FREQUENT RESPIRATORY TRACT INFECTIONS IN CHILDREN

The role of environmental and genetic factors

Jopje Marlies Ruskamp

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FREQUENT RESPIRATORY TRACT INFECTIONS IN CHILDREN; THE ROLE OF ENVIRONMENTAL AND GENETIC FACTORS

Frequente luchtweginfecties in kinderen; de rol van omgevingsfactoren en genetische factoren (met een samenvatting in het Nederlands)

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Jopje Marlies Ruskamp
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Promotoren:	Prof.dr. E.A.M. Sanders
	Prof.dr. B. Brunekreef
Co-promotoren:	Dr. M.O. Hoekstra
	Dr. M.M. Rovers

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Voor Vincent & Lodewijk
Voor mijn ouders

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Chapter 1

General introduction

GENERAL INTRODUCTION

Background

Respiratory tract infections (RTI), presenting as common cold, rhinosinusitis, pharyngitis, tonsillitis, acute otitis media, bronchitis or pneumonia are a major health care problem. This is reflected by the fact that RTI are the primary reason of doctors' visits by children [1]. Every year 466 out of 1000 children aged 0 to 4 years are diagnosed with a RTI by their general practitioner in the Netherlands [2]. Most of these RTI are infections of the upper respiratory tract, i.e. common cold, rhinosinusitis, pharyngitis, tonsillitis, acute otitis media [2]. The peak incidence of physician-diagnosed upper RTI is 5.6 episodes per child-year in children between 12-23 months of age [3]. Furthermore, RTI are the leading indication for antibiotic prescription for children under 18 years of age even though these infections are known to be often predominantly viral [3;4]. Moreover, excessive and irrational use of antibiotic drugs is a world-wide concern due to the alarmingly rapid development of bacterial resistance [5]. Approximately 65% of antibiotic prescriptions for children in Western countries are prescribed for a diagnosed RTI [6]. The total number of antibiotic prescriptions per 1000 children per year did not change between 1999 and 2005 in a significant way; it ranged from 307 in 2001 compared to 282 in 2004. The prevalence of receiving at least one prescription per child varied between 19.3% in 2001 and 17.8% in 2004 [7]. In the Netherlands, surgical interventions are also quite common, especially for recurrent upper RTI, e.g. the insertion of tympanostomy tubes and (adeno) tonsillectomy. Approximately 10% of the children undergo ENT surgery for otitis media, whereas overall 30% of the children undergo one of more surgical ENT interventions irrespectively for which indication before the age of 9 years (based on own data, and [8]).

Doctor visits, the use of antibiotics and ENT surgery for RTI lead to high costs, directly affecting the community. In addition, indirect costs arise by loss of working days incurred by parents, when they have to take care of their sick child. Estimates of direct and indirect costs are high: in the United States, even the direct costs associated with otitis media alone are estimated at approximately $4 billion per year [9]. However, the true impact is probably underestimated because indirect costs may be substantially higher. In another study, indirect costs, accrued primarily by parental loss of working days, accounted for nearly 90% of the total 3-month costs associated with acute otitis media and its medical treatment [10]. In addition to the health economic consequences, RTI have considerable negative impact on the quality of life of children and their caregivers [11]. These effects seem to be proportional to the number of episodes of RTI per year [12].

Unfortunately, there are no tools available to discriminate between children who benefit from antibiotics or ENT interventions and those for whom these are not required, i.e. children with frequently recurring episodes of RTI with significant effect on health and

well-being, in contrast to children with self-limiting episodes who do not need interventions. Therefore, risk factors for occurrence of frequent RTI need to be evaluated.

Furthermore, a prediction rule, combining several risk factors, might help to identify these groups. Prediction rules are algorithms that enable physicians to explicitly interpret combinations of test results (predictors) in terms of an absolute risk of developing a disease [13;14]. Such a rule can be presented and used as a simple sum-score, a risk-stratification chart, or, in the case of a more complicated algorithm, as a formula requiring a calculator. So far no adequate prediction rule exists for frequent RTI. Currently, there are very few prognostic studies available with regard to occurrence of frequent RTI [15-19]. These studies are not suiTable to guide preventive strategies for frequent RTI due to flaws in the study design and analysis including 1) incomplete health outcomes 2) missing predictors, 3) small sample sizes and selection of study populations.

