, smoking and breast-feeding the association with pneumococcal carriage is inconclusive. Coles et al.[9] found an association between pneumococcal carriage and gender and passive smoking. Other could not confirm this association.[10,11] Greenberg et al.[12] found an association between passive smoking and pneumococcal carriage in infants, in contrast to other studies.[9,13] Breast-feeding is protective against respiratory tract infections[14-16] and invasive pneumococcal disease.[17] However, the association between breast-feeding and pneumococcal carriage in human and in vitro studies is largely inconclusive.[18-22]

Large longitudinal studies on dynamics and risk factors of pneumococcal carriage in the first year of life are lacking. Our aim was to study in a population-based prospective cohort the prevalence, dynamics and risk factors for pneumococcal carriage during the first 14 months of life.

Materials and Methods

This study was embedded in the Generation R Study, a population-based prospective cohort study from fetal life until young adulthood.[23-24] Detailed assessments of fetal and postnatal growth and development were conducted 1,232 Dutch pregnant women and their children, referred to as the Generation R Focus Study. Of all approached pregnant women, 79% participated in the Generation R Focus Study. The study was conducted in Rotterdam, the Netherlands from June 2003 till November 2006. The children were planned to visit the research centre at the ages of 1.5 month, 6 months and 14 months. The Medical Ethics Committee of

the Erasmus Medical Center, Rotterdam, has approved the study. Written informed consent was obtained from all participants.

To detect pneumococcal carriage, trained research assistants obtained a single nasopharyngeal swab at each visit. Nasopharyngeal samples were taken with rayon tipped dacron pernasal swabs. The flexible swab was inserted into the anterior nares, gently rubbed on the posterior nasopharyngeal wall, removed, and stored in Amies transport medium at room temperature. Swabs were plated within 6 hours of sampling on a blood agar plate with 5% sheep blood to isolate *S. pneumoniae*. Infants, who used antibiotics 48 hours preceding the visit, were excluded. All pneumococci were serotyped by capsular swelling method (Quellung reaction) with commercially available antisera (Statens Seruminstitut, Copenhagen, Denmark), according to the instructions of the manufacturer. These procedures are in line with the standard method as described by O'Brien et al.[6] and applied by our lab in a previous study.[25] All children were born before the start of the national pneumococcal vaccination program in the Netherlands. The registry of the baby well clinic showed that none of the infants of the cohort was vaccinated. In the currently used conjugate vaccine in children under the age of 2 years 7 serotypes are included (4, 6B, 9V, 14, 18C, 19F and 23F). Based on these 7 serotypes, we divided the pneumococci into vaccine-types (VT) and non-vaccine-types (NVT). The number of swabs were taken as denominator for the prevalence rate of pneumococcal carriage.

Data on gender, parity, birth weight and gestational age were obtained by midwives and hospital registries. Information about siblings, breast-feeding, educational level of mother, smoking and day care attendance was obtained by postal questionnaires at the infants' age of 6 and 12 months. Mothers were asked whether they ever breast-fed their infant (no, yes). The duration of breast-feeding was categorized into the groups of never breast-feeding, less than 3 months, 3 to 6 months and 6 months or longer. The duration of exclusive breast-feeding was estimated by combining the question of duration of breast-feeding and the questions at what age formula feeding, other types of milk and solid food were started in the first 6 months of life. This resulted in three categories: 1. never breast-fed; 2. partially breast-fed (breast-feeding, other milk and/or solid food); and 3. exclusively breast-fed for at least 3 months ('exclusive' indicates only breast-feeding, no other milk, solid food, or fluids other than water). The socio-economic status of the mother was defined as highest followed education according to the classification of Statistics Netherlands and categorized in low, medium and high.[26]

Differences of infant characteristics between infants with and without nasopharyngeal samples were assessed by the independent sample t-test for continuous normal distributed

variables, non-parametric Mann-Whitney test for continuous non-normal distributed variables and the chi-square test for categorical variables. Differences of pneumococcal carriage between groups with missing risk factors and complete data were assessed in the same way. For the longitudinal analyses, the proportion of children with pneumococcal carriage was calculated at the age of 1.5, 6 and 14 months. Because repeated measurements within subjects are correlated, a Generalized estimating equations (GEE) model adjusting for this correlation was applied. Additionally, these regression models were adjusted for the potential confounders birth weight, parity gestational age, gender, siblings, educational level of mother, day care attendance, smoking and duration of breast-feeding. Associations of risk factors with pneumococcal carriage are presented with odds ratios (OR) with their 95% confidence interval (CI). All tests were carried out using a two-sided alpha level of 5%. The repeated measurement analyses were performed using SAS statistical software, version 9.1 (SAS Institute, Cary, NC, USA). The other statistical analyses were performed using the Statistical Package of Social Sciences version 11.0 for Windows (SPSS Inc, Chicago, IL, USA).

Results

Of the 1,232 pregnant women enrolled in the Generation R Focus Study 3 mothers had a stillborn infant. The remaining mothers gave birth to 1,244 live born infants. Of 138 of these children the consent was withdrawn after birth. Twins (n=27) were excluded from the present analyses since they are correlated. Our cohort for analysis consisted of 1,079 infants. The response rates of the visits were 81.8% (n=883) at 1,5 months, 81.6% (n=881) at 6 months and 80.0% (n=863) at 14 months of age. Seventy percent of the infants came to all three visits. The first postnatal measurements in the Generation R Focus Study started June 2003, data collection on pneumococcal carriage started November 2003, so data on pneumococcal carriage in the first 224 participants at 1.5 months of age are missing. Therefore, the number of swabs taken was 627 at 1.5 months, 832 at 6 months and 757 at 14 months of age. The number of swabs were taken as denominator for the prevalence rate of pneumococcal carriage. Characteristics of the infants are presented in Table 1.

Prevalence of pneumococcal carriage increased significantly ($p<0.001$) from 8.3% (52/627) to 31.3% (260/832) to 44.5% (337/757) at the ages of 1.5, 6 and 14 months, respectively. The prevalence of serotypes covered by the 7-valent conjugate vaccine (VT) increased from 3.0% (19/627) to 16.2% (135/832) and 27.2% (206/757), at the different ages. VT-related serotype 6A was found in 1.0% (6/627), 3.4% (28/832) and 5.8% (44/757) infants at the different

Table 1. General and infant characteristics (n=1079).

Birth weight (grams)	3509 (538)
Gestational age (weeks)	40.3 (27.6-43.4)
Parity > 0 (%)	38.0 (420)
Gender girl (%)	48.3 (521)
Maternal smoking (%)	12.7 (85)
Educational level mother (%)	
Primary school	2.0 (21)
Secondary school	33.9 (361)
Higher education	64.1 (683)
Siblings ≥ 1 (%)	36.7 (256)
Day care (%)	
At 6 months	63.7 (611)
At 12 months	69.0 (666)
Breast-feeding (%)	
Never	12.7 (98)
< 3 months	25.4 (196)
3-6 months	22.3 (172)
> 6 months	39.6 (306)

Values of birth weight is mean (standard deviation), gestational age is median (range). Other values are percentages (absolute numbers). Data were missing on parity (n=2), maternal smoking (n=411), educational level of mother (n=14), siblings (n=381), day care attendance at 6 and 12 months of age (n=413 and n=193, respectively) and breast-feeding (n=307).

ages. Differences in prevalence of VT and NVT carriage at different ages were statistically significant ($p<0.05$). Of the 21 children who carried pneumococci at both 1.5 and 6 months of age, five (23.8%) pneumococci were of the same serotype. Of the 116 children who carried pneumococci at both 6 and 14 months of age, 17 (14.6%) pneumococci were of the same serotype. The distribution of serotypes in the infants carrying pneumococci at two ages was comparable to the serotype distribution in the infants with one positive pneumococci sample. Pneumococcal carriage at three sample time points was found in 6 infants. None of these were the same serotype.

Risk factors for pneumococcal carriage at different ages are shown in Table 2. Analyses of missing nasapharyngeal samples showed that infants without nasapharyngeal samples at the age of 1.5 months more often were breast-fed than infants with nasapharyngeal samples (94.0% and 86.8%; p-value<0.001). Infants without nasapharyngeal samples at the age of 14 months had a lower birth weight than infants with nasopharyngeal samples (3.451 grams and

Table 2. Risk factors for pneumococcal carriage at different ages of the infant.

	S. pneumoniae 1.5 months		*S. pneumoniae* 6 months		*S. pneumoniae* 14 months	
	OR	aOR	OR	AOR	OR	aOR
Birth weight	2.69*** (1.51-4.79)	2.93* (1.16-7.37)	1.41* (1.07-1.85)	1.26 (0.82-1.95)	1.34* (1.02-1.175)	1.11 (0.72-1.72)
Gestational age	1.08 (0.99-1.17)	0.98 (0.85-1.12)	1.10* (1.01-1.20)	1.01 (0.88-1.16)	1.13 (0.96-1.33)	1.05 (0.80-1.38))
Gender	1.10 (0.62-1.93)	1.27(0.56-2.85)	1.12 (0.84-1.50)	1.17 (0.77-1.78)	1.11 (0.84-1.48)	1.06 (0.70-1.60)
Educational level mother	1.51 (0.84-2.70)	0.86 (0.35-2.10)	1.77*** (1.31-2.39)	1.09 (0.68-1.75)	1.58** (1.18-2.12)	0.90 (0.56-1.44)
Siblings	9.58*** (3.87-23.71)	8.02*** (2.91-22.06)	2.45*** (1.68-3.58)	1.98** (1.29-3.03)	1.69** (1.16-2.44)	1.46 (0.96-2.23)
Day care attendance	N.A.	N.A.	3.22*** (2.05-5.06)	3.11*** (1.92-5.02)	3.14*** (1.92-5.02)	2.91*** (1.79-4.75)
Duration of breast-feeding						
continuous variable	N.A.	N.A.	1.33** (1.12-1.57)	1.10 (0.88-1.36)	1.24** (1.06-1.46)	1.24 (0.98-1.58)
Never (reference)						
<3 months	N.A.	N.A.	1.15 (0.59-2.25)	1.42 (0.63-3.20)	0.86 (0.47-1.61)	0.50 (0.21-1.19)
3-6 months	N.A.	N.A.	1.71 (0.88-3.32)	1.82 (0.82-4.04)	1.50 (0.81-2.78)	1.10 (0.47-2.55)
>6 months	N.A.	N.A.	2.16 (1.18-3.98)	1.46 (0.67-3.20)	1.59 (0.91-2.81)	1.23 (0.55-2.74)
Exclusive breast-feeding						
formula-fed only (reference)						
partially breast-fed	N.A.	N.A.	1.63 (0.89-2.99)	1.76 (0.84-3.68)	1.31 (0.75-2.29)	0.91 (0.42-1.98)
exclusive breast-fed for at least 3 months	N.A.	N.A.	1.48 (0.77-2.81)	1.19 (0.54-2.65)	1.31 (0.72-2.37)	0.95 (0.42-2.14)
S. pneumoniae at 1.5 months	N.A.	N.A.	2.01* (1.09-3.73))	1.25 (0.50-3.12)	1.02 (0.53-1.95)	0.56 (0.24-1.31)
S. pneumoniae at 6 months	N.A.	N.A.	N.A.	N.A.	2.42*** (1.73-3.39)	2.50*** (1.55-4.03)

Values are crude odds ratios (OR) and adjusted odds ratios (aOR), assessed using a GEE model. Birth weight, gestational age and educational level of mother were included in the models as continuous variables (Table 1). Breast-feeding was included as continuous variable and as categorical variable. Other variables are binary. * p<0.05, ** p<0.01, ***p<0.001.

3.533 grams; p-value=0.02). All other risk factors showed no significant differences between children with and without nasopharyngeal samples at different ages. Analyses of missing risk factors showed that infants with missing data on siblings more often carried pneumococci ($p< 0.05$) Analyses of all other missing risk factors showed no differences in pneumococcal carriage between children with non-missing and missing risk factors.

Discussion

In our study pneumococcal carriage increased during the first year of life from 8.3% to 31.3% to 44.5% at the ages of 1,5, 6 and 14 months of age. This is in line with other studies of comparable populations.[8,10,25,27-29] Interestingly, the prevalence of serotypes that are present in the 7-valent conjugate vaccine was low 3.0% at 1.5 months of age, and increases to 27.2% at 14 months of age. At 1.5 months of age the prevalence as well as the vaccine-type versus non-vaccine-type distribution mirrors that of the adults[25,30], while at 6 and 14 months of age carriage rates and vaccine-type non-vaccine-type distribution showed a similar pattern as found in children.[25] The reason for the difference in carriage rates among adults and children is not yet known. Regev-Yochay et al.[31] speculate that the presence of antibodies to pneumococci in adults might be the explanation. Circulating maternal antibodies in young infants might explain the adult pneumococcal carriage pattern in infants at 1.5 months of age.

Pneumococcal carriage is determined by environmental and host factors. The most important environmental factors in our study were having siblings and day care attendance. This is in line with previous studies.[5] No association was found between pneumococcal carriage and maternal smoking. However, number of mothers who smoke is rather low. The cohort represents a rather healthy population.[24] Detailed data of exposure to passive smoking are missing. We found no association between pneumococcal carriage and duration of breast-feeding or exclusive breast-feeding. The duration of breast-feeding showed a tendency to be a risk factor for pneumococcal carriage in the univariate analysis, but the confounding factors siblings, day care attendance and educational level of mother explained this effect. In vitro studies have shown that human milk and colostrums inhibits the attachment of pneumococci to epithelial cells.[18,19,31] On the other hand, Rosen et al.[22] showed that pneumococcal capsular antibodies in human milk do not protect against carriage. Lower rates of carriage in exclusively breast-fed children were described once, while another study concluded no association between breast-feeding and pneumococcal carriage.[20,21] One study has demonstrated that colostrum-fed infants were more likely to carry pneumococci at 2 months of age

than infants from whom colostrum was withheld.[9] We expected breast-feeding to be protective against pneumococcal carriage because it is shown to protect against otitis media, other respiratory tract infections[14-16], and invasive pneumococcal disease.[17,32] An explanation that breast-feeding does not protect against pneumococcal carriage but does protect against mucosal infections is given by Finn et al.[33] They showed that secretory IgA-associated killing by phagocytes is complement-dependent, but levels of complement at mucosal surfaces are low. It is speculated that in the presence of active mucosal infection, sufficient amounts of complement may exude from plasma into the mucosal site to support killing of pneumococci.

In our study, high birth weight was associated with pneumococcal carriage at 1.5 months of age. To our knowledge this has never been reported before. One might speculate that infants with higher birth weight are exposed to crowding environment more easily, because infants with low birth weight are treated differently by their parents.

We found an independent association between pneumococcal carriage at 6 months of age and carriage at 14 months of age. This was not explained by any of the risk factors or by lengthy carriage of the same serotype. Only 17 (14.6%) out of the 116 pneumococci carried at both 6 and 14 months of age were of the same serotype. The distribution of serotypes in the group carrying pneumococci at two sample times was comparable to the serotype distribution in the infants carrying pneumococci once. This finding implies that repeated pneumococcal carriage is not restricted to specific serotypes. Our findings suggest that, besides the studied environmental factors, other unknown environmental (exposure) factors not evaluated in our cohort or (genetic) host characteristics might influence the risk of being colonized at 14 months of age.

To appreciate the results some methodological issues should be considered. The response in the Generation R Focus Study is above 80%. None of the participating infants had used antibiotics in the previous 48 hours. This suggests that children with active infectious diseases were not attending the scheduled visits and their visits to the research centre were delayed by the parents. Excluding participants with missing data would lead to selection bias if the associations of the risk factors with pneumococcal carriage differ between infants with and without missing data. No difference in the distribution of the risk factors siblings, day care attendance and breast-feeding was found between those with and without data on pneumococcal carriage at any of the ages. Complete data were available for birth weight, gestational age and gender. Infants with missing data on siblings more often carried pneumococci than infants with data on siblings. Due to selective missing data on siblings the observed association of siblings with pneumococcal carriage might be underestimated. No other differences in carriage rates were found between those with and without missing data on all other risk factors.

In summary, having siblings and day care attendance are independent risk factors for pneumococcal carriage, as well as previous pneumococcal carriage. No association between duration and exclusive breast-feeding and pneumococcal carriage was found. In infants 1.5 months of age the prevalence of VT's is significantly lower than in infants of age 6 and 14 months. Besides the environmental factors, additional research is justified to clarify the role of circulating maternal antibodies in infants, mucosal antibody response and genetic predispositions of the infants in pneumococcal carriage.

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Chapter 3

Air pollution (PM_{10}) is associated with pneumococcal carriage in infancy. The Generation R study.

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Submitted

Abstract

Background. *Streptococcus pneumoniae* (pneumococcus) is the most common bacterial respiratory pathogen. All pneumococcal diseases are preceded by asymptomatic colonization of the nasopharynx. Although risk factors for pneumococcal carriage are widely studied, to our knowledge no studies in humans have been performed on the association between air pollution and pneumococcal carriage.

Methods. We investigated in a population-based prospective cohort study the associations between particulate matter (PM_{10}) exposure and pneumococcal carriage in children at 1.5, 6, 14 and 24 months of age. Associations of average PM_{10} concentrations with pneumococcal carriage were assessed by odds ratios (OR). Additionally, regression models were adjusted for possible confounding by gender, educational level of mother, season, temperature and relative humidity (aOR).

Results. An association of PM_{10} exposure in the preceding day with pneumococcal carriage was found at the children's age of 6 months (aOR 1.12, 95% CI 1.00-1.26, $p<0.05$). An association of PM_{10} exposure in the preceding week with pneumococcal carriage was found at the children's age of 14 months (aOR 1.32, 95% CI 1.10-1.59, $p<0.01$).

Conclusion. PM_{10} concentration is associated with pneumococcal carriage as it increases the risk for colonization in early childhood.

Introduction

Streptococcus pneumoniae (the pneumococcus) is the most common bacterial respiratory pathogen. Pneumococci reside on the mucosal surface of the upper respiratory tract, especially in children. Although carriage of pneumococci is clinically asymptomatic, all pneumococcal infections are preceded by colonization. Furthermore, carriage is an important source for horizontal pneumococcal spread in the community. [15] Recently, an important association was found between carriage of airway pathogens in early life and the development of asthma at school age. [36]

Ealier studies have shown that concentrated ambient particles (CAPs) lead to a functional impairment of the antibacterial capacities of murine macrophages and that CAPs diminish bacterial clearance in the lungs of mice. [43, 44] We hypothesize that air pollution, in our study the PM_{10} concentration, may reduce immune mechanisms in humans and hence diminish pneumococcal clearance, favouring pneumococcal carriage. Although risk factors for pneumococcal carriage are widely studied, to our knowledge no study in humans has been performed on the association between PM_{10} concentration and pneumococcal carriage. We explored in a population-based prospective cohort the association between the PM_{10} concentration and pneumococcal carriage in young children.