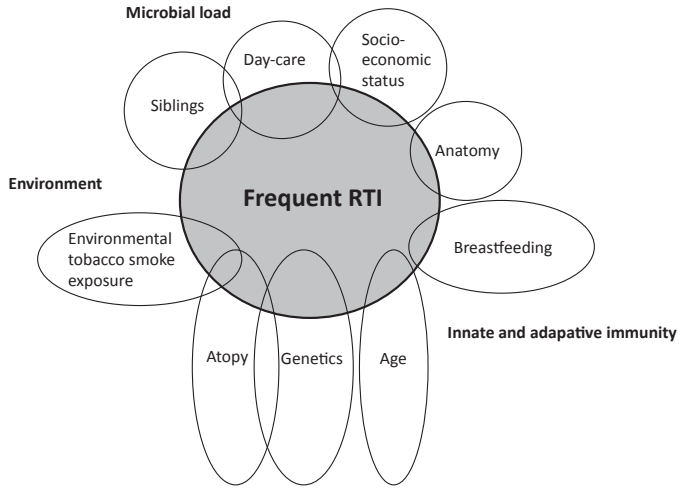
Firstly, the outcomes predicted so far have included a single outcome, e.g. only otitis media, whereas it is known that the common cold, rhinosinusitis, pharyngotonsillitis, otitis media, bronchitis and pneumonia are all closely related entities of RTI. Secondly, only a limited set of possible predictors have been studied, i.e. environmental factors like attending day-care, number of siblings, etc. Other factors known to play an important role in the pathogenesis of RTI such as the genetic predisposition have never been included in the prediction models for frequent RTI. Thirdly, sample sizes required to derive a valid and generally applicable prediction rule are large. However, the sample sizes of the studies performed so far have been small, and often populations studied have been selected rather than random. Moreover, only few studies used a multivariable approach to estimate individual risks for developing the outcome. None of the current studies tested the yield of the prediction rule using different cut-off scores. Lastly, none of the studies assessed the results of a prediction rule in terms of outcomes averted when applied to clinical practice. Prognostic data, i.e. the absolute risk of a healthy child to get frequent RTI, are pivotal for early indication of which child may need preventive or alternative treatment.

In this thesis we studied RTI in a large birth cohort. First various risk factors were evaluated separately, thereafter the studied risk factors were evaluated for their role in the occurrence of frequent RTI in an attempt to develop a prediction rule for these children.

Risk factors in the development of RTI

The pathogenesis of RTI is multifactorial; it results from interactions between environmental factors and the host (Figure 1). Environmental factors relate largely to exposure to viral and bacterial load, such as day-care attendance and the presence of siblings [20-26]. Day-care attendance in the first year of life was associated with doctor-diagnosed upper RTI (adjusted odds ratio [aOR]: 2.7, 95% confidence interval [CI]: 2.1-3.4 for large day-care facility vs. no day-care). Doctor-diagnosed lower RTI was also affected (aOR: 5.6, 95% CI:

Figure 1. Factors influencing the development of frequent RTI.



3.9-7.9 for large day-care facility vs. no day-care) [26]. In children under 1 year of age, the first 6 months of enrolment in day-care was associated with a 69% higher incidence of hospitalizations for RTI compared with children in home care [20].

Another important environmental factor is exposure to environmental tobacco smoke (ETS) [27-30]. Overall there is a consistent picture with odds ratios for either parent smoking of 1.54 (95% CI 1.31-1.80) for lower RTI (bronchitis and/or pneumonia) and 1.48 (95% CI 1.08-2.04) for recurrent otitis media [27;28].

A different air-related environmental factor is traffic-related air pollution. This factor was associated with the incidence of otitis media in the first 2 years of life in a Dutch birth cohort, with odds ratios varying between 1.10 (95% CI 1.00-1.22) and 1.14 (95% CI 1.03-1.27) [31]. Within this cohort traffic-related air pollution was also associated with otitis and other RTI during the first 4 years of life. Adjusted ORs per interquartile pollution range were elevated for ear/nose/throat infections (1.2, 95% CI 1.0–1.3) and flu/serious colds (1.2, 95% CI 1.0–1.4) [32].

Other environmental factors that have been associated with increased occurrence of RTI include lack of breastfeeding and lower socio-economic status [22;33;34].

Host factors include age, immune responses and airway anatomy (e.g. Eustachian-tube dysfunction) [35]. Immune responses can be divided in innate or adaptive immunity, and atopic responses. Atopy is an interesting factor in the development of frequent RTI, however, studies about its role are inconsistent [23;24;26;36-44]. From a pathophysiological view, a relationship between atopy and RTI may be likely. Atopy is reported to up-regulate adhesion factors for respiratory pathogens in atopic inflamed epithelium like increased intracellular adhesion molecule-1 (ICAM-1) expression in the atopic nasal mucosa [45;46].

Furthermore, environmental and host factors like atopy may interact in complex ways in their effect on RTI susceptibility. For instance Koopman et al found that day-care attendance increased the risk of RTI in the first year of life to a greater extent in infants from allergic parents compared with infants from non-allergic parents (aOR 6.1 for attendance of a large day-care facility in children from allergic parents vs aOR 3.8 in children from non-allergic parents) [26]. A similar pattern is seen for the number of allergic parents and having siblings in relation to doctor-diagnosed lower RTI [26]. The association between atopy in the child itself and frequent RTI later in life is still unclear. To study these interactions large study populations are needed with prospective and validated documentation of various risk factors and outcome measures.

Genetics and RTI

Twin studies demonstrate a strong genetic component for frequent RTI, with an estimated heritability of approximately 60% [47-50]. Which genes exactly are involved remains to be clarified, as well as how they interact with each other and the environment. Candidate genes can be found in the immune system. The innate immune system seems to play an important role in the defense against RTI especially at early ages, which is demonstrated by several genetic association studies in this field [51-55]. Mannose-binding lectin (MBL) is a key molecule in innate immunity with the capacity to bind to a broad range of micro-organisms and to subsequently kill them by initiating the lectin pathway of complement activation [56]. As a first line of defense, it seems to be particularly important between 6 and 18 months of age when adaptive immunity is not yet fully developed [57]. Aberrant functional MBL serum levels, caused by *MBL2* gene polymorphisms, frequently occur and are reported to be a potential risk factor for RTI [52;53;58-60]. A 2-fold increased relative risk of acute RTI was found in MBL-insufficient children compared with MBL-sufficient children [52].

Furthermore, it was shown that the lectin pathway of complement activation can also be activated by two ficolins, L-ficolin and H-ficolin [56;61]. Polymorphisms in the ficolin genes *FCN2* and *FCN3* appear to regulate expression as well as function of the ficolins, which may have pathophysiological implications for innate immunity [62-65]. So far, only three studies exploring clinical implications of ficolin polymorphisms have been reported [66-68].

In contrast, several studies on Toll-like receptors (TLRs) and their potential impact on susceptibility to RTI have been published. TLR are key regulators of both innate and adaptive immunity. They recognize a broad range of microbial molecular patterns, which triggers intracellular signals for activation of the immune response. Various genetic variations in TLRs like TLR2 and TLR4 and related CD14 have been associated with altered susceptibility to RTI [54;55;69-72].

However, most of these studies on innate immunity genes are based on small numbers of children and in many studies infections were studied retrospectively or cross-

sectionally, which might have led to a biased effect estimation. Therefore, the real impact of genetic variations on susceptibility to RTI remains to be clarified. For this purpose large study populations, strict definition of phenotypes and standardized assessment of environmental factors are essential. Moreover, gene-gene and gene-environment interactions must be taken into account. Selection of the studied genes should be based on their involvement in important pathways of innate immunity such as the lectin pathway of complement activation, including MBL, ficolins and the TLR pathway. To reduce redundancy and the overall amount of genotyping required, focus should be on haplotypes, defined by common haplotype-tagging single nucleotide polymorphisms (SNPs). A haplotype-tagging SNP acts as a defining marker for a haplotype by virtue of linkage disequilibrium, which is the non-random association of alleles at two or more neighboring loci that are inherited together more often than expected by chance.