Materials en Methods

Design

This study was embedded in the Generation R Study, a population-based prospective cohort study. The Generation R Study was designed to identify early environmental and genetic risk factors for growth, development and health from fetal life until young adulthood and has been described previously in detail. [41, 42] In a subgroup of 1,232 Dutch pregnant women and their children, referred to as the Generation R Focus Study, more detailed assessments of fetal and postnatal growth and development were conducted and nasopharyngeal swabs were taken. Of all approached pregnant women approached, 79% participated in the Generation R Focus Study. All children were born between February 2003 and August 2005. Nasopharyngeal swabs were taken of the children during a visit to the Generation R Focus Study research centre at the age of 1.5, 6, 14 and 24 months. Children who moved out of the Rotterdam study area during follow up were excluded from the present analyses. The Medical

Ethics Committee of the Erasmus Medical Center, Rotterdam, has approved the study. Written informed consent was obtained from all participants.

Measurements and outcome

We obtained data on gender from midwives and hospital registries. Information about maternal educational level was obtained by postal questionnaires.

We previously described how we detected pneumococcal carriage. [45] In short, trained research assistants obtained a nasopharyngeal swab with rayon tipped dacron pernasal swabs. Samples were transported to and processed at the microbiology laboratory of the Erasmus MC. All pneumococci were serotyped by capsular swelling method (Quellung reaction) with the use of commercially available antisera (Statens Seruminstitut, Copenhagen, Denmark), according to the instructions of the manufacturer. All children were born before the start of the national pneumococcal vaccination program in the Netherlands.

Particulate matter (PM_{10}) concentrations were measured at a monitoring station situated in the Rotterdam study area. This monitoring station (Rotterdam-Schiedamsevest) is operating since 1993 within the Dutch National Air Quality Monitoring Network by the National Institute for Public Health and the Environment (RIVM, 2001). [46] The station is set up to assess the urban background concentration level. Daily average (24-hour) monitoring data of temperature and relative humidity were obtained from the Rotterdam meteorological monitoring station operated by The Royal Netherlands Meteorological Institute (KNMI). The Environmental Protection Agency Rijnmond (DCMR) supplied the 24-hour average monitoring data collected during the follow-up period. Based on these data average PM_{10} concentrations, temperature and relative humidity were derived for specific periods of time prior to the date of pneumococcal carriage assessment. The date of sampling was also used to determine the season at time of sampling. Since the data on PM_{10} concentrations cover the whole study period we have no missing data .For temperature, relative humidity and season we also have complete data.

To assess the relation between PM_{10} and pneumococcal carriage, we used average PM_{10} concentration during the last 47 days prior to carriage assessment, because the average duration of carriage with pneumococci at this age was shown to be this long. [47] Secondly, time periods of one week and one day were studied, since a murine model showed an effect of CAPs on pneumococcal clearance in shorter time periods.

Analyses

Associations of average PM_{10} concentration, during different periods of time (one day, one week and 47 days), and pneumococcal carriage were assessed by odds ratios (OR). Additionally, regression models were adjusted for possible confounding by gender, educational level of mother, season, temperature and relative humidity (aOR). Subgroup analyses for pneumococcal vaccine (VT) and non-vaccinetype (NVT) carriage and PM_{10} were performed at the different time periods.

The statistical analyses were performed using the Statistical Package for the Social Sciences version 15.0 for Windows (SPSS Inc, Chicago, IL, USA).

Results

We obtained a nasopharyngeal sample of the children at the age of 1.5, 6, 14 and 24 months. Our cohort for analysis consisted of 576, 774, 757 and 642, respectively. Of the infants 48.5% (n=482) were girls. Educational level of mother was low in 1.9% (n=19), medium 34.3% (n=337) and high in 63.8% (n=627). In Table 1 we present the pneumococcal carriage rate and average PM_{10} concentration at the children's age of 1.5, 6, 14 and 24 months.

We found an association of PM_{10} exposure in the preceding day with pneumococcal carriage at the children's age of 6 months (aOR 1.12, 95% CI 1.00-1.26, $p<0.05$). An association of PM_{10} exposure in the preceding week with pneumococcal carriage was found at the children's age of 14 months (aOR 1.32, 95% CI 1.10-1.59, $p<0.01$ (Table 2). We repeated all analyses for carriage separated in VT and NVT. The results were similar for all pneumococci, VT and NVT (data not shown). Since there was no significant difference in outcome between the univariate analyses and the adjusted analyses, we only present the aOR.

Table 1. Pneumococcal carriage and average PM_{10} concentration at different ages.

	1.5 months	6 months	14 months	24 months
	(n=635)	(n=735)	(n=622)	(n=642)
S. pneumoniae carriage % (n)	8,3 (53)	31,5 (244)	43.9 (273)	38,6 (248)
PM10, µg/m3 (range)				
47 days	-	33.1 (25.4-46.6)	32.6 (25.4-46.9)	31,1 (25,7-41,7)
7 days	30.0 (14.3-110.1)	30.5 (15.0-110.1)	29.9 (13.7-110.1)	29,8 (15,2-76,7)
1 day	28.0 (13.4-104.7)	30,0 (13,7-101,1)	30.0 (12.2-110.1)	31,6 (12,2-92,6)

Value of PM10 µg/m3 is median with range

Table 2. Adjusted odds ratio's for pneumococcal carriage.

time periods before carriage assessment	aOR 1.5 months	aOR 6 months	aOR 14 months	aOR 24 months
47 days	NA	0.95 (0.85-1.06)	1.03 (0.91-1.16)	0.75 (0.53-1.06)
7 days	0.87(0.79-1.36)	0.95 (0.85-1.06)	1.32**(1.10-1.59)	1.00 (0.84-1.18)
1 day	1.06 (0.86-1.29)	1,12* (1,00-1,26)	1.01 (0.96-1.47)	0.99 (0.86-1.15)

Values are odds ratio's for pneumococcal carriage adjusted for gender, educatinal level of mother, season, temerature and relative humidity. * $p<0.05$, ** $p<0.01$

Discussion

We observed an increased risk for pneumococcal carriage in children at the age of 6 months of approximately 10% with every 10μg/m^3 rise in average PM_{10} during the preceding day before pneumococcal carriage assessment. Moreover, we observed an approximately 30% increased risk for pneumococcal carriage per 10 mg/m^3 rise in average PM_{10} for children at the age of 14 months in the week preceding carriage assessment.

A previous study has demonstrated *in vitro* that CAPs reduce lung macrophage antibacterial activity. [44] Sigaud et al. has shown reduced clearance of pneumococci from lungs of mice caused by impairment of macrophages. [43] Although we assessed nasopharyngeal carriage and not lung infection, a comparable dysfunction of macrophages in the nasopharynx induced by PM_{10} might result in reduced clearance of pneumococci from the nasopharynx. This mechanism might explain the increased risk for pneumococcal carriage we observed in our study. Macrophages have shown to appear in the nasopharynx three days after colonization in a mouse model, this could explain the association between pneumococcal carriage and PM_{10} at the week prior to carriage assessment. [48]

Another explanation for our findings might arise from the microbial pattern recognition Toll-like receptor 2 (TLR2), since TLR2 is known to be involved in the response of airway epithelial cells to PM. [49, 50]

Lawther et al. has shown inhibition of growth of various bacteria isolated from the human respiratory tract by extracts of atmospheric pollutants [51]. However, more recently no direct effect of PM_{10} on growth of *S. pneumoniae* was demonstrated. [52] Hence, a direct effect of PM_{10} on pneumococcal growth is not likely to explain the results of our findings.

We did not observe an association between PM_{10} concentration and pneumococcal carriage at the infant's age of 1.5 and 24 months. Pneumococcal carriage at 1.5 months of age is low (8.3%), Therefore the lack of power might play a role in limiting the effect. Pneumococcal carriage shows a yet unexplained trend with age; carriage rates increase in the first years of

life and decrease after the first few years of life. [15] At different ages different mechanisms of clearing pneumococci from the nasopharynx might be involved. The susceptibility to PM_{10} concentration might be age related and this could explain why we did not find an association at 24 months of age.

We observed no difference in the association between PM_{10} and pneumococcal carriage separated in VT and NVT. This suggests that the observed association is pneumococcal serotype independent.

To appreciate the results some limitations should be considered. The response in the Generation R Focus Study is above 80%. We were able to asses PM_{10} concentrations, temperature, relative humidity and season for all pneumococcal carriage assessment moments. We measured PM_{10} concentrations at one point in the city, while concentrations might be variable at different places in the city. A more accurate measurement of PM_{10} concentrations might even show a larger effect of PM_{10} concentrations on pneumococcal carriage. The percentages of mothers with lower socio-economic status (low educational level) and the percentages of mothers or children with medical complications are lower among the participants than expected from the population figures in Rotterdam. [42] This selection towards a more affluent and healthy study population may affect the pneumococcal carriage rate. Potentially, this might introduce some selection bias and may hamper the generalisability of the results.

In conclusion, our data clearly demonstrate an association between PM_{10} concentration and pneumococcal carriage. This unique finding is highly relevant, since pneumococcal carriage as well as air pollution is associated with the development of asthma and respiratory tract infections. Further research is warranted to elucidate the underlying mechanisms that associate pneumococcal carriage and PM_{10} and its implications for respiratory diseases in children.

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Chapter 4

Dynamics and determinants of *Staphylococcus aureus* carriage in infancy: the Generation R Study.

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ABSTRACT

Serial nasal swabs were collected at the age of 1.5, 6 and 14 months in 443 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: 52.1% at the age of 1.5 months to 12.9% at 14 months. Persistent carriage, as defined by continuous carriage of the same S. aureus strain at the three sampling moments, was hardly detected in early infancy.

PAPER

Staphylococcus aureus is a human commensal as well as a cause of a wide range of infections (5,8,19). A significant fraction of the human population is colonized with S. aureus on epithelial surfaces, of which the anterior nares are the most frequent carriage sites (3,11,13,19,20). Longitudinal studies distinguish three carriage patterns among healthy adult individuals (1,6,15,18,21). Persistent carriage occurs in about 20% of the adult population, 30% is intermittent carrier and 50% of the individuals are non-carriers (19,15,10,13). Persistent carriers usually carry the same strain for extended periods of time, whereas intermittent carriers tend to host different strains over time (19,18,13).

Children and adolescents under 20 years of age seem to have higher persistent carriage rates than adults (19,1,14, 4). 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 (1). The highest *S. aureus* carriage rate was observed in infants aged 3 months or younger (17).

Several determinants have been suggested to influence carriage rate in healthy children. Number of older siblings and family size (<5 and ≥5 people) as well as breastfeeding and passive smoking were found to be associated with *S. aureus* nasal carriage (2,16).

The objective of the present investigations is to study the dynamics of *S. aureus* nasal carriage in the first year of life, as well as its human and microbial determinants.

This project was embedded in the Generation R Study, a population-based prospective cohort study of pregnant women and their children from fetal life onwards. Detailed assessments of fetal and postnatal growth and development were conducted in 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 (34,35).

The Medical Ethics Committee of the Erasmus Medical Center, Rotterdam, has approved the study. Written informed consent was obtained from all participants (34). 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=884), 6 months (n=882) and 14 months (n=863), during these visits 627 got a swab taken at 1.5 month, 832 at 6 months and 757 at 14 months; 758 infants attended all visits and 443 provided us with three swabs to use for longitudinal analysis. Infants with antibiotic usage in the preceding 48 hours were excluded from nasal sampling (7,9).

Trained research nurses obtained a nasal swab (Copan Dacron Swabs, Brescia, Italy) for *S. aureus* isolation at each visit. Nasal samples were taken using a sterile transport swab suitable for aerobes and anaerobes. The swab was rubbed gently through both nostrils and transported in Amies transport medium to the medical microbiology laboratory of the Erasmus MC within 6 hours of sampling and 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*. Infants with antibiotic usage in the preceding 48 hours were excluded from nasal sampling (n=0).

S. aureus strains from samples of infants who were positive twice or more were genotyped, using pulsed field gel electroforese (PFGE). Plugs for PFGE were prepared in 1% low melting agarose and kept for 3-4 hours 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 four hours with endonuclease *Sma*I (50U). After digestion of the DNA, PFGE, performed using a Chef Mapper (BioRad, 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).

Multinomial logistic regression analysis was performed to report on the association of *S. aureus* carriage pattern with gender, birth weight, gestational age, breastfeeding, 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 multinomial logistic regression analysis. The statistical analyses were performed using the Statistical Package of Social Sciences version 11.0 for Windows (SPSS Inc, Chicago, IL, USA).

Of all mothers of the non-colonized infants, 223 (63,9%) attended higher education as compared to 63 (70.8%) mothers of the colonized infants. The median birth weight of the non-colonized infants was 3580 grams (5-95% range 2751 - 4305) and 3520 grams (5-95% range 2385 – 4605) in the colonized infants. Median gestational age was equal for the non-colonized and colonized infants, 40.3 weeks. 170 infants (48.0%) of the non-colonized infants were male, as compared to 55 (61.8%) in the colonized group. 39.5% (n=131) of the non-colonized infants had at least one sibling, in the colonized group 40.9% (n=36). Of all infants in

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

Determinants of *Staphylococcus aureus* carriage in the first year of life				
	Not colonized (0-1) (n=354)	Colonized (2>) (n=89)	OR (95% CI)	aOR (95% CI)
Gender				
- Female	184 (52.0%)	34 (38.2%)	1.00	1.00
- Male	170 (48.0%)	55 (61.8%)	**1.75 (1.09 – 2.82)**	**2.09 (1.17 – 3.72)**
Gestational age	40.3 (37.7 – 42.0)	40.3 (37.0 – 42.4)	0.91 (0.78 – 1.06)	0.94 (0.76 – 1.17)
Birth weight	3580 (2751 – 4305)	3520 (2385 – 4605)	1.00 (1.00 – 1.00)	1.00 (1.00 – 1.00)
Breast feeding at 6 months				
- No	237 (69.1%)	56 (63.6%)	1.00	1.00
- Yes	106 (30.9%)	32 (36.4%)	1.28 (0.78 – 2.09)	1.36 (0.75 – 2.47)
Mother educational level				
- Higher education	223 (63.9%)	63 (70.8%)	1.00	1.00
- Lower/intermediate education	126 (36.1%)	26 (29.2%)	0.73 (0.44 – 1.21)	0.52 (0.26 – 1.05)
Mother prenatal smoking				
- No	319 (94.4%)	79 (94%)	1.00	1.00
- Yes	19 (5.6%)	5 (6%)	1.06 (0.34 – 2.93)	5.35 (0.86 – 33.40)
Mother postnatal smoking				
- No	260 (87.8%)	70 (88.6%)	1.00	1.00
- Yes	36 (12.2%)	9 (11.4%)	0.93 (0.43 – 2.02)	0.25 (0.05 – 1.33)
Siblings				
- No	201 (60.5%)	52 (59.1%)	1.00	1.00
- Yes	131 (39.5%)	36 (40.9%)	1.06 (0.66 – 1.71)	1.03 (0.57 – 1.87)
Day care attendance				
- No	67 (22.5%)	25 (31.6%)	1.00	1.00
- Yes	231 (77.5%)	54 (68.4%)	0.63 (0.36 – 1.08)	0.53 (0.27 – 1.01)

Values are means (SDS) or medians (5-95% range) for variables with skewed distribution. 443 infants provided nasal swabs at all three collection moments. Data were missing on breastfeeding (n=12), educational level of the mother (n=5), maternal smoking prenatal (n=21), maternal smoking postnatal (n=68), siblings (n=23), daycare attendance (n=66).

the non-colonized group, 30.9% (n=106) received breastfeeding up until 6 months compared to 36.4% (n=32) in the colonized group. 231 non-colonized infants (77.5%) attended day care in the first year of life, 68.4% (n=54) of the colonized infants. (Table1)

The prevalence of *S. aureus* carriage significantly decreased from 52.1% (231 of 443) at the age of 1.5 months to 21.7% (96 of 443) at the age of 6 months and 12.9% (57 of 443) at the age of 14 months (p value <0.001). (Figure 1)

A group of 36.3% (161 of 443) of the infants was never found positive for *S. aureus*, 2.9% (13 of 443) were found positive at all 3 moments. The largest group consisted of infants with one positive swab 43.6% (193 of 443). 76 infants had two nasal swabs positive for *S. aureus* (17.2%).

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 (3 missing: one lab number was missing, one swab got lost, 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 3 different strains for 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 a great variety of different genotypes. (Figure 2)

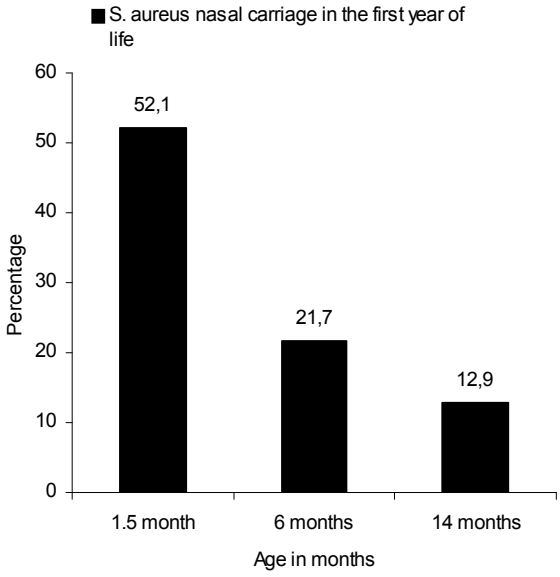

Figure 1. *S.aureus* carriage in the first year of life. We found a significant decrease in *S.aureus* nasal carriage in the first year of life. P<0.001, for the difference between *S.aureus* carriage rates at 1.5, 6 and 14 months of age.

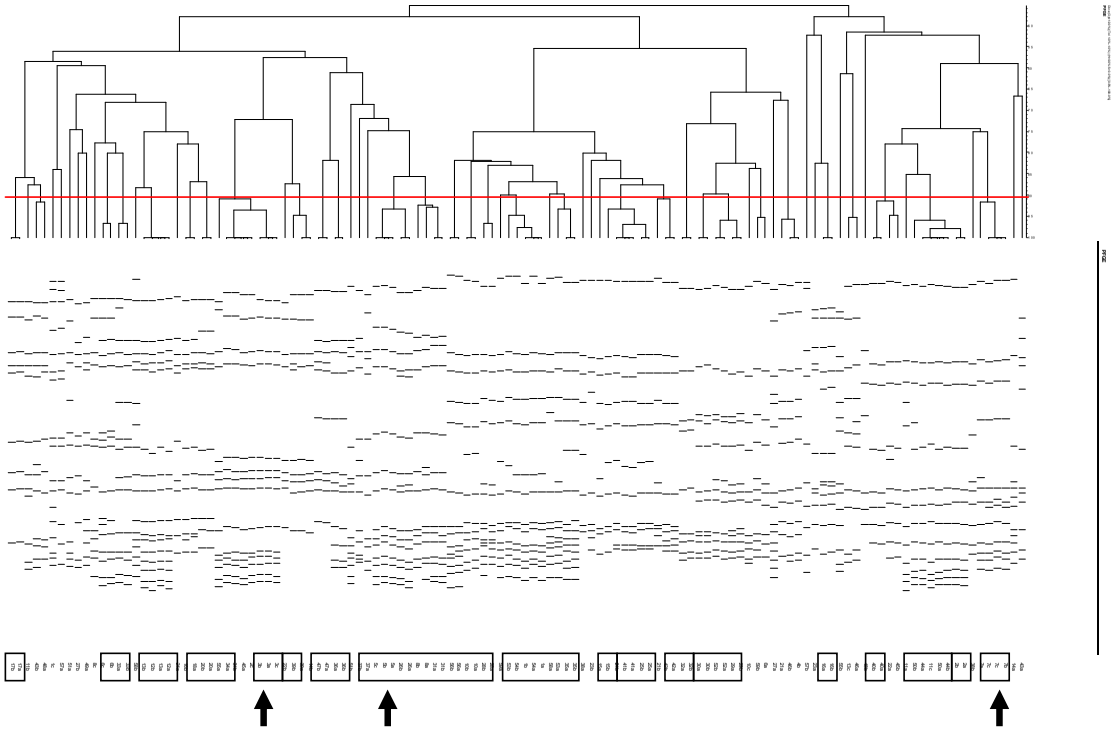

Figure 2. *S. aureus* dendrogram.
Pulsed field gel electrophoretic analysis of all strains derived from infants with two or more cultures positive for S. aureus. The banding patterns are shown in the central portion of the figure. On the left, strain relatedness percentage is indicated in the form of 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).