Design of the study

In the present thesis we were able to study the associations between environmental factors, host factors and genetic variants and frequency of RTI in a large group of children. Our study was part of the Prevention and Incidence of Asthma and Mite Allergy (PIAMA) study [73]. This large prospective birth cohort study (n=4146) has been conducted in three regions of the Netherlands. Questionnaire data are available for each year from birth till the age of 8 years and DNA was collected in a subgroup of the children (Figure 2).

Information about frequency of RTI was collected from annual questionnaires from 1 till 8 years of age by the following question: "How often did your child have serious respiratory tract and/or ear-nose-throat infections such as flu, infection of the throat, middle ear or sinuses, bronchitis or pneumonia during the last 12 months?" Answers were recorded in 3 classes: 0, 1-2 times, or ≥ 3 times". Frequent RTI is defined as ≥ 3 RTI per year reported in 3 or 4 of the four annual questionnaires collected at age 1-4 years, or ≥ 3 RTI per year reported in 2, 3 or 4 of the four annual questionnaires collected at age 5-8 years. These definitions are based on the 95 percentile of frequency of RTI, i.e. with these definitions the 5% of the children with highest reported frequency of RTI were selected. Information on day-care attendance, siblings, breastfeeding, parental smoking and various potential confounders in the study population is also based on yearly questionnaires.

Apart from this, genetic analysis of selected single nucleotide polymorphisms (SNPs) in genes encoding the MBL, ficolin and TLR pathway of innate immunity was performed. A SNP is a DNA variant that represents variation in a single base at a frequency of ≥1%. DNA was collected successfully from 1037 children. From these children, 987 were of Dutch ancestry and used for analysis. Fifty children of non-Dutch origin were excluded to avoid effects resulting from population stratification. SNPs were selected based on associations or functional consequences found in the literature. This selection was com-

pleted with haplotype tagging SNPs selected from the publicly available database of the International HapMap Project (International HapMap Consortium, 2005; www.hapmap.org).

Objectives and outline of the current thesis

The work presented in this thesis addresses three major questions concerning frequent RTI.

1. What is the role of selected environmental and host factors in the occurrence of frequent RTI?
2. Can we identify genetic factors in the innate immunity which are associated with frequent RTI?
3. Can we predict frequent RTI with a combination of environmental, genetic and other host factors?

Ad question 1: What is the role of selected environmental and host factors in the occurrence of frequent RTI?

In **Chapter 2**, we investigate the interaction between tobacco smoke exposure and neonatal total IgE in relation to the risk of frequent RTI.

In **Chapter 3**, we study the association between atopy, as defined by IgE serum levels (specific IgE and total IgE) and skin prick test, and occurrence of frequent RTI at different ages.

Ad question 2: Can we identify genetic factors in the innate immunity which are associated with frequent RTI?

In **Chapter 4**, the results of a systematic review of the effect of mannose-binding lectin polymorphisms and RTI occurrence in children and adolescents are presented, whereas the actual associations between polymorphisms in mannan-binding lectin gene and frequent RTI within our birth cohort are described in **Chapter 5**.