Boys have a significantly higher risk to be positive twice or more times as compared to girls (aOR 2.09; 95% CI 1.17-3.72; p-value 0.012). We did not find a significant association between *S. aureus* carriage and presence of siblings (aOR 1.03 95%CI 0.57-1.87; p-value 0.919), nor with breastfeeding, day care attendance, maternal smoking (pre- and postpartum), birth weight or gestational age. (Table 1)

We documented a significant decrease in prevalence of *S. aureus* carriage in the first year of life ($p< 0.001$), which is in line with literature data (2,16). A possible explanation for this drop may be found in the competition between *S. aureus* and *S. pneumoniae*. Bogaert et al found an inverse prevalence of these pathogens in slightly older children, the same could occur in young infants (2). In our cohort, Labout et al found an increased level of pneumococcal carriage in the first year of life (12). The significant decrease in 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 (work in progress).

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 hardly found infants carrying the same strain over time. Previous studies show a higher prevalence of persistent carriage amongst older children and adolescents up to the age of 20, compared to adults (1,14,4). 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 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 colonization 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 recolonization with the strain from the mother or other family members, who might be a persistent carriers in 20% of the cases (19,10).

The main difference in our finding as compared to the study on determinants of carriage by Bogaert et al and Peacock et al, is the lack to identify family size, passive smoking or breastfeeding as significant determinants of carriage (2,16). However, we seem to be more precise with our data on breastfeeding with very little missing data and furthermore our data

covers a larger cohort of children in the same age group (first year of life), as compared to 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 hardly occurs among infants and the incidence of carriage drops enormously in the first year of life. Whether these differences are a result of immune-modulation or other biological phenomena is subject to further investigation.

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Chapter 5

Interactions between respiratory pathogens during colonization in the first months of life. The Generation R Study.

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Submitted

Abstract

Background. Microbe-microbe as well as microbe-host-microbe interactions are considered to play an important role in the microbial colonization dynamics of the nasopharynx.

Methods. We investigated in a population-based prospective cohort study the microbial dynamics of *Streptococcus pneumoniae, Staphylococcus aureus*, *Haemophilus influenzae* and *Moraxella catarrhalis* during colonization in infancy.

Results. In consecutive 1.5, 6 and 14 months samples, we observed increasing carriage rates of *S. pneumoniae, H. influenzae* and *M. catarrhalis*, whereas the carriage rate of *S. aureus* decreased. Regression models showed a negative association between carriage of *S. pneumoniae* and *S. aureus* at 1.5 months (odds ratio [OR] 0.43, 95% CI 0.24-0.76) and 14 months (adjusted odds ratio [aOR] 0.56, 95% CI 0.37-0.98) of age. In the adjusted regression models the same trend was found. Strikingly, this association was predominantly caused by non-vaccine-type pneumococci (aOR 0.20, 95% CI 0.06-0.63 at 1.5 months of age). Finally, a positive association between carriage of *S. pneumoniae* and *H. influenzae* was observed at 1.5 months (OR 3.27, 95% CI 1.51-7.08), 6 months (OR 1.66, 95% CI 1.19-2.34) and 14 months (OR 1.91, 95% CI 1.39-2.61).

Conclusions. Our data show a negative interaction between carriage of pneumococci, in particular non-vaccine-type pneumococci, and *S. aureus*, and a positive association between carriage of pneumococci and *H. influenzae*. The latter observation could not be conclusively related to pneumococcal serotypes.

Introduction

The pathogenic species *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Haemophilus influenzae* and *Moraxella catarrhalis* are important colonizers and coinhabitants of the human nasopharynx. Asymptomatic colonization of the nasopharynx is considered to be the first step in the pathogenesis of infections caused by these bacteria. In addition, healthy carriers are thought to be primarily responsible for the person-to-person bacterial transmission of these pathogens.

Competitive and cooperative microbe-microbe as well as microbe-host-microbe interactions are considered to play an important role in the microbial colonization dynamics of the nasopharynx. [14] Pneumococcal conjugate vaccination also affects pneumococcal colonization of the nasopharynx. [53-57] However, the exact consequence of pneumococcal conjugate vaccination on multiple species colonization is at present poorly understood.

Several studies have reported associations between pneumococcal carriage and *S. aureus* carriage. However, most of these were cross-sectional studies, studies in older children and adults or studies in specific patient populations. Furthermore, most studies did not analyze vaccine and non-vaccine-type pneumococci separately or did not correct for well-known confounding variables. [7, 9, 16, 17, 19, 22] Only two longitudinal studies on bacterial carriage in infancy have been performed. First, a study in Western Australia investigated 79 Aborigional and 88 non-Aborigional children from birth till the age of 24 months. [17] No significant association was found between pneumococcal and *S. aureus* carriage, but a positive association was observed between carriage of pneumococci and *H. influenzae*, and of pneumococci and *M. catarrhalis* carriage [8]. Secondly, a prospective cohort of 212 healthy children 6 to 36 months of age was monitored for 1 year. [58] Nasopharyngeal samples were taken during upper respiratory tract infection. A negative association was observed between carriage of *S. pneumoniae* and *S. aureus*, between *S. pneumoniae* and *H. influenzae* and between *S. aureus* and *H. influenzae*.

Our aim was to study the associations between *S. pneumoniae, S. aureus, H. influenzae* or *M. catarrhalis* carriage in a large longitudinal study in healthy infants, in the pre-7-valent pneumococcal conjugate vaccine era. Importantly, known risk factors for carriage were taken into account and we studied the role pneumococcal serotypes play in this dynamic interactive process.

Materials and Methods

This study was embedded in the Generation R Study, a population-based prospective cohort study from fetal life until young adulthood. The Generation R Study has been described in detail previously. [41, 42] Detailed assessments of fetal and postnatal growth and development were conducted in 1,232 Dutch pregnant women and their children. All children were born between February 2003 and August 2005. After excluding still birth (n=3), withdrawn of consent (n=138), and twins (n=27), the cohort for analysis consisted of 1079 infants. The children were invited to visit the Generation R Focus Study research centre at the age of 1.5 month (response rate 82%), 6 months (response rate 82%) and 14 months (response rate 80%). The Medical Ethics Committee of the Erasmus Medical Center, Rotterdam, has approved the study. Written informed consent was obtained from all participants.

To detect nasal and nasopharyngeal bacterial carriage, samples were taken at the infants' age of 1.5, 6, and 14 months. Nasopharyngeal samples were taken using rayon tipped dacron pernasal swabs (Copan Italia, Brescia, Italy). The swabs were transported in Amies transport medium and plated within 6 hours after sampling on blood agar plates with 5% sheep blood (BA), chocolate agar plates (CHOC) and *Haemophilus* selective agar plates (HAEM). The BA, CHOC and HAEM plates were incubated at 35°C using 5% CO_2 conditions for two days. Bacterial growth was determined daily; BA was used to culture *S. pneumoniae, M. catarrhalis* and *S. aureus,* CHOC to grow *S. pneumoniae, M. catarrhalis, H. influenzae* and *S. aureus*, and HAEM to culture *H. influenzae*. To detect *S. aureus* carriage, nasal samples were taken using sterile swabs, which were then transported in Amies transport medium (Copan Italia, Brescia, Italy) and incubated directly in dehydrated phenol red mannitol broth (Becton Dickinson, Le Pont de Claix, France) at 35°C to culture *S. aureus*. Species identification was performed using standard microbial methods. All pneumococci were serotyped by capsular swelling method (Quellung reaction) using antisera from Statens Seruminstitut, in Copenhagen, Denmark, according to the instructions of the manufacturer.

We obtained data on gender, birth weight and gestational age from midwives and hospital registries. Information concerning siblings, breast-feeding, educational level of the mother, maternal smoking and day care attendance was obtained by postal questionnaires at the infants' age of 6 and 12 months.

Because repeated measurements across periods are likely to be correlated, a statistical model adjusting for this correlation was applied. We used a Generalized estimating equations (GEE) model with logit link, binomial error structure and fully parameterized cluster structure of the log odds ratio used to model the association of the responses from subjects

for binary data. Associations between the different bacteria were assessed by odds ratios (OR). Additionally, regression models were adjusted for the potential confounders gender, birth weight, gestational age, educational level of mother, siblings, day care attendance, duration of breast-feeding and maternal smoking. Next, we assessed, using similar analyses, whether the interactions of pneumococcal carriage with the other bacteria was different in vaccine-type (VT) and non-vaccine-type (NVT) pneumococci. All tests were carried out using a two-sided alpha level of 5%. The statistical analyses were performed using SAS statistical software, version 9.1 (SAS Institute, Cary, NC, USA).

Table 1. General characteristics of the study population.

cohort for analyses n=1079	Girls n=521	Boys n=558
Birth weight (grams)	3467 (537)	3548 (537)
Gestational age (weeks)	40.3 (37.3-42.1)	40.3 (37.0-42.2)
Siblings ≥1 (%)		
no	62.3 (213)	64.3 (229)
yes	37.7 (129)	35.7 (127)
Smoking mother (%)		
no	86.8 (283)	87.7 (300)
yes	13.2 (43)	12.3 (42)
Educational level mother (%)		
Primary school	2.7 (14)	1.3 (7)
Secondary school	30.8 (158)	36.8 (203)
Higher education	66.5 (341)	62.0 (342)
Day care at 6 months		
no	33.7 (110)	38.8 (132)
yes	66.3 (216)	61.2 (208)
Day care at 12 months		
no	28.0 (122)	34.0 (153)
yes	72.0 (314)	66.0 (297)
Breast-feeding (%)		
Never	11.8 (45)	13.5 (53)
< 3 months	23.7 (90)	27.0 (106)
3-6 months	22.9 (87)	21.7 (85)
> 6 months	41.6 (158)	37.8 (148)

Values of birth weight is mean (standard deviation), gestational age is median (90% range). Other values are percentages (absolute numbers). Data were missing for siblings (n=381), smoking (n=411), educational level mother (n=14), day care at 6 months (n= 413), day care at 12 months (n=193) and breast-feeding (n=307).

Results

Characteristics of the infants according to gender are presented in Table 1. Mean birth weight of the girls was 3467 grams and 3548 grams for boys. Median gestational age was 40.3 weeks for both sexes. 37.7% of the girls and 35.7% of the boys had at least one sibling. Educational level of the mother showed the same distribution for girls and boys (2.7%, 30.8% and 66.5% for girls and 1.3%, 36.8%, 62.0% for boys for the groups of primary, secondary and higher education, respectively). At 6 and 12 months of age 66.3% and 72.0% of the girls visited a day care centre at least once a week; for boys this was 61.2% and 66.0%, respectively. Of all girls, 11.8% never received breast-feeding, 23.7% of the mothers stopped with breast-feeding before the girls' age of 3 months, 22.9% stopped between the girls' age of 3 to 6 months, 41.6% stopped after the girls' age of 6 months. For boys this was 13.5%, 27.0%, 21.7% and 37.8%, respectively.

In the consecutive 1.5 months, 6 months and 14 months samples, we observed an increasing carriage rate of *S. pneumoniae* (8.9%, 31.0% and 43.5%, respectively), *H. influenzae* (7.0%, 24.7% and 31.7%, respectively) and *M. catarrhalis* (11.6%, 30.5% and 29.6%, respectively), whereas the carriage rate of *S. aureus* decreased (52.3%, 20.1% and 14.5%, respectively) (Figure 1). At 1.5 months 6 months and 14 months of age we found 36.5%, 53.6% and 62.2% of the pneumococci were vaccine-type pneumococci, respectively. [59]

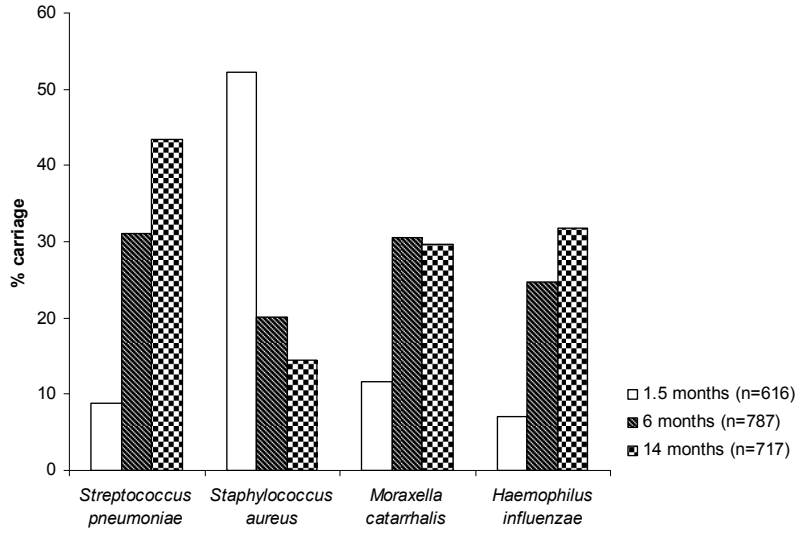

Figure 1. Prevalence of carriage of *S. pneumoniae*, *S. aureus*, *M. catarrhalis* and *H. influenzae* at different ages.

Table 2. Associations in carriage of the different bacterial species at 1.5, 6 and 14 months of age.

1.5 months of age						
	H. influenzae		*M. catarrhalis*		*S. aureus*	
	OR	aOR	OR	aOR	OR	aOR
S. pneumoniae	3.27*** (1.51-7.08)	2.33 (0.79-6.83)	0.73 (0.28-1.90)	0.74 (0.46-1.19)	0.43** (0.24-0.76)	0.49(0.23-1.02)
H. influenzae	_	_	1.41 (0.59-3.36)	0.54 (0.13-2.29)	1.08 (0.59-1.95)	1.31 (0.59-2.88)
M. catarrhalis	_	_	_	_	0.67 (0.45-0.99)	0.51* (0.27-0.98)
6 months of age						
	H. influenzae		*M. catarrhalis*		*S. aureus*	
	OR	aOR	OR	aOR	OR	aOR
S. pneumoniae	1.66** (1.19-2.34)	1.31 (0.79-2.20)	1.10 (0.79-1.52)	0.93 (0.31-2.80)	0.93 (0.64-1.34)	0.98 (0.52-1.83)
H. influenzae	_	_	1.11 (0.78-1.57)	0.66 (0.39-1.12)	0.84 (0.56-1.27)	0.80 (0.43-1.50)
M. catarrhalis	_	_	_	_	0.62 (0.38-1.02)	0.81 (0.48-1.36)
14 months of age						
	H. influenzae		*M. catarrhalis*		*S. aureus*	
	OR	aOR	OR	aOR	OR	aOR
S. pneumoniae	1.91** (1.39-2.61)	1.69** (1.07-2.68)	1.39* (1.01-1.91)	1.39 (0.88-2.20)	0.56* (0.37-0.89)	0.69 (0.41-1.17)
H. influenzae	_	_	1.60** (1.14-2.24)	1.32 (0.82-2.12)	0.78 (0.49-1.24)	0.89 (0.45-1.74)
M. catarrhalis	_	_	_	_	1.06 (0.67-1.66)	1.03 (0.55-1.93)

Values are crude odds ratios (OR) and adjusted odds ratios (aOR). Adjustment was made for birth weight, gestational age, gender, educational level of mother, siblings, daycare attendance and smoking. * p<0.05, ** p <0.01, ***p <0.001.

In Table 2 the univariate and the multivariate analyses of the associations between the different bacteria at the different time points are presented. In the multivariate analyses we adjusted for gender, birth weight, gestational age, educational level of mother, siblings, day care attendance, maternal smoking, and duration of breastfeeding.

Pneumococcal carriage was negatively associated with *S. aureus* carriage in the univariate analysis at the age of 1.5 (odds ratio [OR] 0.43 95% CI 0.24-0.76) and 14 months (aOR 0.56 CI 0.37-0.89). After correcting for confounding factors the same trend was found. Pneumococcal carriage was associated with *H. influenzae* carriage at 1.5 months (OR 3.27, 95% CI 1.51-7.08), 6 months (OR 1.66, 95% CI 1.19-2.34) and 14 months (OR 1.91, 95% CI 1.39-2.61). Pneumococcal carriage was not associated with carriage of *M. catarrhalis*. In the adjusted analyses no associations between *S. aureus* , *M. catarrhalis* and *H. influenzae* were observed.

In Table 3 the association between pneumococcal vaccine-type and non-vaccine-type carriage and carriage of *S. aureus*, *M. catarrhalis* and *H. influenzae* at the different time points is presented. We observed a negative association between *S. pneumoniae* and *S. aureus* and, surprisingly, this association was predominantly caused by non-vaccine-type pneumococci (at 1.5 months aOR 0.92 CI 0.45-1.87) for VT and aOR 0.20 CI 0.06-0.63 for NVT). We observed no serotype-related association between pneumococci and *M. catarrhalis*. The positive association observed between *S. pneumoniae* and *H. influenzae* could not be attributed to particular groups of serotypes.

Discussion

Associations between carriage of different microbial species has been extensively described in literature. Consistent with previous studies, we observed an increasing carriage rate with age of *S. pneumoniae* (8.9%, 31.0% and 43.5%, at 1.5, 6 and 14 months of age, respectively), *H. influenzae* (7.0%, 24.7% and 31.7%, respectively) and *M. catarrhalis* (11.6%, 30.5% and 29.6%, respectively), whereas the carriage rate of *S. aureus* decreased with age (52.3%, 20.1% and 14.5%, respectively). [6, 8-10, 60-64]

We observed a negative association between pneumococcal carriage and *S. aureus* carriage at the infants' age of 1.5 monts and 14 months in the univariate analysis. A similar trend was observed in the multivariate analysis, in particular at the infants' age of 1.5 and 14 months. Regev-Yochay et al. have reported a possible explanation for the negative interaction between pneumococci and *S. aureus* by demonstrating in an *in vitro* model pneumococcus-mediated hydrogen peroxide-mediated (H_2O_2) killing of *S. aureus*. In humans this mechanism

Table 3. Associations between pneumococcal vaccine-type (VT) and non-vaccine-type (NVT) carriage, and carriage of *S. aureus*, *M. catarrhalis* and *H. influenzae* at 1.5, 6 and 14 months of age.