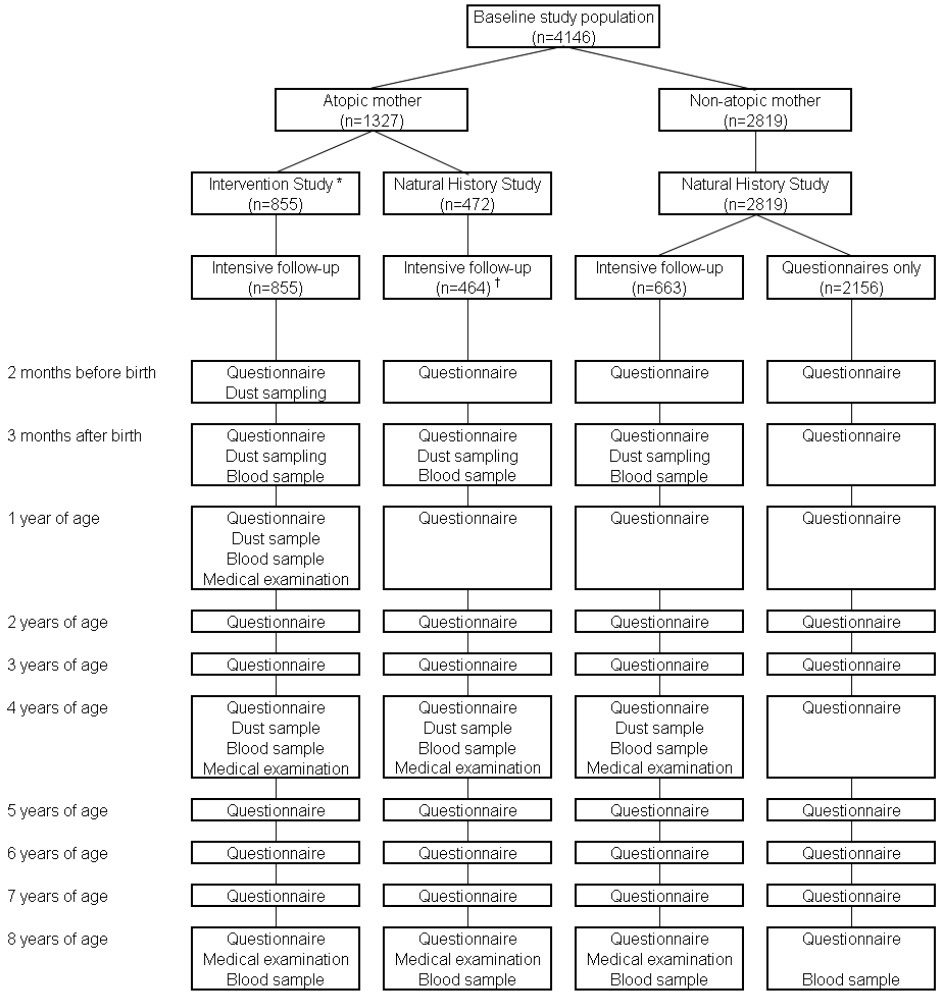
Similarly, we explore the associations of polymorphisms in ficolin genes (**Chapter 6**) and the TLR pathway (**Chapter 7**) with frequent RTI.

Ad question 3: Can we predict frequent RTI with a combination of environmental, genetic and other host factors?

In **Chapter 8**, we study the predictive value of host and environmental factors in frequent RTI and the additional predictive value of genetic factors.

In **Chapter 9**, a summarizing discussion completes the thesis. The results of our study are discussed in the light of actual clinical practice. In addition the research questions raised in this introduction are addressed and some recommendations are made for future research.

Figure 2. Flow chart of the data collection at different ages in the PIAMA study. Based on the thesis by Brussee, JE (2005)[74].



* Intervention measures were applied in 810 subjects; 416 subjects received active mattress covers and 394 subjects received placebo mattress covers; † Children who had dropped out of the study population before the age of 3 months were not selected for intensive follow-up.

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Chapter 2

Elevated neonatal total IgE enhances the risk of frequent respiratory tract infections in children with intrauterine tobacco smoke exposure

Jopje M. Ruskamp
Henriette Smit
Maroeska M. Rovers
Maarten O. Hoekstra
Anne G.M. Schilder
Bert Brunekreef
Alet H. Wijga
Marjan Kerkhof
Johan C. de Jongste
Elisabeth A.M. Sanders

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ABSTRACT

Background: Exposure to environmental tobacco smoke (ETS) is known to increase the risk of respiratory tract infections (RTI). Some children, however, may be more susceptible to harmful effects of ETS than others. We examined whether early atopic status (defined by elevated neonatal total IgE (tIgE) or symptoms of atopic dermatitis (AD)) modified the association between ETS exposure and RTI.