	1.5 months of age		**6 months of age**		**14 months of age**	
	S. aureus		*S. aureus*		*S. aureus*	
	OR	aOR	OR	aOR	OR	aOR
S. pneumoniae						
VT	0.66 (0.26-1,67)	0.92 (0.45-1.87)	1.21 (0.78-1.88)	1.30 (0.36-4.65)	0.70 (0.43-1.13)	0.71 (0.35-1.44)
NVT	0.30** (0.14-0.67)	0.20** (0.06-0.63)	0.66 (0.38-1.15)	0.94 (0.37-2.41)	0.40* (0.19-0.84)	0.68 (0.43-1.33)
	M. catarrhalis		*M. catarrhalis*		*M. catarrhalis*	
	OR	aOR	OR	aOR	OR	aOR
S. pneumoniae						
VT	0.42 (0.06-3.13)	0.73 (0.09-5.88)	1.01 (0.67-1.54)	0.77 (0.41-1.43)	1.17 (0.81-1.69)	1.04 (0.62-1.75)
NVT	0.70 (0.20-2.42)	0.72 (0.16-3.24)	1.24 (0.80-1.90)	0.72 (0.39-1.30)	1.36 (0.88-2.12)	1.38 (0.73-2.61)
	H. influenzae		*H. influenzae*		*H. influenzae*	
	OR	aOR	OR	aOR	OR	aOR
S. pneumoniae						
VT	2.77 (0.71-10.84)	3.33 (0.62-17.96)	1.54* (1.00-2.38)	1.19 (0.61-2.33)	1.85* (1.29-2.65)	1.63 (0.97-2.74)
NVT	4.27** (1.72-10.58)	2.35 (0.59-9.29)	2.02** (1.30-3.14)	1.58 (0.85-2.96)	2.09*** (1.35-3.25)	1.90 (0.97-3.71)

Values are crude odds ratios (OR) and adjusted odds ratios (aOR). Adjustment was made for birth weight, gestational age, gender, educational level of mother, siblings, daycare attendance and smoking. * $p<0.05$, ** $p<0.01$, ***$p<0.001$.

of interaction between pneumococci and *S. aureus* might have either a comparable direct bactericidal effect or, alternatively, could occur by microbe-host-microbe interaction via host cell signalling pathways activated by H_2O_2. [65] However, in a recent study they could not conclusively explain the interaction between pneumococci and *S. aureus* as a result of varying H_2O_2 production of pneumococcal strains observed in their cohort study. [66] Furthermore, they have found no significant role for different *S. aureus* strains. [66] However, Selva et al. have observed that lysogenic strains of *S. aureus* are highly sensitive to H_2O_2, whereas nonlysogenic strains are resistant. [67] Park et al. have hypothesized that oxidant defence by catalase produced by *S. aureus* might play a role in promoting *S. aureus* carriage. The authors have shown that expression of catalase contributes significantly to the survival of *S. aureus* in the presence of *S. pneumoniae* both *in vitro* and in a murine model of nasal co-colonization. [68]

Madhi et al. have found evidence for microbe-host-microbe interactions when studying bacterial carriage in HIV-infected and HIV-unaffected children. [69] They have demonstrated a negative association between pneumococci and *S. aureus* in HIV-uninfected children, but not in HIV-infected children, suggesting that the negative association might be caused through interference by host (immunological) factors.

The observed negative association between pneumococcal carriage and *S. aureus* carriage was, in contrast to previous studies [7, 9], predominantly caused by non-vaccine-type pneumococci rather than by vaccine-type pneumococci. Several studies have reported associations between carriage of pneumococci and *S. aureus*. [7, 9, 16, 17, 19, 22] The question was raised whether vaccination against pneumococci might result in increased carriage rates of *S. aureus*, and consequently, in *S. aureus* related diseases. A pneumococcal vaccination study in children with a history of recurrent acute otitis media has suggested an increased incidence of *S. aureus*-related acute otitis media as a result pneumococcal conjugate vaccination. [70, 71] However, others have shown no association between pneumococcal carriage and *S. aureus* cariage in pneumococcal conjugate-vaccinated nor in non-vaccinated children. [70, 71] A negative association between pneumococcal carriage and *S. aureus* carriage was observed in 790 children younger than 40 months of age at primary care clinics with respiratory tract infections. [9] This association was predominantly caused by vaccine-type pneumococci. However, the negative association between *S. pneumoniae* and *S. aureus* carriage disappeared after correction for confounding factors. Moreover, no association between pneumococcal carriage and *S. aureus* carriage in adults was found. Other studies investigating the interaction between *S. pneumoniae* carriage and *S. aureus* carriage did not study vaccine-type-related interactions. A cross sectional study in the Czech Republic

reported a negative association between pneumococcal and *S. aureus* carriage in 425 healthy day care attendees aged three to six years, but they did not correct for confounding factors nor investigated the association for groups of vaccine and non-vaccine-type pneumococci separately. [22] A study in South African children, hospitalized for severe pneumonia, aged 1 to 60 months found a negative association in HIV-negative children but not in HIV-positive children. [19] Bogaert et al. have confirmed the absence of an association in a cohort of 245 HIV-positive adults. [16] Jacoby et al. have demonstrated in a longitudinal study on bacterial carriage in children in Western Australia followed from birth till the age of 24 months, no significant association between pneumococcal and *S. aureus* carriage. [17] Pettigrew et al. have described colonization of the same four bacteria during upper respiratory tract infection in young children. [58] Similar to our and the abovementioned studies, the authors observed a negative association between pneumococcal and *S. aureus* carriage.

Taken together, although scientific literature presents conflicting data on the type of interaction, it is apparent that in children, whether healthy or suffering from respiratory tract infections, there is a negative association between pneumococcal carriage and *S. aureus* carriage. The role of different serotypes in the negative association between carriage of *S. pneumoniae* and *S. aureus*, remains inconclusive. This might be due to the fact that the cohorts studied so far are still limited in size, and consequently, do not allow for the analysis at serotype-specific level. Perhaps the interaction between both species is serotype-independent after all.

We observed a positive association between pneumococcal carriage and *H. influenzae* carriage. *In vitro* studies, murine experimental studies and human studies on the interaction between pneumococci and *H. influenzae* are contradictory. Two studies have shown *in vitro* inhibition of growth of *H. influenzae* by *S. pneumoniae*. [20, 21] In contrast, Lysenko et al. have shown in a murine model of co-colonization the clearance of *S. pneumoniae* from the upper respiratory tract by *H. influenzae*. [18] Jacoby et al. have found a positive association between pneumococcal and *H. influenzae* carriage in humans. [17] Our large-cohort study confirms these observations. In addition, we showed that both vaccine-type and non-vaccine-type pneumococci showed an interaction with *H. influenzae*. The detailed information on pneumococcal serotypes did not give us specific clues on the serotype(s) primarily responsible for this interaction. In contrast to the observation of Jacoby et al. and our observation, Pettigrew et al. observed a negative association between pneumococcal and *H. influenzae* carriage. However, in contrast to our study, the study of Pettigrew and co-workers has been performed among children with upper respiratory tract infections, which might influence the dynamics within the nasopharyngeal microflora.

In conclusion, our data show a negative interaction between carriage of pneumococci, in particular non-vaccine-type pneumococci, and *S. aureus*. In addition, a positive association between pneumococcal carriage and carriage of *H. influenzae* as observed, which could not be conclusively related to pneumococcal serotypes. The important question whether microbe-microbe or microbe-host-microbe interactions (or both) play an important role in the microbial interactions in the nasopharynx remains subject of further investigation.

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Part 2

The consequences of carriage of *S. pneumoniae*, *H. influenzae* and *M. catarrhalis* and otitis media, and the consequences of carriage of *S. aureus* and atopic dermatitis in young children

Chapter 6

Risk factors for otitis media in children with special emphasis on the role of colonization with bacterial airway pathogens: the Generation R study

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Abstract

Objective

Acute otitis media is the most frequent diagnosis in children visiting physicians' offices. Risk factors for otitis media have been widely studied. Yet, the correlation between bacterial carriage and the development of otitis media is not entirely clear. Our aim was to study in a population-based prospective cohort the risk factors for otitis media in the second year of life with special emphasis on the role of colonization with *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Moraxella catarrhalis*.

Patients and methods

The study was embedded in the Generation R Study. Data on risk factors and doctor-diagnosed otitis media were obtained by midwives, hospital registries and postal questionnaires in the whole cohort (n=7295). Nasopharyngeal swabs were obtained at the age of 1.5, 6 and 14 months in the focus cohort (n=1079).

Results

Of these children, 2515 (47.2%) suffered at least one period of otitis media in their second year of life. The occurrence of otitis media during the follow-up period in the first 6 months of life and between 6 and 12 months of age was associated with the risk of otitis media in the second year of life (aOR, 1.83 95% CI 1.24-2.71 and aOR 2.72, 95% CI 2.18-3.38, respectively). Having siblings was associated with an increased risk for otitis media in the second year of life (aOR 1.42, 95% CI 1.13-1.79). No associations were found between bacterial carriage in the first year of life and otitis media in the second year of life.

Conclusions

In our study, otitis media in the first year of life is an independent risk factor for otitis media in the second year of life. Surprisingly, bacterial carriage in the first year of life did not add to this risk. Moreover, no association was observed between bacterial carriage in the first year of life and otitis in the second year of life.

Introduction

Acute otitis media is one of the most common childhood infections and the leading cause for children to visit a doctor. [72] Most children experience at least one episode of otitis media; in 50-85% of the children acute otitis media is diagnosed at least once in the first 3 years of life. [73, 74] Risk factors for otitis media have widely been studied. In addition to host factors like birth weight, gestational age and craniofacial abnormalities, environmental factors like passive smoking, day care attendance, socioeconomic status, pacifier use and breast-feeding have been studied. Frequency of colonization with bacterial airway pathogens was shown to be associated with otitis media in the first years of life. [8, 75-78] *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Moraxella catarrhalis* are the most frequently cultured pathogens in acute otitis media. Early age of colonization seems to increases the risk of otitis media. [8, 32, 78] Syrjänen et al. have suggested that carriage of newly acquired bacteria is associated with otitis media. [33]

Our aim was to study in a population-based prospective cohort the risk factors for otitis media in children with special emphasis on the role of colonization with *S. pneumoniae*, *H. influenzae* and *M. catarrhalis* in the first year of life.

Patients and methods

Design

This study was embedded in the Generation R Study, a population-based prospective cohort study from fetal life until young adulthood. The Generation R Study has been described in detail previously. [41, 42] The cohort includes 9778 mothers and their children living in Rotterdam, the Netherlands. Detailed assessments of fetal and postnatal growth and development were conducted in 1,232 Dutch pregnant women and their children in the Generation R Focus Study. The Medical Ethics Committee of the Erasmus Medical Centre, Rotterdam, has approved the study. Written informed consent was obtained from all participants.

Measurements and outcome

We obtained data on birth weight, gestational age and gender from midwives and hospital registries. Information about maternal smoking, siblings, educational level of the mother, day care attendance, breast-feeding, pacifier use and otitis media in the first year of life was obtained by postal questionnaires at the infants' age of 6, 12 and 24 months. Mothers were

asked whether their children suffered from fever in the prior period (no, yes), and subsequently whether this period of fever was accompanied by earache and whether they had visited a doctor. The diagnosis otitis media in the period from 0 to 6 months of age and the period from 6 to 12 months of age was defined as having at least one period of fever accompanied by earache for which a doctor was visited during any part of the 6 months before reaching the age of respectively 6 and 12 months. Likewise, the diagnosis of otitis media in the second year of life was defined as having at least one period of fever accompanied by earache for which a doctor was visited during any part of the year before reaching the age of 24 months. Furthermore, otitis-proneness was defined as having four or more episodes by 1 year of age, six or more by 2 years of age, or placement of myringotomy tubes, similar to the definition used by Faden et al. [77] Risk factors for otitis media in the second year of life were studied in the whole cohort.

In the Focus cohort, samples were taken at the infants' age of 1.5, 6, and 14 months to detect nasopharyngeal bacterial carriage. Nasopharyngeal samples were taken with rayon tipped dacron pernasal swabs (Copan Italia, Brescia, Italy) and transported in Amies transport medium and plated within 6 hours of sampling on a blood agar plate with 5% sheep blood, a chocolate agar plate and a Heamophilus selective agarplate. The plates are kept at 35°C in a CO_2 rich environment for two days. Bacterial growth was determined daily. All bacteria were determined by use of standard methods.

Analyses

In the whole cohort associations of birth weight, gestational age, gender, maternal smoking, siblings, educational level of mother, day care attendance, duration of breast-feeding, pacifier use and otitis media in the first year of life with otitis media in the second year of life were assessed by logistic regression models resulting in odds ratios (OR) with their 95% confidence interval (CI). Additionally, a multivariate logistic regression model was performed to assess the association of each risk factor separately with otitis media in the second year of life was adjusted for all the other risk factors resulting in adjusted odds ratios (aOR). In the focus cohort association of bacterial carriage in the first year of life with otitis media in the second year of life was assessed for the airway pathogens *S. pneumoniae*, *M. catarrhalis* and *H. influenzae*. The analyses were performed using frequency of specific bacterial carriage in the first year of life, and at ages of first bacterial carriage. Furthermore, we did the same analysis with the three bacteria grouped as any airway pathogen. Additionally, regression models were adjusted including all determinants presented in Table 1. Furthermore, possible interaction between otitis media in the first year of life and bacterial carriage and the risk of

otitis media in the second year of life was tested. According to the national Dutch vaccination policy all children were vaccinated against *H. influenzae B*, but non of the children were vaccinated against pneumocci

Differences of infant characteristics between infants with and without data on otitis media in the second year of life were assessed by the independent sample t-test for continuous normal distributed variables, non-parametric Mann-Whitney test for continuous non-normal distributed variables and the chi-square test for categorical variables. Differences of frequency of otitis media in the second year of life between groups with missing risk factors and complete data were assessed in the same way. All tests were carried out using a two-sided alpha level of 5%. The statistical analyses were performed using the Statistical Package of Social Sciences version 11.0 for Windows (SPSS Inc, Chicago, IL, USA).

Results

In the Generation R Cohort, 7295 children, with a delivery date from April 2002 until January 2006, participated in the postnatal phase of the study. Data on otitis media in the second year of life was available in 5323 (response rate 73%) children. Characteristics of the children of the whole cohort and their association with otitis media in the second year of life are presented in Table 1. Having siblings is associated with an increased risk of otitis media in the second year of life (aOR 1.42, CI 1.13-1.79). Furthermore, the occurrence of otitis media during the follow-up period between 0 to 6 months and the occurrence of otitis media during the follow-up period between 6 to 12 months of age was associated with the occurrence of otitis media in the second year of life (respectively, aOR 1.83, CI 1.24-2.71 and aOR 2.72, CI 2.18-3.38).

Bacterial carriage was studied in the focus cohort, in which 1079 children, with a delivery date from February 2003 until August 2005, participated. The children were planned to visit the Generation R Focus Study research centre at the age of 1.5 month (response rate 81.8%), 6 months (response rate 81.6%) and 14 months (response rate 80.0%). Of the infants 379 (43.3%) had at least one episode of otitis media in the second year of life.

In Table 2 the associations between the frequency of bacterial carriage in the first year of life and otitis media in the second year of life are presented. We analysed the association between bacterial carriage in the first year of life and otitis media in the second year of life in two different ways. First, we defined carriage by adding the three sampling time points and assessed whether the frequency of bacterial carriage was associated with otitis media.

Table 1. Population descriptive.

		Otits media 12-24 months of age			
	n=5323	no n=2808 (52,8%)	yes n=2515 (47,2%)	OR	aOR
Birthweight	3438 (574)	3422 (577)	3456 (570)	**1,11* (1,01-1,22)**	1,07 (0,84-1,37)
gestational age	40,0 (25,3-43,4)	40,0 (25,3-43,3)	40,1 (27,1-43,4)	1,02 (0,99-1,05)	1,04 (0,97-1,12)
Gender					
Male (ref)	2643 (49,7)	48.4%	51.0%		
Female	2680 (50,3)	51.6%	49.0%	0,90 (0,81-1,00)	0,89 (0,72-1,11)
smoking					
No (ref)	3110 (84,9)	85.1%	84.6%		
Yes	554 (15,1)	14.9%	15.4%	1,04 (0,87-1,25)	1,16 (0,86-1,55)
siblings					
No (ref)	2159 (58,9)	62.9%	54.1%		
Yes	1508 (41,1)	37.1%	45.9%	**1,44*** (1,26-1,65)**	**1,42* (1,13-1,79)**
educational level mother					
low	292 (5,8)	4.7%	7.0%		
middle	1911 (37,8)	37.3%	38.4%	**0,69** (0,54-0,88)**	0,84 (0,36-1,95)
high	2851 (56,4)	58.1%	54.5%	**0,62*** (0,49-0,80)**	1,03 (0,44-2,40)
day care					
No (ref)	730 (22,3)	21.3%	23.5%		
Yes	2543 (77,7)	78.7%	76.5%	0,88 (0,75-1,04)	0,86 (0,66-1,13)
breastfeeding					
never (ref)	450 (19,0)	17.7%	20.4%		
less than 3 months	1031 (43,5)	46.3%	40.3%	**0,76* (0,61-0,95)**	0,86 (0,63-1,16)

		Otits media 12-24 months of age			
	n=5323	no n=2808 (52,8%)	yes n=2515 (47,2%)	OR	aOR
more than 3 months	890 (37,5)	36.0%	39.3%	0,95 (0,76-1,19)	1,14 (0,83-1,55)
Pacifier use					
No (ref)	1242 (34,4)	34.5%	34.4%		
Yes	2364 (65,6)	65.5%	65.6%	1,01 (0,88-1,16)	0,95 (0,75-1,20)
Otits from 0 to 6 months					
No (ref)	3309 (91,9)	94.7%	88.5%		
Yes	292 (8,1)	5.3%	11.5%	**2,34*** (1,82-3,00)**	**1,83** (1,24-2,71)**
Otits from 6 to 12 months					
No (ref)	2808 (52,8)	67.4%	45.8%		
Yes	2515 (47,2)	32.6%	54.2%	**2,44*** (2,16-2,76)**	**2,72*** (2,18-3,38)**

Values of birth weight is mean (standard deviation), gestational age is median (range). Other values are absolute numbers (percentages). Data were missing on birth weight (n=8), gestational age (n=1), maternal smoking (n=1659), siblings (n=1656), educational level of mother (n=269), day care attendance (n=2050), breast feeding (n=2952), pacifier use (n=1717), otitis from 0 to 6 months (n=1722) and otitis from 6 to 12 months (n=815). No data was missing on gender. OR univariate model, aOR full model. Birthweight entered in analyses in Kg. *p<0,05, **p<0,01, ***p<0,001

Table 2. Association between bacterial carriage in the first year of life and otitis media in the second year of life.

		Otits media 12-24 months of age			
	n (%)	no (n=384)	yes (n=287)	OR	aOR
Streptococcus pneumoniae					
never (reference)	316 (47,1)	47.1%	47.0%	reference	reference
once	241 (35,9)	35.4%	36.6%	1,04 (0,74-1,45)	0,64 (0,35-1,17)
2 or more	114 (17,0)	17.4%	16.4%	0,94 (0,61-1,45)	0,72 (0,33-1,57)
Moraxella catarrhalis					
never (reference)	354 (52,8)	54.7%	50.2%	reference	reference
once	240 (35,8)	33.1%	39.4%	1,30 (0,93-1,81)	1,57 (0,88-2,80)
2 or more	77 (11,5)	12.2%	10.5%	0,93 (0,56-1,54)	0,91 (0,38-2,5)
Haemophilus influenzae					
never (reference)	381 (56,8)	58.3%	54.7%	reference	reference
1X	224 (33,4)	31.5%	35.9%	1,22 (0,87-1,69)	1,08 (0,60-1,96)
once	66 (9,8)	10.2%	9.4%	0,99 (0,58-1,68)	1,16 (0,44-3,08)
Airway pathogen					
never (reference)	136 (20,3)	20.6%	19.9%	reference	reference
once	163 (24,3)	25.3%	23.0%	0,94 (0,59-1,50)	0,58 (0,25-1,35)
2 or more	372 (55,4)	54.2%	57.1%	1,09 (0,73-1,63)	0,86 (0,40-1,84)

Or univariate model, aOR full model including all risk factors as shown in table 1.

Second, we assessed whether early age of bacterial carriage was associated with otitis media (data not shown). Both analyses were done for the bacteria separately and for any airway pathogen. No associations were observed. In addition to this, we assessed whether bacterial carriage in the first year of life was associated with otitis-proneness. Again, no associations were observed (data not shown).

Possible interaction between otitis media in the first year of life and bacterial carriage was tested. The interaction term was not significant. In Table 3 we present the associations between otitis media in the first year of life (6-12 months) and otitis media in the second year of life stratified for different bacterial carriage states in the first year of life. Bacterial carriage did not significantly change the increased risk for otitis media in the second year of life in case previous otitis media (6-12 months) was registered.

Table 3. Association between otitis media in the first year of life (6-12 months) and otitis media in the second year of life stratified by carriage state of different bacteria.

			Otits media (12-24 months of age)			
		n (%)	no (n=308)	yes (n=217)	OR	aOR
Never *S. pneumoniae*	otitis media 6-12 months of age					
	no	176 (71,5)	81.9%	56.9%	refrence	refrence
	yes	70 (28,5)	18.1%	43.1%	3,44 (1,93-6,14)	2,81 (1,14-6,92)
Once or more *S. pneumoniae*	otitis media 6-12 months of age					
	no	192 (68,8)	78.0%	55.7%	refrence	refrence
	yes	87 (31,2)	22.0%	44.3%	2,83 (1,68-4,77)	2,81 (1,27-6,24)
Never *M. catarrhalis*	otitis media 6-12 months of age					
	no	194 (70,3)	79.5%	56.4%	refrence	refrence
	yes	82 (29,7)	20.5%	43.6%	3,01 (1,76-5,12)	2,26 (1,00-5,12)
Once or more *M. catarrhalis*	otitis media 6-12 months of age					
	no	174 (69,9)	80.3%	56.1%	refrence	refrence
	yes	75 (30,1)	19.7%	43.9%	3,19 (1,82-5,60)	3,19 (1,31-7,74)
Never *H. influenzae*	otitis media 6-12 months of age					
	no	197 (68,2)	77.3%	54.7%	refrence	refrence
	yes	92 (31,8)	22.7%	45.3%	2,82 (1,70-4,70)	2,90 (1,34-6,28)
Once or more *H. influenzae*	otitis media 6-12 months of age					
	no	171 (72,5)	83.1%	58.0%	refrence	refrence
	yes	65 (27,5)	16.9%	42.0%	3,56 (1,96-6,48)	3,25 (1,30-8,12)
Never airway pathogen	otitis media 6-12 months of age					
	no	77 (72,6)	84.1%	55.8%	refrence	refrence
	yes	29 (27,4)	15.9%	44.2%	4,20 (1,70-10,37)	4,20 (0,43-41,32)
Once or more airway pathogen	otitis media 6-12 months of age					
	no	291 (69,5)	78.8%	56.3%	refrence	refrence
	yes	128 (30,5)	21.2%	43.7%	2,88 (1,88-4,42)	2,76 (1,50-5,09)

Or univariate model, aOR full model including all risk factors as shown in table 1.

Discussion

We observed a positive association between having siblings and otitis media. Also, we observed a tendency that low education of the mother might attribute to the risk for otitis media, while breast-feeding might be a protective factor. No associations were observed between birth weight, gestational age, gender, maternal smoking, day care attendance or pacifier use and otitis media in the second year of life. In a meta-analysis of risk factors for acute otitis media by Uhari et al., day care attendance was shown to be the most significant risk factor. [79] Furthermore, similar to the trend we observed, Uhari et al. showed a positive association between siblings and otitis media and a negative association between breast feeding and otitis media. This study, in contrast to our results, also showed that parental smoking and pacifier use was associated with otitis media.

We observed that otitis media in the first year of life is a risk factor for otitis media in the second year of life. Corbeel has stated that the first period of otitis media determines the risk for recurrence because inflammation causes dysfunction of the Eustachian tube in young children due to the small caliber and the horizontal direction and is, therefore, predisposing for a high risk for recurrent of otitis media. [80]

Next, we analyzed in the focus cohort the association between bacterial carriage and otitis media in the second year of life, and otitis-proneness. No associations were observed between frequency of bacterial carriage, age of first bacterial carriage or the different species of bacterial carriage and otitis media or otitis-proneness. In addition, we observed no role for bacterial carriage in recurrent otits media. Bacterial carriage in the first year of life is not associated with otitis media later on. In other studies the frequency of carriage with different bacteria was shown to be associated with otitis media. [8, 75, 77, 78] Faden et al. have shown that carriage of *S. pneumoniae, H. influenzae* and *M. catarrhalis* was associated with otitis-proneness in 17 otitis prone children vs 17 non-otitis prone children, during healthy periods and during periods of otitis media, in children aged 0-36 months in. The association in healthy periods was only significant for *H. influenzae* carriage but not for the other two bacteria studied. [77] In another study in 157 infants, an association between carriage of *H. influenzae*, measured at 13 routine visits in the first year of life, and otitis-proneness was shown. [78] In another study in 306 children aged 0 to 24 months, an association between *M. catarrhalis* and otitis-proneness was observed. [75] In this population they also showed an association between the frequency of carriage of the three airway pathogens and the frequency of otitis media episodes. [8] Prellner et al. did not show an increased risk for otitis media when *S. pneumoniae, H. influenzae* or *M. catarrhalis* were present. [81] Early age of first

colonization was also shown to be associated with the risk for otitis media. [8, 32, 78] But, Syrjänen et al. have shown in their study on the temporal association between pneumococcal carriage and otitis media that not preceding pneumococcal carriage state, but newly acquired carriage is associated with otitis media. [33] Their data support the hypothesis that viral respiratory infection might enhance the acquisition of pneumococci. Recently, Revai et al. have shown an association between presence of pathogenic bacteria in the nasopharynx during upper respiratory tract infections and the risk for otitis media following the upper respiratory tract infection. [82] Taken together, it appears that the presence of newly acquired pathogenic bacteria in combination with (viral) upper respiratory tract infections increases the risk for otitis media, rather than the presence of pathogenic bacteria itself increases the risk for otitis media.

To appreciate the results some limitations of our study had to be considered. In contrast to other studies, our definition of otitis media was based on parental reported questionnaire data, we do not have a doctor verified diagnosis of otitis media. We observed that 49 (7.2%) of the children had at least one period of otitis media in their first six months of life, 247 (31.3%) had otitis media in their second six months of life and 379 (43.3%) of the children had at least one period of otitis media in their second year of life. 511 (71.8%) of the children had at least one period of otitis media by the age of 24 months. This might be an overestimation of the number of children experiencing otitis media, resulting in less contrast between children with and without otitis media. This might explain that we did not find significant risk factors for otitis media. On the other hand, children with fever and earache were only classified as otitis "yes" if they visited a doctor. To our opinion this makes our definition of otitis media reliable.

Conclusion: In our study, otitis media in the first year of life is an independent risk factor for otitis media in the second year of life. Surprisingly, bacterial carriage in the first year of life did not add to this risk. Moreover, no association was observed between bacterial carriage in the first year of life and otitis in the second year of life.

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Chapter 7

Atopic Dermatitis in Infants: an Important Role for *Staphylococcus aureus* Nasal Colonization. The Generation R Study.

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Abstract

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

Design Population-based prospective cohort study of pregnant women and their children.

Setting This project was embedded in the Generation R Study in Rotterdam, the Netherlands

Participants Postnatal, 1,079 Dutch children participated in the Focus Cohort.

Main exposures: Nasal swabs for *Staphylococcus aureus* cultivation were taken at the age of 1.5, 6 and 14 months.

Outcome Questionnaires on atopic dermatitis and confounders (parental eczema, birth weight, gestational age, gender and previous period of atopic dermatitis) were obtained at 6, 12 and 24 months. The outcome was atopic dermatitis in the second year of life.

Results First positive culture of *Staphylococcus aureus* at 6 months was associated with atopic dermatitis in the second year of life (aOR 2.77 95%CI 1.27–6.02), after adjustment for confounders. Moreover, infants colonized at 6 months have an increased risk to suffer from moderate to severe (aOR 2.84 95%CI 1.18–6.82) atopic dermatitis in the second year of life.

Conclusion *Staphylococcus aureus* colonization at 6 months 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) [83-85]. AD is an inflammatory skin disease which usually presents in the first years of life [86, 87]. As reported in many studies, *S. aureus* is the most important pathogen associated with AD. Skin colonization with *S. aureus* is known to be related with AD disease severity [88].

A significant fraction of the healthy population is colonized with *S. aureus* on epithelial surfaces, of which the anterior nares are the most frequent carriage sites [83, 89, 90]. Longitudinal studies distinguish three types of carriage patterns among healthy adult individuals, non carriers, intermittent carriers and persistent carriers [91-94]. Persistent carriers have a well documented higher risk of acquiring *S. aureus* infection, but they barely exist in infancy [95-98]. The anterior nares may serve as an important endogenous reservoir for involvement in AD, reaching an colonization incidence of 39-82% in adult patients with AD [99, 100]. *S. aureus* might play a role in the chronicity and severity of AD through its release of superantigenic exotoxins [39]. Specifically, colonization 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 [101, 102]. S. aureus enterotoxins [103] increase the inflammation in atopic dermatitis and provoke the generation of enterotoxins specific IgE which correlates with the severity of the disease [37-39].

Longitudinal data on nasal colonization with *S. aureus* in infancy was recently described [98]. Additionally we aim to assess the risk of developing atopic dermatitis following nasal colonization 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 [41, 42]. The Medical Ethics Committee of the Erasmus Medical Center, Rotterdam, has approved the study. Written informed consent was obtained from all participants [41].

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 6 weeks, 832 at 6 months and 757 at 14 months. 758 infants attended all visits of whom 443 provided us with three swabs to use for longitudinal analysis. The amount of infants with a swab at 1.5 months is significantly 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.

***S. AUREUS* ISOLATION** Research nurses obtained a nasal swab for *S. aureus* isolation at each visit. Nasal samples were taken using a swab that was rubbed through then nostrils. The methods of sampling were described in more detail previously [98].

S. aureus colonization 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* colonization in the first year of life on the development of atopic dermatitis, three groups were created; infants whom were never positive, positive once and positive twice or more.

EXPOSURES AND COVARIATES 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 maternal smoking (pre and postpartum) and parental eczema complaints was obtained by questionnaires at the age of 2, 6 and 12 months and analysed in a dichotomous way.

ATOPIC DERMATITIS Information regarding AD was obtained using an age-adapted version of the questionnaire of the "International Study of Asthma and Allergies in Childhood" (ISAAC) at the age of 12 and 24 months [104]. 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 second year of life. The severity score was based on questions regarding the level of suffering. Questions related to continuous or intermittent rash, rash clearance and whether infants were kept awake because of the itchy rash. This resulted in three groups: no AD, mild AD (episode of rash without additional complaints) and moderate/severe AD (episode of rash with additional complaints as mentioned above).

DATA ANALYSES We used chi-square tests to confirm equal variances among 443 infants with all three swabs available versus the total cohort of 1,079 infants who were available

for postnatal analysis. To study the association between colonization and AD we performed univariate and multivariate binary logistic regression, adjusted for important confounders such as gender, gestational age, birth weight, parental eczema complaints and previous episode of eczema complaints. To study the association between colonization and severity of AD we conducted multinomial logistic regression analyses, both univariate and multivariate. We conducted complete cases analyses and thus removed the infants with missing data in the outcome (11.2% missings in the outcome). 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 complaints in the parents occurred in 3.4% of the mothers (n=32) and 4.4% of the fathers (n=39). The absolute number of smoking mothers prepartum (n=91) and postpartum (n=90) remained equal.

In the period of 6-12 months of age, 259 of 1079 infants (26.8%) suffered from AD complaints. A total of 273 of 1079 infants (28.5%) infants suffered from AD in the second year of life, of whom 55 (20.1%) suffered from moderate to severe AD and 218 from a mild phenotype (79.9%). (Table 1)

S. aureus colonization at 6 months was clearly associated with AD in the second year of life (aOR 1.76 95%CI 1.09 – 2.84). (Table2) 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 complaints in the second year of life (aOR 2.77 95%CI 1.27–6.02). Infants with a higher frequency of colonization in the first year of life (twice or more) have an increased risk to suffer from AD in the second year of life (OR 2.00 95%CI 1.10–3.63), however after adjustment, specifically for previous episode of AD, the significant effect disappears (aOR 1.74 95%CI 0.78 – 3.86).

S. aureus colonization was also correlated with severity of AD. (Table3) Infants colonized at 6 months had an increased risk to suffer from moderate/severe atopic dermatitis in the second year of life (aOR 2.84 95%CI 1.18–6.82). Frequent *S. aureus* colonization in the first year of life (twice or more) was univariately found associated with moderate/severe atopic

Table 1. Population descriptive

		Atopic Dermatitis			
	N= 1,079	No (n=685)	Yes (n=273)	OR	aOR
Birth weight	3509 (538)	3523 (532)	3507 (554)	1.00 (1.00-1.00)	1.00 (1.00-1.00)
Gestational age	40.3 (37.1-42.1)	40.3 (37.1-42.1)	40.3 (36.7-42.1)	0.96 (0.89-1.04)	0.96 (0.80-1.15)
Gender					
- Female (ref)	521 (48.3%)	332 (48.5%)	133 (48.7%)	1.00	1.00
- Male	558 (51.7%)	353 (51.5%)	140 (51.3%)	0.99 (0.75-1.31)	1.05 (0.65-1.67)
Maternal smoking prenatal					
- No (ref)	921 (91.0%)	594 (92.4%)	251 (93.7%)	1.00	1.00
- Yes	91 (9.0%)	49 (7.6%)	17 (6.3%)	0.82 (0.46-1.45)	0.39 (0.1- 1.54)
Maternal smoking postnatal					
- No (ref)	606 (87.1%)	418 (86.7%)	158 (89.3%)	1.00	1.00
- Yes	90 (12.9%)	64 (13.3%)	19 (10.7%)	0.79 (0.46-1.35)	0.97 (0.41-2.30)
Eczema complaints in mother					
- No (ref)	897 (96.6%)	573 (97.1%)	221 (95.7%)	1.00	1.00
- Yes	32 (3.4%)	17 (2.9%)	10 (4.3%)	1.53 (0.69-3.38)	1.05 (0.36-3.07)
Eczema complaints in father					
- No (ref)	848 (95.6%)	552 (96.7%)	211 (91.7%)	1.00	1.00
- Yes	39 (4.4%)	19 (3.3%)	19 (8.3%)	2.62 (1.36-5.04)*	1.34 (0.50-3.59)
Eczema complaints 6-12 months					
- No (ref)					
- Yes	706 (73.2%)	545 (83.6%)	116 (45.1%)	1.00	1.00
	259 (26.8%)	107 (16.4%)	141 (54.9%)	6.19 (4.49-8.54)*	6.03 (3.73-9.75)*

Data were missing on maternal smoking prenatal (n=67), maternal smoking postnatal (n=383), mother eczema complaints (n=150), father eczema complaints (n=192), atopic dermatitis between 6-12 months (n=114), atopic dermatitis at 2 years of age (n=121).

Results are presented as crude odds ratios (OR) and adjusted odds ratios (aOR) corrected for all other variables.

* p-value<0.05

Table 2. The association between *S. aureus* colonization and atopic dermatitis in infancy

	Atopic dermatitis in the second year of life			
	No (n=685)	Yes (n=273)	OR	aOR
S. aureus 1.5 month				
- no (ref)	204 (48.0%)	61 (42.7%)	1.00	1.00
- yes	221 (52.0%)	82 (57.3%)	1.24 (0.85 – 1.82)	1.19 (0.71 – 1.98)
S. aureus 6 months				
- no (ref)	448 (81.9%)	151 (70.9%)	1.00	1.00
- yes	99 (18.1%)	62 (29.1%)	1.89 (1.29 – 2.68)*	1.76 (1.09 – 2.84)*
S. aureus 14 months				
- no (ref)	438 (85.7%)	161 (83.0%)	1.00	1.00
- yes	73 (14.3%)	33 (17.0%)	1.23 (0.79 – 1.93)	1.42 (0.80 – 2.51)
First positive culture				
- never (ref)	118 (27.3%)	30 (18.4%)	1.00	1.00
- 1.5 month	221 (51.0%)	82 (50.3%)	1.46 (0.91 – 2.35)	1.31 (0.69 – 2.50)
- 6 months	54 (12.5%)	38 (25.3%)	2.77 (1.56 – 4.93)*	2.77 (1.27 – 6.02)*
- 14 months	40 (9.2%)	13 (8%)	1.28 (0.61 – 2.69)	1.55 (0.61 – 3.93)
S. aureus ¶				
- never	163 (42.9%)	41 (32.5%)	1.00	1.00
- once	172 (45.3%)	61 (48.4%)	1.18 (0.69 – 1.99)	0.97 (0.48 – 1.98)
- twice or more	45 (11.8%)	24 (19.0%)	2.00 (1.10 – 3.63)*	1.74 (0.78 – 3.86)

Of the total group (n=1,079), 452 infants did not get a swab at 1.5 month, 247 infants missed the swab at 6 months and 322 infants missed the swab at 14 months.

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

¶ Only 443 infants with 3 swabs available were analysed.
* p-value<0.05

dermatitis complaints in the second year of life (OR 4.67 95%CI 1.17-18.70). After adjustment for confounders this significant effect disappears (aOR 2.86 95%CI 0.47–17.44).

Differences in characteristics for infants, for whom three swabs were and were not available, were assessed by chi-square tests. The 443 infants with three bacterial cultures available did not differ from the total cohort of 1,079 infants with respect to the main determinants. Of the children with all three swabs available, less mothers smoked during pregnancy (5.7% versus 11.4%, p=0.002). Moreover, there is a slight significant difference in the outcome of AD in the second year of life, 31.8% (n=170) of the infants without three swabs available suffered from atopic dermatitis in the second year of life, as compared to 24.3% (n=103) in the group of infants with all three swabs available (p=0.01).