Methods: Data of 2863 children from the Prevention and Incidence of Asthma and Mite Allergy birth cohort were collected till 4 years. Neonatal tIgE was collected from a subset of 914 children, and clinical information by yearly parental questionnaires. The effect of pre-and/or postnatal ETS exposure, early atopic status and interaction between these factors was studied for various RTI.

Results: Children with elevated tIgE or AD and prenatal ETS exposure have a strongly increased risk of frequent RTI (aOR respectively 6.18, 95% CI 1.45-26.34; and 5.69, 2.01-16.04; interaction p-values respectively 0.006 and 0.14) compared to non-atopic children without prenatal ETS exposure. Similar results were seen for LRTI and otitis. This effect was less evident for postnatal ETS.

Conclusion: Early atopic status enhances the risk of RTI in children with prenatal ETS exposure. This suggests that host factors modify the association between ETS and RTI.

INTRODUCTION

Respiratory tract infections (RTI) are among the most common infectious diseases, especially in preschool children, and put a serious burden on the affected children and their parents [1]. There is growing awareness that the cause of RTI is multifactorial and that it results from interactions between environmental factors and the host. Some of the most evident environmental factors influencing susceptibility to RTI are exposure to environmental tobacco smoke (ETS) and exposure to other children like day-care attendance and presence of siblings [2-5]. Recognized host factors are for instance airway anatomy, immunological factors and atopy [1,3,6-9]. Exposure to ETS is consistently associated with increased risk of RTI in children [10-15]. However, some children might be more susceptible to the effects of environmental factors on RTI than others. Previously, the association between exposure to other children and RTI in children with and without allergic parents was reported in the Prevention and Incidence of Asthma and Mite Allergy (PIAMA) birth cohort in the first year of life [16]. Contacts with other children (day-care attendance or presence of siblings) increased the risk of RTI to a greater extent in infants with atopic parents compared with infants of non-atopic parents.

Next to the effect of exposure to other children, the effect of exposure to ETS on respiratory infections might also be influenced by host factors like atopy. We therefore studied whether the association between ETS exposure and RTI is indeed modified by parental allergy or atopic factors in the child itself, as indicated by the presence of elevated (≥0.50 IU/ml) neonatal total IgE (tIgE) or clinical symptoms of atopic dermatitis (AD). This was studied in the same cohort now prospectively followed from birth till 4 years of age.

METHODS

Study population

Children participated in the PIAMA birth cohort study. Details of the study design have been published previously [17]. Recruitment took place in the years 1996 and 1997. A screening questionnaire was filled in by 10 232 pregnant women visiting one of 52 prenatal clinics in The Netherlands [18]. Based on this screening, 7862 women were invited, and 4146 agreed and gave informed consent. After birth the baseline study population consisted of 3963 children. Questionnaires for parental completion, partly based on the ISAAC core questionnaires were sent to the parents during pregnancy, at the child's ages of 3 and 12 months, and yearly thereafter up to the age of 4 years [19]. Complete questionnaire information was available for 2863 children. All allergic mothers (n=1327) and a random sample of non-allergic mothers (n=663) were selected for more extensive investigation, including measurement of neonatal tIgE level in the blood spot on filter

paper that was, as a common practice, left over from routine neonatal screening in the first week of life. In this way, informed consent was obtained from 1755 parents and tIgE was actually measured in heel prick blood from 1280 infants. Data on the studied risk factors and neonatal tIgE levels were obtained from 914 infants. The study was approved by the Institutional Review Board and all parents gave written informed consent.

Outcome variables

Primary outcome

Information on the frequency of RTI in children 1–4 years of age was collected from annual parental questionnaires using the following question: "How often did your child have serious respiratory tract and/or ear-nose-throat infections, such as flu, infection of the throat, middle ear, or sinuses, bronchitis or pneumonia, during the last 12 months?"