Table 3. The association between *S. aureus* colonization and atopic dermatitis severity in infancy

	Atopic dermatitis in the second 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	1.09 (0.73–1.65)	1.13 (0.67 – 1.92)	2.37 (0.97–5.80)	1.71 (0.48 – 6.16)
S. aureus 6 months				
- no (ref)	1.00	1.00	1.00	1.00
- yes	1.57 (1.05–2.35)*	1.60 (0.97 – 2.65)	3.39 (1.77–6.49)*	2.84 (1.18 – 6.82)*
S. aureus 14 months				
- no (ref)	1.00	1.00	1.00	1.00
- yes	1.43 (0.89–2.28)	1.66 (0.94 – 2.96)	0.51 (0.15–1.72)	0.23 (0.03 – 1.87)
S. aureus ¶				
- never (ref)	1.00	1.00	1.00	1.00
- once	1.06 (0.61–1.86)	1.00 (0.48 – 2.10)	2.19 (0.57–8.42)	0.82 (0.13 – 5.26)
- twice or more	1.70 (0.90–3.22)	1.60 (0.69 – 3.70)	4.67(1.17–18.70)*	2.86 (0.47 – 17.44)

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

¶ Only 443 infants with 3 swabs available were analysed.
* p-value<0.05

DISCUSSION

Bacterial colonization is considered an important factor in the pathophysiology involved in AD [105]. We found a clear association, after adjustment for important confounders, between the prevalence of *S. aureus* nasal colonization at 6 months and the occurrence of AD in the second year of life in a healthy infant cohort. 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 [37]. Other studies reported on increased levels of antistaphylococcal IgE and staphylococcal toxins A-E in the serum of AD patients [102, 106]. No other studies ever reported on nasal colonization of *S. aureus* as risk factor preceding AD in infants.

Colonization 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 colonization and the development of AD in childhood.

We found severity of AD to be associated with *S. aureus* nasal colonization. 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. This is additional to data on severity presented by others [37]. Several studies describe an association between colonization and a higher eczema severity score [37, 107, 108].

Our study provides data on nasal colonization of *S. aureus* preceding AD adjusted for several confounders, supporting a direct link between colonization and AD in one of the largest birth-cohorts, with the smallest number of 443 in infants with all three swabs available, which is still large as compared to other studies. A larger sample was studied for the individual swab results. The missing data in the outcome, more infants suffering from atopic dermatitis in the group of infants with less than 3 swabs available, may lead to an underestimation of the effect we find.

We adjusted for previous episode of atopic dermatitis, allowing us to draw conclusions of AD following bacterial colonization with *S. aureus*, rather than the other way around, bacterial colonization following AD.

AD complaints are not confirmed by a clinician in our study. However the questionnaire used was validated and age-adapted. 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 colonization status.

We did not study methicillin-resistant *Staphylococcus aureus* (MRSA) colonization 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 [109]. Studying MRSA in a Dutch population cohort is thus not very important.

Nasal colonization and colonization of the affected skin in patients with AD are strongly associated, which may explain a pathophysiological role for *S. aureus* nasal colonization and AD [110]. One can speculate a systemic release of enterotoxins specific IgE against superantigens of *S. aureus* to lead to atopic dermatitis.

Our results are in line with, and in addition to, literature suggesting at least an effect-modifying role or a pathophysiological role for *S. aureus* [37, 105]. Further studies are required to clarify the pathophysiological role of *S. aureus* colonization in relation to AD.

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Part 3

The risk factors associated with upper respiratory tract infections in young children, in particular the socioeconomic status of the parents.

Chapter 8

Social disadvantage and upper respiratory tract infections in early childhood; contribution of prenatal factors.

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(submitted)

Abstract

OBJECTIVE: To test the hypothesis that 1) toddlers of low socioeconomic status are more susceptible to upper respiratory tract infections (URTI) than those of high socioeconomic status, and that 2) part of this increased susceptibility is explained by prenatal or perinatal circumstances, including indicators of prenatal growth, perinatal health or prenatal exposure to psychosocial stressors.

PARTICIPANTS AND METHODS: Using data from 5554 children and their mothers participating in a population-based cohort study in Rotterdam, The Netherlands, we analyzed the associations of two socioeconomic indicators, i.e. maternal educational level (high, mid-high, mid-low, and low) and household income level (>2200, 1200-2200, and <1200 euros per month), with URTI in early childhood. Through questionnaires, we obtained information on the occurrence of URTI between 0 and 6 months of age, between 7 and 12 months, and between 13 and 24 months.

RESULTS: At all ages, there was a graded inverse relationship between both maternal educational level and household income level with the risk for upper respiratory tract infections. Adjusted for confounders and factors related to exposure to infectious agents, toddlers of mothers with a low educational level had a 62% (OR: 1.62; 95% CI:1.26,2.08) higher susceptibility to URTI between 13 and 24 months than toddlers of mothers with a high educational level. Independent of postnatal factors, prenatal financial difficulties, prenatal psychiatric symptoms and poor prenatal family functioning explained 21% of this increased susceptibility. Indicators of prenatal growth and perinatal health did not contribute to the explanation. We found comparable results for the association between household income and URTI.

CONCLUSIONS: Toddlers of low socioeconomic status are more susceptible to URTI than toddlers of high socioeconomic status. Independently of postnatal circumstances, part of this increased susceptibility is due to adverse intrauterine circumstances, in particular prenatal exposure to maternal psychosocial stressors.

Introduction

Socioeconomic circumstances affect children's health: children from families with a low socioeconomic status generally have poorer health than those from families with a high socioeconomic status. This socioeconomic gradient has been demonstrated for different dimensions of child health, including mortality[111, 112], general health status[113, 114], injuries and accidents[115], mental health[116], and specific childhood diseases such as infectious diseases[73, 117, 118]. Recent evidence suggests that socioeconomic differences in health become larger as children get older, and that they may contribute to the origins of health differences in adult life [113, 119]. This underlines the importance of research on the nature of socioeconomic differences in health in early life.

Despite previous efforts to explain the mechanism underlying the socioeconomic gradient in child health[113, 114, 120], these mechanisms remain poorly understood. On the basis of the 'fetal origins' hypothesis, which highlights the importance of experiences in the womb for health later in life, researchers' attention has shifted to the possible role of the intrauterine environment in explaining the socioeconomic gradient in child health. Recently, Dowd investigated whether maternal health status and health behaviors during pregnancy and during early infancy could explain the relationship between family income and overall health status of 3-year old children; this was not the case. However, the influence of measures of the child's prenatal and perinatal health, such as birth weight or apgar score, was not explored in this study. Furthermore, information on prenatal psychosocial factors, which have been implicated in explaining socioeconomic inequalities in adult health[121, 122], was not available.

The present study was conducted to examine the socioeconomic inequalities in health among infants and toddlers up to 2 years of age, and the extent to which prenatal or perinatal circumstances contribute to these inequalities. The outcome of interest was occurrence of upper respiratory tract infections, the most frequent diseases in early childhood that can affect the quality of life of both the children and their families[123]. Using two indicators of family socioeconomic status, i.e. maternal educational level and average household income, we estimated socioeconomic inequalities in 'susceptibility' to upper respiratory tract infections by controlling for any differences in exposure to infectious agents[124]. We hypothesized that toddlers of low socioeconomic status are more susceptible to upper respiratory tract infections than those of high socioeconomic status, and that, independently of postnatal circumstances, part of this increased susceptibility is explained by prenatal or perinatal circumstances, such as by indicators of prenatal growth or exposure to prenatal psychosocial stress.

Methods

The Generation R Study

This study was embedded within the Generation R Study, a population-based prospective cohort study from fetal life until young adulthood that has previously been described in detail[41, 42]. Ideally, enrolment took place in early pregnancy, but was possible until the birth of the child. All children were born between April 2002 and January 2006 and form a prenatally enrolled birth-cohort that is currently being followed-up until young adulthood. Of all eligible children in the study area, 61% participated in the study[42]. The study was conducted in accordance with the guidelines proposed in the World Medical Association Declaration of Helsinki[125] and has been approved by the Medical Ethical Committee of the Erasmus MC, University Medical Center Rotterdam. Written consent was obtained from all participating parents.

Population for analyses

Of the 7893 mothers and their children in the postnatal cohort, 6969 had been included prenatally. Of these 6969 participants, 6559 gave consent for receiving questionnaires postnatally. We excluded twins (n=137) from the analyses, since data were correlated. For the same reason, of the mothers who participated in the study with more than one child, we excluded data from the second (n=459) or third child (n=9). We also excluded participants who lacked information on maternal educational level (n=400), leaving a study population of 5554 mothers and their children.

Maternal educational level and household income level

On the basis of a questionnaire during pregnancy, we established the highest education each mother had achieved, and the net household income, i.e. the net income of the mother ánd her partner if present. Maternal educational level was categorized into: 1.) high (university or higher), 2.) mid-high (higher vocational training), 3.) mid-low (more than three years of general secondary school, or intermediate vocational training completed, or first year of higher vocational training), and 4.) low education (no education, primary school, lower vocational training, intermediate general school, or three years or less of general secondary school) [126]. Household income was categorized into 1.) >2200 euros per month, 2.) 1200-2200 euros per month, and 3.) <1200 euros per month. The cut-off for the lowest income category was based on the national poverty level as established in 2005 for a family consisting of one parent and one child[127].

Upper respiratory tract infections

When the children were 6, 12 and 24 months old, we obtained information on the occurrence of upper respiratory tract infections through postal questionnaires. Parents were asked whether their child had suffered from a serious cold, an ear infection or a throat infection in the preceding period (i.e. from 0-6 months, from 7-12 months, and from 13-24 months), and whether they had visited a physician for this infection. When parents reported at least one of these infections, independent of whether they had visited a physician, their children were considered to have had an upper respiratory tract infection.

Covariates

Ethnicity of the mother, age of the mother, and age of the child at which the questionnaire was completed, were considered potential confounders in the associations between educational/income level and upper respiratory tract infections in early childhood; these variables may be related to both socioeconomic status and to parent-reported upper respiratory tract infections[128, 129], but are not in the causal pathway[130].

The variables listed below, which are known to be associated with respiratory tract infections in childhood[73, 131-133] were hypothesized to be in the pathway from family socioeconomic status to susceptibility for upper respiratory infections in early childhood. These so-called explanatory variables were divided into prenatal/perinatal factors and postnatal factors. Unless stated otherwise, information on these variables was obtained using questionnaires. Categories are indicated between parentheses.

Prenatal/perinatal factors

We collected information on possible sources of maternal psychosocial stress during pregnancy. These included: *single motherhood* (yes, no); financial difficulties (yes, no); presence of *psychiatric symptoms* (including depression and anxiety) as measured using the Global Severity Index (score in tertiles, the higher the worse) of the Brief Symptom Inventory[134]; presence of *long-lasting difficulties* (score in tertiles, the higher the worse) as measured using a 12 item-checklist covering financial problems, social deprivation, neighborhood problems and problems in relationships[135]; and (*poor*) *family functioning* as measured with the family assessment device (score in tertile, the higher the worse)[136].

In early, mid and late pregnancy, we obtained information on whether the mother *smoked during pregnancy* (no, yes).

Birth weight, *date of birth* and *apgar score after 1 minute* (<7, ≥7) were obtained from midwife and hospital charts. *Gestational age* was established by fetal ultrasound examination[137]. Using a questionnaire 2 months after birth, we established whether the infant had been *hospitalized in the first week after birth* (yes, no).

Postnatal factors

When the child was 2 months old, we used the Global Severity Index (score in tertiles, the higher the worse) of the Brief Symptom Inventory[134] to establish presence of *maternal psychiatric symptoms postnatally.* Presence of *postnatal financial difficulties* was established at child age of 24 months.

We established whether the child was receiving *breastfeeding at the age of 6 months* (yes, no) and whether the child was *exposed to tobacco smoke at the age of 24 months* (yes, no).

The presence of older *siblings* was established at the age of 6 months of the infant. Information on *day care attendance* was collected at the age of 24 months.

Multiple imputation and statistical analyses

Because missing data on the outcome variables were not completely random (see below), complete-case-analysis was likely to introduce biased results[138]. Imputation of outcome variables using the predictors under study minimizes this bias[139]. Therefore, we imputed missing values in the outcome variables and the covariates using 'multiple imputation'; this simulation-based approach creates a number of imputed (completed) data sets by "filling in" plausible values for the missing data[140]. Using the PROC MI procedure in SAS 9.1.3, five imputed data sets were created, in which imputations were based on the relationships between all the variables included in this study.

After multiple imputation, we evaluated the occurrence of upper respiratory tract infections from 0-6 months, from 6-12 months, and from 12-24 months in the whole study population, and that stratified by educational level and by household income level.

Next, logistic regression analysis was used to quantify the association between educational/income level and the risk for upper respiratory tract infections from 13-24 months, adjusted for the potential confounders (model 1). The highest educational/income level was set as reference. Then, the factors related to exposure to infectious agents, i.e. siblings and day care attendance, were included in the model (model 2), which we considered to reflect the differences in susceptibility for upper respiratory infections.

The extent to which prenatal/perinatal circumstances, independently of postnatal circumstances, contributed to the explanation of the effect of socioeconomic status on susceptibility for upper respiratory tract infections, was analyzed in two stages. First, each potential mediator was added separately to model 2. For each adjustment, the percentage change in OR for the educational level/income level with an increased risk for upper respiratory tract infections was calculated ($100x[OR_{model\ 2} - OR_{+mediator}]/[OR_{model\ 2} - 1]$). Only those variables that individually produced at least 10% change in the OR for the educational/income level with the highest risk were selected for the next stage.

In the second stage, we used a method previously described by Stronks et al.[141] to assess the independent contribution of prenatal/perinatal factors. The following three models were fitted:

- Model 2 + selection of prenatal/perinatal factors (= model 3)
- Model 2 + selection of postnatal factors (= model 4)
- Model 2 + selection of prenatal/perinatal and postnatal factors (= model 5)

Then, the contribution of prenatal/perinatal factors, independently of postnatal factors was established by calculating the percentage reduction due to the inclusion of prenatal/perinatal factors to a model already containing postnatal factors (model 5 compared to model 4). The independent contribution of postnatal factors was established by calculating the percentage reduction due to the inclusion of postnatal factors to a model already containing prenatal/perinatal factors (model 5 compared to model 3).

Furthermore, we tested interaction terms between the socioeconomic indicators and confounders and explanatory factors. There was only an indication that the effect of a low education was stronger among the Turkish mothers (p=0.053). However, we found this insufficient support to present the analyses stratified by each ethnic group. Results in this paper are therefore based on models including main effects only.

Statistical analyses were performed using Statistical Package of Social Sciences version 15.0 for Windows (SPSS Inc, Chicago, IL, USA) and the Statistical Analysis System (SAS) for Windows (SAS Institute Inc, USA), version 9.1.3.

Results

Of the 5554 children, 25.8% of their mothers had a high educational level, and 23.1% of their mothers had a low educational level (table 1). About 59% had a net household income of more than 2200 euros per months, 16.8% had a household income of less than 1200 euros

per month. Appendix 1 and 2 show the associations of educational level and income level with the covariates included in this study.

Table 1. Characteristics of the study population (n=5554)*.

Maternal characteristics	
Age at enrolment (years)	30.3 (5.0)
Single motherhood (%)	12.9
Educational level	
HIgh (%)	25.8
Mid-high (%)	21.3
Mid-low (%)	29.9
Low (%)	23.1
Household income (euros/month)	
>2200 (%)	59.4
1200-2200 (%)	23.8
<1200 (%)	16.8
Ethnicity	
Dutch (%)	53.7
Capeverdian (%)	4.0
Moroccan (%)	5.5
Dutch Antillean (%)	2.6
Surinamese (%)	8.1
Turkish (%)	8.2
Other European (%)	8.1
Other (%)	9.8
Child characteristics	
Birth weight (grams)	3425.8 (548.6)
Gestational age at birth (weeks)	40.1 (36.0,42.4)
Breastfeeding at 6 months (%)	29.8
Child care attendance at 24 months (%)	70.5
Exposure to tobacco smoke at 24 months (% yes)	18.1
Siblings (% yes)	33.1

* Values are percentages in case of categorical variables, or means (with standard deviation) or medians (with 95% range) in case of continuous variables.
Data were missing on parity (n=6), single motherhood (n=59), ethnicity (n=10), household income (n=827), birth weight (n=3), gestational age at birth (n=1), breastfeeding at 6 months (737), child care attendance at 24 months (n=1690), exposure to tobacco smoke at 24 months (n=1362), siblings (n=2149).

Parent-reports on upper respiratory tract infections at the ages 0-6 months, 7-12 months and 13-24 months were available in respectively 61%, 74% and 75% of the study population (table 2). Compared with responders, among the group of non-responders, mothers were younger, were more often in the lower educational and income levels, were more often of non-Dutch origin, and were more often a single mother; the infants among the group of non-responders had a lower birth weight (data not shown). After multiple imputation the incidences of upper respiratory tract infections were somewhat higher than those based on the original data: 43.0% from 0-6 months, 64.2% from 7-12 months and 73.3% from 13-24 months (table 2).

At all ages, there was a graded inverse relationship between both maternal educational level and household income level with the risk for upper respiratory tract infections (figures 1 and 2); the lower the educational or income level, the higher the risk.

Table 2. Parent -reported upper respiratory tract infections at the ages 0-6 months, 7-12 months and 13-24 months before and after multiple imputation.*

	Before imputation	After imputation
Upper respiratory tract infections (%)		
0-6 months	39.1 (1325/3393)	43.0 (2386/5554)
7-12 months	60.9 (2519/4136)	64.2 (3564/5554)
13-24 months	70.2 (2926/4169)	73.3 (4070/5554)

* Values are percentages, with between parentheses the numerator and denominator.

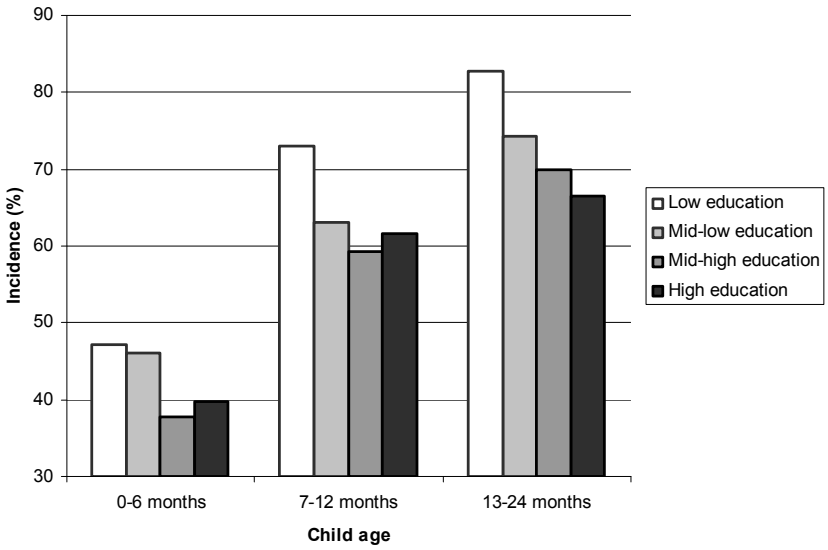

Figure 1. Incidence of parent-reported upper respiratory tract infections from 0-6 months, from 7-12 months and from 13-24 months, stratified by maternal educational level

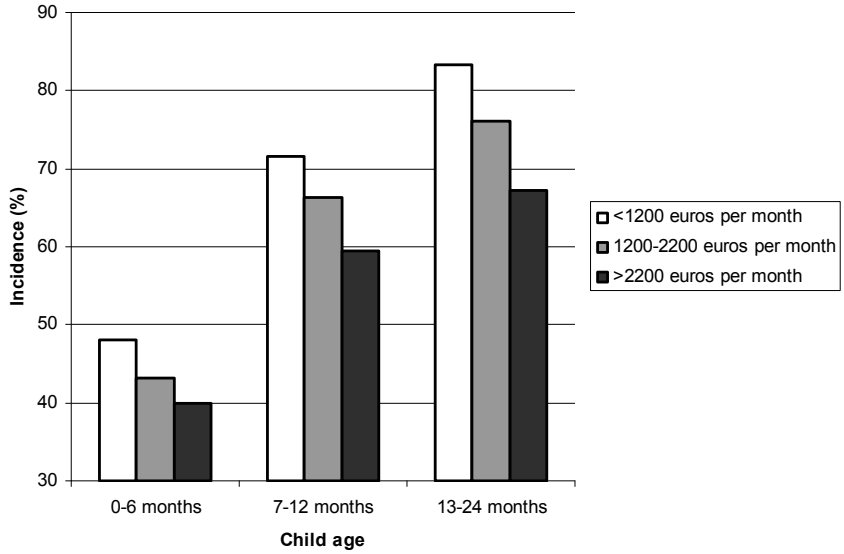

Figure 2. Incidence of parent-reported upper respiratory tract infections from 0-6 months, from 7-12 months and from 13-24 months, stratified by household income level.

Maternal educational level and upper respiratory tract infections

Table 3 presents the associations of maternal educational level and household income level with upper respiratory tract infections from 13-24 months, expressed in odds ratios. After adjustment for the potential confounders, children of mothers with a low educational level had a higher risk of having an upper respiratory tract infection compared with those of mothers with a high educational level (OR: 1.50 (95% CI: 1.19,1.88). After additional adjustment for presence of siblings and day care attendance the OR was 1.62 (95% CI: 1.26,2.08).

Individual adjustment for prenatal financial difficulties, prenatal psychiatric symptoms, prenatal family functioning, postnatal psychiatric symptoms, and breastfeeding at 6 months attenuated the OR of 1.62 for low education by at least 10%; these factors were included in the next phase of the analyses (table 4).

Table 5 shows that adjustment for the selected prenatal factors reduced the OR for low education to 1.44. This implies that these factors explained 29% (model 3 compared to model 2: 1.62-1.44/0.62) of the increased susceptibility for upper respiratory tract infections. The *independent* contribution of these factors was somewhat lower: 21% (1.62-1.40/0.62).

The total contribution of postnatal factors was 35% (model 4 compared to model 2: 1.62-1.44/0.62), while the independent contribution of postnatal factors was 27% (1.44-1.27/0.62). Together, prenatal/perinatal and postnatal factors explained 56% (1.62-1.27/0.62) of the effect of low education. The OR in the final model (model 5) was no longer statistically significant.

Table 3. Logistic regression analyses: associations of maternal educational level and household income level with upper respiratory tract infections between 13-24 months of age.*

Socioeconomic indicator		**Crude OR (model 0)**		**Adjusted for confounders§ (model 1)**		**Adjusted for confounders and exposure variables¶ (model 2)**
Maternal educational level						
High		**Ref**		Ref		**Ref**
Mid-high		1.09 (0.99,1.39)		**1.08 (0.91,1.27)**		1.11 (0.93,1.31)
Mid-low		**1.45 (1.19,1.76)**		**1.07 (0.87,1.31)**		1.13 (0.92,1.38)
Low	*1*	*2.41 (1.97,2.96)*	*2*	*1.50 (1.19,1.88)*	*3*	*1.62 (1.26,2.08)*
Household income		***4***		**5**		***6***
7 >2200 (euros/week)	*8*	*Ref*		Ref		**Ref**
9 1200-2200 (euros/week)	*10*	*1.53 (1.89,3.14)*		**1.19 (0.98,1.44)**		**1.26 (1.03,1.53)**
11 < 1200 (euros/week)	*12*	*2.44 (1.28,1.83)*		**1.48 (1.14,1.44)**		**1.56 (1.19,2.03)**

* Values are odds ratios with associated 95% confidence intervals. Analyses with maternal educational level are based on 5554 subjects; those with household income are based on 4727 subjects.

§ Potential confounders are ethnicity mother, age mother, age child at which 24-months questionnaire was filled in.

¶ Exposure variables are childcare attendance at 24 months and siblings.

Table 4. Change in odds ratios related to the associations of maternal educational level and household income level with upper respiratory infections between 13-24 months of age after individual adjustment for potential mediators.

Models	**OR (95%CI) 'Low education' versus 'high education'**	Change* 1	**OR (95%CI) Household income '<1200' versus '>2200'**	Change* 2
Model 2 (includes ethnicity mother, age mother, age child at which 24-months questionnaire was filled in, childcare attendance at 24 months and siblings)	**1.62 (1.26,2.08) (10% change range: 1.56,1.68)**		**1.56 (1.19,2.03) (10% change range: 1.50,1.62)**	
13 Prenatal/perinatal factors				
14 Model 2 + single motherhood	1.58 (1.21, 2.06)	-6%	1.53 (1.08,2.18)	-5%
15 Model 2 + prenatal financial difficulties	1.50 (1.16,1.94)	-19%	1.31 (1.00,1.73)	-45%

Models	**OR (95%CI) ' Low education' versus ' high education'**	Change* 1	**OR (95%CI) Household income ' <1200' versus '>2200'**	Change* 2
Model 2 (includes ethnicity mother, age mother, age child at which 24-months questionnaire was filled in, childcare attendance at 24 months and siblings)	**1.62 (1.26,2.08) (10% change range: 1.56,1.68)**		**1.56 (1.19,2.03) (10% change range: 1.50,1.62)**	
16 Model 2 + prenatal psychiatric symptoms	1.55 (1.21,2.00)	-11%	1.45 (1.10,1.92)	-20%
Model 2 + prenatal family functioning	1.54 (1.20,1.98)	-13%	1.46 (1.11,1.93)	-18%
Model 2 + prenatal long lasting difficulties	1.59 (1.25,2.03)	-5%	1.47 (1.12,1.92)	-16%
Model 2 + Maternal smoking during pregnancy	1.61 (1.23,2.10)	-2%	1.54 (1.17,2.03)	-4%
17 Model 2+ birth weight	1.62 (1.26,2.08)	-0%	1.55 (1.19,2.02)	-2%
18 Model 2+ gestational age at birth	1.60 (1.25,2.05)	-3%	1.55 (1.18,2.02)	-2%
19 Model 2 + apgar score 1 minute	1.62 (1.26,2.08)	-0%	1.56 (1.19,2.03)	-0%
20 Model 2 + hospitalisation in 1st week	1.60 (1.24,2.05)	-3%	1.53 (1.18,2.00)	-5%
21 Postnatal factors				
22 Model 2 + postnatal psychiatric symptoms	1.50 (1.15,1.96)	-19%	1.35 (0.93,1.98)	-38%
23 Model 2 + postnatal financial difficulties	1.57 (1.22,2.03)	-8%	1.50 (1.13,1.98)	-11%
Model 2 + Breastfeeding at 6 months	1.56 (1.20,2.01)	*-10% (9.7)*	1.57 (1.21,2.05)	+2%
Model 2 + Exposure to environmental tobacco smoke	1.57 (1.19,2.06)	-8%	1.51 (1.15,1.99)	-9%

* Change 1 and change 2 represent the change in odds ratio relative to model 2 for respectively 'low education' versus 'high education' and for household income '<1200 euros/month' versus '>2200 euros/month', after individual adjustment for the potential mediators ($100x[OR_{model\ 2} - OR_{model\ 2\ +mediator}]/[OR_{model\ 2} - 1]$).

Table 5. Logistic regression models fitted on association between maternal education and parent-reported upper respiratory tract infections between 13-24 months of age.*

	Model 2 OR (95% CI)	Model 3 OR (95% CI)	Model 4 OR (95% CI)	Model 5 OR (95% CI)
Maternal education				
High (ref)	**1.00**	1.00	1.00	1.00
Mid-high	1.11 (0.93,1.31)	1.07 (0.90,1.27)	1.09 (0.90,1.32)	1.06 (0.95,1.69)
Mid-low	1.13 (0.92,1.38)	1.05 (0.85,1.28)	1.07 (0.86,1.32)	1.00 (0.80,1.25)
Low	*24 1.62 (1.26,2.08)*	*25 1.44 (1.11,1.86)*	**1.40 (1.07,1.84)**	1.27 (0.95,1.69)
Prenatal financial difficulties				
No (ref)		**1.00**		1.00
Yes		1.33 (1.08,1.63)		**1.36 (1.02,1.48)**
Prenatal psychiatric symptoms				
26 27 Lowest tertile (ref) *28*		1.00		1.00
Middle tertile		**1.27 (1.06,1.51)**		**1.23 (1.02,1.48)**
Highest tertile		**1.40 (1.17,1.68)**		**1.30 (1.04,1.61)**
Prenatal family functioning				
29 Lowest tertile (ref)		1.00		1.00
30 **Middle tertile** *31*		1.17 (0.98,1.41		1.18 (0.97,1.42)
32 **Highest tertile** *33*		1.14 (0.94,1.38)		1.13 (0.93,1.38)
Postnatal psychiatric symptoms				
34 Lowest tertile (ref)			1.00	1.00
Middle tertile			**1.38 (1.15,1.64)**	**1.26 (1.05,1.51)**
Highest tertile			**1.51 (1.26,1.81)**	**1.29 (1.04,1.60)**
Breastfeeding at 6 months				
No (ref)			1.00	1.00
Yes			**0.76 (0.65,0.88)**	**0.74 (0.63,0.86)**

* Values are odds ratios with associated 95% confidence intervals

Model 2: Adjusted for ethnicity mother, age mother, age child at which 24-months questionnaire was filled in, childcare attendance at 24 months and siblings.

Model 3: Model 2 + prenatal financial difficulties, prenatal psychiatric symptoms, prenatal family functioning

Model 4: Model 2 + postnatal psychiatric symptoms and breastfeeding at age 6 months

Model 5: model 2 + prenatal financial difficulties, prenatal psychiatric symptoms, prenatal family functioning, postnatal psychiatric symptoms and breastfeeding at age 6 months

Adjusted for all the other factors in this final model, prenatal financial difficulties, prenatal psychiatric symptoms, postnatal psychiatric symptoms, and breastfeeding at 6 months were significantly associated with the risk for upper respiratory tract infections at the age of 13-24 months.

Household income level and upper respiratory tract infections

Adjusted for the potential confounders, the lowest household income level was associated with a 48% increased risk for upper respiratory tract infections at the age of 13-24 months compared with the highest level (OR 1.48; 95% CI: 1.14,1.44; table 3). After additional adjustment for presence of siblings and day care attendance this was 56% (OR: 1.56; 95% CI: 1.19,2.03).

The following factors fitted the criterion to be included in the next phase: prenatal financial difficulties, prenatal psychiatric symptoms, prenatal family functioning, prenatal long lasting difficulties, postnatal psychiatric symptoms and postnatal financial difficulties (table 4).

Table 6 shows that, in total, the selected prenatal/perinatal factors explained 59% (1.56-1.23/0.56) of the increased susceptibility to upper respiratory tract infections associated with the lowest income level. However, the independent contribution of prenatal/perinatal factors was 38% (1.34-1.13/0.56). The total contribution of postnatal factors was 39% (model 4 compared to model 2: 1.56-1.34/0.56), while the independent contribution was 21% (1.44-1.27/0.62). In total, 77% of the increased susceptibility could be explained by the joint adjustment for prenatal/perinatal and postnatal factors. While the effect of a low income level was no longer significant in the final model, those of prenatal financial difficulties, prenatal psychiatric symptoms, and postnatal psychiatric symptoms were significant.

Table 6. Logistic regression models fitted on association between household income level and parent-reported upper respiratory tract infections between 13-24 months of age.*

	Model 2 OR (95% CI)	**Model 3 OR (95% CI)**	**Model 4 OR (95% CI)**	**Model 5 OR (95% CI)**
Household income level				
>2200 euros/week (ref)	1.00	1.00	1.00	1.00
1200-2200 euros/week	**1.26 (1.03,1.53)**	1.11 (0.90,1.36)	1.19 (0.96,1.47)	1.09 (0.87,1.37)

	Model 2 OR (95% CI)	Model 3 OR (95% CI)	Model 4 OR (95% CI)	Model 5 OR (95% CI)
<1200 euros/week	**1.56 (1.19,2.03)**	1.23 (0.93,1.64)	1.34 (0.91,1.99)	1.13 (0.78,1.65)
Prenatal financial difficulties				
No (ref)		**1.00**		1.00
Yes		1.35 (1.03,1.73)		**1.46 (1.07,2.00)**
Prenatal psychiatric symptoms				
35 36 Lowest tertile (ref) *37*		1.00		1.00
Middle tertile		**1.25 (1.02,1.53)**		1.22 (0.97,1.52)
Highest tertile		**1.37 (1.12,1.67)**		**1.28 (1.02,1.60)**
Prenatal family functioning				
38 Lowest tertile (ref)		1.00		1.00
39 **Middle tertile** *40*		1.18 (0.99,1.40)		1.19 (1.00,1.42)
41 **Highest tertile** *42*		1.16 (0.96,1.40)		1.14 (0.93,1.40)
Prenatal long lasting difficulties				
43 Lowest tertile (ref)		1.00		1.00
Middle tertile		1.06 (0.90,1.25)		1.07 (0.90,1.28)
Highest tertile		1.09 (0.83,1.43)		1.06 (0.81,1.38)
Postnatal psychiatric symptoms				
44 Lowest tertile (ref)			1.00	1.00
Middle tertile			**1.38 (1.22,1.65)**	**1.26 (1.01,1.53)**
Highest tertile			**1.47 (1.22,1.76)**	**1.26 (1.01,1.56)**
Postnatal financial difficulties				
No (ref)			1.00	1.00
Yes			1.01 (0.79,1.30)	0.88 (0.67,1.16)

* Values are odds ratios with associated 95% confidence intervals

Model 2: Adjusted for ethnicity mother, age mother, age child at which 24-months questionnaire was filled in, childcare attendance at 24 months and siblings.

Model 3: Model 2 + prenatal financial difficulties, prenatal psychiatric symptoms, prenatal family functioning, prenatal long lasting difficulties

Model 4: Model 2 + postnatal psychiatric symptoms, postnatal financial difficulties

Model 5: model 2 + prenatal financial difficulties, prenatal psychiatric symptoms, prenatal family functioning, prenatal long lasting difficulties, postnatal psychiatric symptoms and postnatal financial difficulties

Discussion

The present study indicates that toddlers of low socioeconomic status, as measured by either a low maternal educational level or a low household income level, are more susceptible to upper respiratory tract infections than toddlers of high socioeconomic status. This is in line with previous reports[73, 117, 118]. The novelty of our study lies in the demonstration that, independently of postnatal circumstances, part of this increased susceptibility was explained by adverse prenatal circumstances, in particular prenatal psychosocial stress.

In both adults and children, a low socioeconomic status has been associated with a higher incidence of respiratory infections[73, 117, 118, 124, 142]. Theoretically, this can be attributed to an increased exposure to infectious agents, and/or to a decreased host resistance, i.e. susceptibility to infections[124]. Viral challenge studies have provided evidence that adults of low socioeconomic status are indeed more susceptible to develop upper respiratory tract infections[124]. Our study suggests the same for toddlers. Furthermore, a substantial part of the increased susceptibility to these infections in toddlers of low socioeconomic status seems to be explained by an increased exposure to both prenatal and postnatal psychosocial stressors. Family stress measured postnatally has previously been shown to increase children's susceptibility to infections. For example, Drummond et al.[143] found that psychosocial stress is related to recurrent upper respiratory tract infections in children, possibly through decreased mucosal immunity. More recently, Wyman et al.[132] demonstrated that children of parents with higher levels of psychiatric symptoms in the context of family stressors had more febrile illnesses. However, while our results suggest that stress during pregnancy also has an independent effect on susceptibility to upper respiratory tract infections in early childhood, we found no other studies that investigated such an association. It has been speculated, though, that stress during pregnancy may dampen the fetal immune system through changes in the HPA-axis[144], which supports the possibility that prenatal stress increases a child's susceptibility to infections through an intra-uterine effect. Further support is provided by the observed correlation between both a low socioeconomic status and depressive symptoms in the mother with higher salivary cortisol levels in children[145].

While socioeconomic status is strongly related to birth weight and perinatal morbidity[146-149], these factors hardly contributed to the explanation of the observed socioeconomic differences in upper respiratory tract infections between 13 and 24 months of age, suggesting that a low socioeconomic status does not influence a child's susceptibility to these infections through its link with fetal growth and health at birth.

Methodological considerations

In this study, a major concern is the self-reported nature of the data, which might have introduced recall and reporting bias. Parents' reports of their children's health status might be affected by their socioeconomic status and by their own psychological state[150, 151]. If mothers of lower socioeconomic status and those with more psychosocial stress are more likely to consider their children as being in poor health, this might have overestimated the socioeconomic differences in upper respiratory tract infections, as well as the contribution of psychosocial-stress factors to the explanation of these differences. However, in contrast to our results regarding upper respiratory tract infections, preliminary analyses showed that mothers of low socioeconomic status reported less asthma-related symptoms compared with those of high socioeconomic status, a finding that concurs with previous reports[152]. This supports the internal validity of the results presented here. Using data from physicians or laboratory data may not be a good alternative to parent-reported data: patterns of consultation do not necessarily reflect socioeconomic variations in upper respiratory tract infections, since the decision to seek help from a doctor is dependent on access to health care and on health behaviour.

Our study was conducted in an exclusively urban population, and, although the participation rate in the Generation R Study was relatively high (61%), there was some selection towards a study population that was relatively highly educated and more healthy[42]. This limits the generalizability of our findings. Non-participation would have lead to selection bias if the associations of family socioeconomic status with upper respiratory tract infections in early childhood differed between participants and non-participants. This seems unlikely, but is difficult to ascertain. One should also take into account potential bias due to missing information on maternal educational level (6.7%) and household income (21%). Compared with mothers with available data on their socioeconomic status, those without information on educational level or income level were younger, more often of non-Dutch ethnicity, were more often smokers and were more likely to have financial difficulties and a high score on psychopathology (data not shown), thus making these mothers more likely to be of low socioeconomic status. Upper respiratory tract infections were also more prevalent in this subgroup (data not shown). Therefore, missing data is more likely to have resulted in an underestimation rather than an overestimation of our effect estimates. By using multiple imputation, we have minimized any bias that would have resulted from missing data on the outcome.

In conclusion, our study adds to the small body of literature concerning the contribution of early life factors to socioeconomic inequalities in child health. Although upper respiratory tract infections are generally relatively mild, the excess in respiratory infections attributable to social disadvantage results in a higher disease burden and an impaired quality of life in children of low socioeconomic status[153]. Furthermore, these infections have social implications, leading to for example more job absence and medical costs[123]. There is evidence that the increased susceptibility to respiratory infections associated with low socioeconomic status in early life may persist into adulthood[142], further underlining the importance of interventions to reduce these socioeconomic inequalities early in life. Our results suggest that a reduction may be accomplished by interventions aimed at active tracking and counselling of pregnant women exposed to psychosocial stressors.