Four answers were possible: never, 1–2 times, 3–5 times, and ≥6 times. On the basis of the answers given on the 4 consecutive annual questionnaires, we defined frequent RTI as ≥3 RTI per year reported on 3 or 4 annual questionnaires.

Secondary outcomes

Information on the frequency of lower RTI (LRTI) and otitis in children 1–4 years of age was collected from annual parental questionnaires using the following questions: "Did a doctor diagnose pneumonia and/or bronchitis in your child in the last 12 months?" and "Did a doctor diagnose infection of the middle ear in your child in the last 12 months?".

On the basis of the answers given on the 4 consecutive annual questionnaires, we defined LRTI as ≥1 doctor-diagnosed pneumonia and/or bronchitis during year 1-4, and otitis as ≥1 doctor-diagnosed episode of infection of the middle ear during year 1-4.

Exposures and confounders

The following (dichotomous) exposures were defined, based on questionnaires during pregnancy, at the child's ages of 3 and 12 months: 1. Maternal smoking during pregnancy: any smoking after the 4th week of pregnancy, data collected during pregnancy; 2. Postnatal ETS exposure at 3 months: ≥1 cigarette / cigar / pipe per day smoked in the house by the mother, father, others or by visitors (visitors: at least 1 day/week), data collected at 3 months; 3. Postnatal ETS exposure at 1 year: combination of exposure to ≥1 cigarette / cigar / pipe per day in the house and/or when the child stayed with family, neighbours, acquaintances, host parents, day-care centre or elsewhere, data collected at 1 year.

Previously, it was shown that agreement between reported smoking and air nicotine concentrations within the PIAMA study was good, supporting the validity of the smoking data reported in the questionnaire [20].

Based on previous literature, potential confounders for susceptibility to RTI were analyzed as well, i.e. duration of pregnancy, gender, birth weight, body mass index (BMI) (ac-

cording to standard international age-and gender-specific definitions), breastfeeding (at 12 weeks), day-care attendance (at least 4 hours/week), number of siblings (at 3 months), frequent wheeze (≥4 episodes of wheeze in year 1), parental allergy (presence of allergy in either 1 or both parents according to validated questionnaire) and parental education [18].

Effect modification by early atopic status

We studied elevated neonatal tIgE, AD and parental allergy as potential effect modifiers. All were taken as early markers of atopic constitution [21,22]. Total IgE was measured in plasma obtained from a heel prick blood spot, as described previously [21]. Definition of AD (based on ISAAC questionnaire) was defined at 3 months of age as a history of an itchy rash which was coming and going on flexural sites (the folds of the elbows or behind the knees), around ears or eyes or in front of the ankles [19]. Parental allergy was based on the presence of allergy in either 1 or both parents according to validated questionnaire [18].

Statistical analysis

The associations between pre-and postnatal ETS exposure and early markers of atopic constitution with the respiratory infectious outcomes at 4 years were analysed by logistic regression analyses. To evaluate potential confounding, we compared at baseline whether the groups with and without exposure differed in relation to the potential confounders. If that was the case, we used Mantel Haenszel to check if the aOR differed more than 10% from the crude OR, which was our cut-off point for actual confounding. Adjusted odds ratios (aOR) with 95% confidence intervals (CI) were estimated after selection of the actual confounding factors: frequent wheeze (≥4 episodes of wheeze in year 1), breastfeeding (at 12 weeks), level of parental education and parental allergy. To investigate the independent effect of pre- and postnatal exposure to ETS, we adjusted the OR of maternal smoking for postnatal ETS exposure and vice versa (next to breastfeeding, parental education and parental allergy). Potential effect modification on an additive scale was investigated for elevated neonatal tIgE, AD and parental allergy by stratification of the study population. Interaction on a multiplicative scale by these factors was tested by using interaction terms in logistic regression analyses.

All analyses were performed with SPSS statistical software version 15.0 (SPSS Inc, Chicago, IL).