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Chapter 9

Summary and future prospects

Samenvatting

Dankwoord

About the author

List of publications

PhD Portfolio

Summary and future prospects

For the investigations presented in part 1 and part 2 of this thesis we used data on bacterial carriage collected in the Generation R focus cohort. The carriage rate of *S. pneumoniae* (8.3%, 31.3% and 44.5%, measured at 1.5 months, 6 months and 14 months, respectively), *H. influenzae* (7.2%, 23.8% and 31.7%, respectively) and *M. catarrhalis* (11.8%, 29.9% and 29.7%, respectively), increased in infancy, whereas the carriage rate of *S. aureus* decreased (53.0%, 20.4% and 14.5%, respectively).

In Part 1 the aim was to determine the prevalence, dynamics and risk factors for carriage of *S. pneumoniae, H. influenzae*, *M. catarrhalis* and *S. aureus* in infancy.

Main findings.

In **chapter 2** we presented that in infants 1.5 months of age the prevalence of vaccine type pneumocci is significantly lower than in infants of age 6 and 14 months. Our population represents a genetically heterogeneous group of pneumococci. At 1.5 months of age the prevalence of the different bacteria, as well as the vaccine-type versus non-vaccine-type distribution of *S. pneumoniae*, mirrors that of adults. Crowding, in our study defined by family size and day care attendance was a risk factors for carriage of pneumococci. Previous pneumococcal carriage was a risk factor for subsequent carriage. In **chapter 3** we presented that air pollution (PM_{10}) is associated with pneumococcal carriage at children's age of 6 and 14 months, as it increases the risk of pneumococcal carriage. In **chapter 4** the main finding was that in contrast to the adult population, persistent nasal carriage of *S. aureus* was hardly detected in infancy.

In **chapter 5** we observed a negative association between carriage of *S. pneumoniae* and *S. aureus* at 1.5 months and 14 months of age, primarily observed with non-vaccine type pneumococci. Furthermore, we observed a positive association between carriage of *S. pneumoniae* and *H. influenzae* at 1.5 months and 14 months of age, present for both vaccine type and non-vaccine type pneumococci. Finally, no association was observed between *S. pneumoniae* and *M. catarrhalis* carriage.

Future prospects.

The role of mucosal and systemic antibodies needs to be elucidated. Since we collected blood samples of children and their mothers, representing pre- and postnatal stages, and saliva samples of the children, the Generation R cohort provides an excellent opportunity to investigate this role. Using modern high-throughput multiplex methods (e.g. Luminex) the response to multiple bacterial antigens can be determined in very small amounts of sample. Genetic predisposition might also influence bacterial carriage. The Generation R cohort provides an excellent opportunity, since we collected DNA of the participants, so single nucleotide polymorphism (SNP) analyses and even genome wide association studies (GWAS) can be performed.

Further research is warranted to disclose the underlying mechanisms of the association between air pollution and pneumocccal carriage. It is important to confirm our observation in an independent cohort study, preferably a more detailed study using more detailed measurements of air pollution. Next to that, a possible effect of air pollution on carriage of other bacterial species should be investigated. The implications of the association between air pollution and pneumococcal carriage for respiratory diseases in children need to be subject for future studies as well.

The question whether microbe-microbe and/or microbe-host-microbe interactions play a crucial role in the microbial interactions in the nasopharynx should be subject of further investigation. Since the nasopharyngeal niche consists of many more bacterial species as well as viral species, studies on additional interactions between other bacterial and viral species than those studied up till now are of great interest. Such studies should take host, pathogen and environmental factors into account. These issues can all be excellently investigated in the Generation R cohort.

In part 2 the aim was to investigate some consequences of 1) carriage of *Streptococcus pneumoniae, Haemophilus influenzae* and *Moraxella catarrhalis* and otitis media in young children; 2) carriage of *Staphylococcus aureus* and atopic dermatitis in young children.

Main findings.

In **chapter 6** we presented that of the children investigated, 2515 (47.2%) suffered at least once of otitis media in their second year of life. The occurrence of otitis media in the first 6

months of life and between 6 and 12 months of age increased the risk of otitis media in the second year of life. Surprisingly, bacterial carriage in the first year of life did not add to this risk. Having siblings was associated with an increased risk for otitis media in the second year of life. No associations were found between bacterial carriage in the first year of life and otitis media in the second year of life.

In **chapter 7** we observed that first positive culture of *S. aureus* at 6 months was associated with atopic dermatitis in the second year of life. Moreover, infants colonized at 6 months had an increased risk to suffer from moderate to severe atopic dermatitis in the second year of life. So we conclude that *S. aureus* colonization at 6 months is associated with atopic dermatitis and its severity in young children.

Future prospects.

Future research on the association between pathogens like *S. pneumoniae*, *H. influenzae* and *M. catarrhalis*, and the development of infections such as otitis media, pneumonia, sepsis, meningitis should take into account the duration of carriage of these bacteria as well as co-occurrence of viral infections. Host, pathogen and environmental determinants should be taken into account in such studies as well. Of particular interest is the immune response of the host against bacterial carriage and the possible protection for subsequent (invasive) diseases.

Bisgaard et al were the first to show an association between bacterial carriage in early life and the development of asthma at the age of 5. Currently, in the Generation R study detailed measurements of longfunction, astma symptoms, bacterial colonisation and immune status are conducted in all 5 year old children of the cohort. Furthermore, GWA studies are currently being performed. The Generation R study provides an excellent opportunity to further investigate the development of asthma in the context of these clinical, microbiological, immunological and genetic parameters.

In part 3 the aim was to investigate which risk factors are associated with upper respiratory tract infections in young children and explain socioeconomic inequalities in upper respiratory tract infections.

Main findings.

In **chapter 8** we observed a graded inverse relationship between both maternal educational level and household income level with the risk for upper respiratory tract infections. Adjusted for confounders and factors related to exposure to infectious agents, toddlers of mothers with a low educational level had a 62% increased susceptibility to upper respiratory tract infections in the second year of life compared to toddlers of mothers with a high educational level. Independent of postnatal factors, prenatal financial difficulties, prenatal psychiatric symptoms and poor prenatal family functioning explained 21% of this increased susceptibility. Indicators of prenatal growth and perinatal health did not contribute to the explanation. We found comparable results for the association between household income and upper respiratory tract infections. Thus, toddlers of low socioeconomic status are more susceptible to upper respiratory tract infections than toddlers of high socioeconomic status. Independently of postnatal circumstances, part of this increased susceptibility is due to adverse intrauterine circumstances, in particular prenatal exposure to maternal psychosocial stressors.

Future prospects.

Within Generation R we are able to follow up the children until young adulthood. It will be of great interest to investigate the relationship between socioeconomic status and respiratory tract infections at older age.

Samenvatting

In dit proefschrift worden verschillende studies naar de prevalentie, risicofactoren en consequenties van dragerschap van *Streptococcus pneumoniae* (pneumococcus), *Haemophilus influenzae*, *Moraxella catarrhalis* en *Stapylococcus aureus* bij jonge kinderen gepresenteerd. Infectieziekten zijn de grootste oorzaak van doktersbezoek bij jonge kinderen in de westerse wereld en de grootste oorzaak van kindersterfte wereldwijd. De in dit proefschrift bestudeerde bacteriën worden beschouwd als belangrijke ziekteverwekkers in deze leeftijdscategorie. Bijzonder is dat deze bacteriën op jonge leeftijd veelvuldig de neus-keelholte koloniseren, zonder dat daarbij ziekte ontstaat. Echter, dit asymptomatisch dragerschap is wel de eerste stap in de pathogenese van ziekte veroorzaakt door deze organisme. Daar komt nog bij dat dragerschap de oorzaak is van verspreiding van de bacteriën tussen personen. Daarom zijn studies naar dragerschap van deze bacteriën bij gezonde kinderen van belang.

De in dit proefschrift beschreven studies werden uitgevoerd in het kader van het Generation R onderzoek, een prospectieve cohortstudie vanaf het foetale leven in Rotterdam, Nederland. In meer detail werd het onderzoek verricht in de Generation R focus groep.

In **Deel 1** van dit proefschrift was ons doel om de prevalentie, dynamiek en risicofactoren van dragerschap van *S. pneumoniae*, *H. influenzae*, *M. catarrhalis* en *S. Aureus* te onderzoeken. De prevalentie van dragerschap van *S. pneumoniae* (8.3%, 31.3% and 44.5%, gemeten op de leeftijd van 1.5 maand, 6 maanden en 14 maanden, respectievelijk), *H. influenzae* (7.2%, 23.8% en 31.7%, respectievelijk) and *M. catarrhalis* (11.8%, 29.9% en 29.7%, respectievelijk), nam toe gedurende de eerste maanden van het leven, terwijl de prevalentie van dragerschap van *S. aureus* afnam (53.0%, 20.4% and 14.5%, respectievelijk). In **hoofdstuk 2** presenteren we dat bij kinderen op de leeftijd van 1.5 maand de prevalentie van vaccin type pneumokokken significant lager is dan bij kinderen van 6 en 14 maanden oud. De populatie pneumokokken die wij vonden was genetisch zeer divers. De prevalentie van de verschillende bacteriën, als ook de vaccin type versus non vaccin type pneumokokken distributie, lijkt op de leeftijd van 1.5 maand sterk op de distributie bij volwassenen. Vergelijkbaar met eerdere onderzoeken hebben wij ook gevonden dat crowding, in onze studie gedefinieerd door familie grootte, en het bezoeken van een kinderdagverblijf onafhankelijke risicofactoren zijn voor pneumokokken dragerschap. Verder hebben wij gevonden dat eerder dragerschap van pneumokokken een onafhankelijke risicofactor is voor subsequent dragerschap. In **hoofdstuk 3** presenteren we dat luchtverontreiniging (PM_{10}) geassocieerd is met verhoogd risico op pneumokokken dragerschap bij kinderen op de leeftijd van 6 en 14 maanden. In **hoofdstuk 4** is de belangrijkste bevinding dat, in tegenstelling tot bij volwassenen, persisterend dragerschap van *S. aureus*

nagenoeg niet voor komt bij jonge kinderen. In **hoofdstuk 5** presenteren we een negatieve associatie tussen dragerschap van *S. pneumoniae* en *S. aureus* op de leeftijd van 1.5 en 14 maanden, vooral in non vaccin type pneumokokken. Verder observeerden we een positieve associatie tussen dragerschap van *S. pneumoniae* en *H. influenzae* op de leeftijd van 1.5 en 14 maanden leeftijd. Dit was zowel voor vaccin type als in non vaccin type pneumokokken het geval. Tenslotte observeerden we geen associatie tussen *S. pneumoniae* en *M. catarrhalis* dragerschap.

In **deel 2** van dit proefschrift was ons doel om de consequenties van dragerschap te onder zoeken van 1) *Streptococcus pneumoniae, Haemophilus influenzae* en *Moraxella catarrhalis* op oorontsteking bij jonge kinderen en 2) dragerschap van *Staphylococcus aureus* op atopische dermatitis bij jonge kinderen. In **hoofdstuk 6** presenteren we dat 2515 (47.2%) kinderen op zijn minst 1 episode van oorontsteking doormaakten in hun eerste 2 levensjaren. We observeerden dat het doormaken van oorontsteking in de eerste 6 maanden en in de tweede 6 maanden van het leven een onafhankelijk risico factor is voor oorontsteking in het tweede levensjaar. Verwonderlijk is dat bacterieel dragerschap in het eerste levensjaar niet bijdraagt aan dit risico. Verder observeerden we dat het hebben van een of meer broertjes of zusjes is geassocieerd met een toegenomen risico op oorontsteking in het tweede levensjaar. We vonden geen associatie tussen bacterieel dragerschap in het eerste levensjaar en oorontsteking in het tweede levensjaar. In **hoofdstuk 7** concluderen we dat dragerschap van *S. aureus* op de leeftijd van 6 maanden is geassocieerd met een verhoogd risico op atopische dermatitis en de ernst van atopische dermatitis bij jonge kinderen.

In **deel 3** van dit proefschrift was ons doel om te onderzoeken welke risicofactoren geassocieerd zijn met bovenste luchtweg infecties bij jonge kinderen en om een verklaring te vinden voor socio-economische verschillen in bovenste luchtweg infecties bij jonge kinderen. In **hoofdstuk 8** observeerden we een gegradeerde omgekeerde relatie tussen zowel opleiding niveau van moeder als het inkomen van het huishouden met het risico op bovenste luchtweg infecties. Geadjusteerd voor confounders en factoren die gerelateerd zijn aan blootstelling aan infectieuze agenten, hebben kleuters van moeders met een laag opleiding niveau een 62% verhoogde vatbaarheid voor bovenste luchtweg infecties in hun tweede levensjaar in vergelijking tot kleuters van moeders met een hoog opleiding niveau. Onafhankelijk van postnatale factoren, prenatale financiële problemen, prenatale psychiatrische symptomen en prenataal slecht functioneren van het gezin verklaarde 21% van deze toegenomen vatbaarheid. Indicatoren van prenatale groei en perinatale gezondheid droegen niet bij aan de verklaring. We vonden vergelijkbare resultaten voor de associatie tussen het inkomen van het huishouden en bovenste luchtweginfecties. Kortom, kleuters met een lage sociaal

economisch status zijn vatbaarder voor bovenste luchtweg klachten dan kinderen met een hoge sociaal economische status. Onafhankelijk van postnatale factoren is een gedeelte van deze toegenomen vatbaarheid te verklaren door intra-uteriene factoren, vooral prenatale blootstelling aan maternale psychologische stress.

In **hoofdstuk 9** presenteren we de belangrijkste bevindingen en bespreken we vooruitzichten voor toekomstig onderzoek.

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About the author

Joost Adriaan Maria Labout was born in 's-Hertogenbosch, The Netherlands, on February 17th, 1975. He passed his secondary school exam (VWO) in 1996 at the DAC in 's-Hertogenbosch. In the same year, he started to study medicine at the Erasmus University Rotterdam (Erasmus Medical Centre). During his study he had participated in a Comparative study of different methods to genotype hepatitis C virus type 6 variants at the Department of Virology, Erasmus Medical Centre, Rotterdam and the Department of Medical Microbiology, Chulalangkorn University, Bangkok, Thailand (head Prof. dr. Osterhaus and Prof. dr. Y. Poovorawan). After obtaining his medical degree, he started with the research project presented in this thesis in July 2004 at the Erasmus MC Sophia childrens hospital in Generation R under supervision of Prof. dr. H.A. Moll and Prof. dr. P.W.M. Hermans. He was a member of the AIO committee and the education committee of the Molecular Medicine postgraduate school, Erasmus Medical Centre. He obtained a Master of Science degree in Clinical Epidemiology from the Netherlands Institute for Health Sciences (Nihes) in Rotterdam in 2007. From April 2008, he combined his research training with the following residencies: Pediatrics (ANIOS) at the Amphia Hospital, Breda, head dr. A.A.P.H. Vaessen-Verberne (April 2008-March 2009). Pediatrics (ANIOS) at the ErasmusMC Sopihia Childrenshospital, Rotterdam, head dr. M. de Hoog (April 2009-January 2010). Intensive care (ANIOS) at the Amphia Hospital, Breda, head dr. B.J.M. van der Meer (February 2010-June 2010). Currently he is doing a residency Anesthesiology (AIOS) at the Erasmus MC, head Prof. dr. R.J. Stolker.

Joost Labout is married to Anouk Labout-van Hulten and togheter they are the proud parents of Imme en Gijsje.

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Joost A.M. Labout, MD, Liesbeth Duijts, MD, Debby Bogaert, MD, PhD, Lidia R. Arends, PhD, Vincent W.V. Jaddoe, MD, PhD, Albert Hofman, MD, PhD, Ronald de Groot, MD, PhD, Henri A. Verbrugh, MD, PhD, Henriëtte A. Moll, MD, PhD, Peter W.M. Hermans, PhD (submitted)

Air pollution (PM_{10}) is associated with pneumococcal carriage in infancy. The Generation R study.
Joost A.M. Labout, MD, Yvonne de Kluizenaar, MsC, Frank Pierik, PhD, Vincent W.V. Jaddoe, MD, PhD, Albert Hofman, MD, PhD, Henri A. Verbrugh, MD, PhD, Peter W.M. Hermans, PhD, Henk M.E. Miedema, PhD, Henriëtte A. Moll, MD, PhD. (submitted)

Social disadvantage and upper respiratory tract infections in early childhood; contribution of prenatal factors.
LM Silva, JAM Labout, HA Moll, EAP Steegers, VWV Jaddoe, A Hofman, JP Mackenbach, H Raat. (submitted)

PhD Portfolio

Name PhD student:	Joost Adriaan Maria Labout
Erasmus MC Department:	Paediatrics
Research School:	NIHES
PhD period:	July 2004 –December 2010
Promotoror:	Prof. dr. H.A. Moll and Prof. dr. P.W.M. Hermans

1. PhD training

	Year	Workload (ECTS)
General research skills		
- English language	2005	1.4
- Working with SPSS for windows	2005	0.15
- A first glance at SPSS for windows	2005	0.15
Specific courses		
- Principles of Research in Medicine	2005	0.7
- Searching Genes in Complex Disorders	2005	0.7
- Genetic Epidemiology of Complex Diseases	2005	1.4
- Introduction to Genomics and Bioinformatics	2005	0.7
- Principles of Genetic Epidemiology	2005	0.7
- Study design	2005	4.3
- Classical Methods for Data-analysis	2006	5.7
- Modern Statistical Methods	2006	4.3
- Genetic-Epidemiologic Research Methods	2006	5.7
- Advances in Population-based Studies of Complex Genetic Disorders	2006	1.4
- Genetic Linkage Analysis: Model-based Analysis	2006	1.4
- Genetic Linkage Analysis: Model-free Analysis	2006	1.4
- SNP's and Human Diseases	2006	1.4

	Year	Workload (ECTS)
(Inter) national presentations at conferences and congresses		
- ISPPD, posterpresentation: Risk factors for pneumococcal carriage in healthy dutch infants. The Generation R study.	2006	1.4
- NvMM, posterpresentation: Risk factors for pneumococcal carriage in healthy dutch infants. The Generation R study.	2007	1.4
- Europneumo, posterpresentation: Interactions between respiratory pathogens during colonization in the first months of life. The Generation R study.	2008	1.4
- NvMM, oralpresentation: Interactions between respiratory pathogens during colonization in the first months of life. The Generation R study.	2008	1.4
- ISPPD, posterpresentatie: Air pollution is associated with pneumococcal carriage in infancy. The Generation R study.	2008	1.4
- ESPID, oralpresentation: Interactions between respiratory pathogens during colonization in the first months of life. The Generation R study.	2008	1.4
2. Teaching		
- Supervisor M. Blommaart, Life sciences, Hogeschool Zeeland. Bachelor Thesis Topic: Dragerschap van *Streptococcus pneumoniae* in het Generation R focus cohort.	2008	2.0
- Supervisor A. Lebon, Clinical Epidemiology, NIHES. Master Thesis Topic: Dynamics and determinants of *Staphylococcus aureus* carriage in infancy: the Generation R Study	2008	2.0