RESULTS

Study population

From the 4146 included mothers, 183 (4%) dropped out before returning the first postnatal questionnaire due to various reasons (e.g. stillbirth, language barrier, not interested, moved). Of the 3963 remaining children, complete data on outcomes, exposures

and confounders were available in 2863 children (72%). Table 1 shows characteristics of the study population. The children without complete data were more likely than the children with complete data to have one or two allergic parents (44% and 15% versus 40% and 9%), to have a mother who smoked during pregnancy (25% versus 15%), and to be exposed to environmental tobacco smoke (at 1 year 35% versus 24%). The characteristics of the children with neonatal tIgE data (n=914) were similar to those of the complete population of our study (n=2863), except for parental allergy which was more prevalent in the first group as a results of the original PIAMA study design (at least one allergic parent in 70% versus 49%) [17].

Association between RTI, ETS exposure and early atopic status

The prevalence of frequent RTI (≥3 RTI per year reported by the parents in 3 or 4 years) during first 4 years of life was 4% in our study population. At least 1 episode of doctor-diagnosed LRTI was reported in 27% and 1 episode of doctor-diagnosed otitis in 49% of the children in 4 years. The primary outcome measure frequent RTI was associated with the secondary outcomes LRTI and otitis, OR (95% CI) respectively 6.33 (4.27-9.38) and 4.68 (2.95-7.41). Maternal smoking during pregnancy was present in 15% and postnatal ETS exposure (at age 3 months) in 24% of the study population. Only prenatal ETS exposure without postnatal exposure (at 3 months) was present in 118 children (4%), whereas only postnatal exposure was present in 368 children (12%). Elevated neonatal tIgE and AD at 3 months was reported in 9% and 8% of the study population.

Frequent RTI, LRTI and otitis were not associated with maternal smoking during pregnancy on its own; aOR respectively 1.46 (0.85-2.49), 1.23 (0.97-1.56) and 1.24 (0.95-1.62) (Table 2, results for LRTI and otitis not shown). Neither was postnatal ETS exposure at 3 months or 1 year associated with any of the infectious outcomes. A strong association was found for frequent RTI and frequent wheeze in year 1, aOR 4.05 (2.55-6.44) (Table 2). Atopic dermatitis at 3 months, parental allergy, breastfeeding <12 weeks and low parental level of education were to a lesser extent associated with increased risk of frequent RTI (Table 2). By contrast, elevated neonatal tIgE was not associated with frequent RTI, aOR 1.11 (0.41-3.01).

Effect modification by early atopic status

Maternal smoking during pregnancy on its own was overall not significantly associated with increased risk of RTI, but a strong association with RTI was found in children with prenatal ETS exposure and elevated neonatal tIgE or AD (Table 3). In children with elevated neonatal tIgE maternal smoking during pregnancy was strongly associated with frequent RTI (aOR 6.18, 1.45-26.34) (Table 3). The interaction between maternal smoking during pregnancy and elevated neonatal tIgE in relation to frequent RTI was found to be significant on a multiplicative scale (p-value 0.006). The effect of prenatal exposure on

Table 1. General characteristics of the study population

	Children with complete questionnaire data (n=2863)		Children with complete questionnaire data and neonatal total IgE (n=914)	
	n	%	n	%
Gender (male)	1462	51	465	51
Duration of pregnancy (<37 weeks)	135	5	35	4
Maternal smoking during pregnancy (>4 weeks from start)	441	15	126	14
Birth weight				
<2500 g	89	3	20	2
≥2500 g	2766	97	893	98
Breastfeeding ≥12 weeks	1478	52	479	52
Born by caesarean section	243	8	84	9
Contact with other children outside home*				
No contact	1745	61	548	60
Contact small no of children (<5)	424	15	146	16
Contact large no of children (≥5)	691	24	219	24
Siblings†				
No other children	1419	50	467	51
1 or 2 other children	1344	47	421	46
≥3 other children	98	3	26	3
Environmental tobacco smoke (ETS) exposure at 3 months	691	24	191	21
Day-care at age 1	692	24	219	24
Parental allergy‡				
No parents allergic	1442	50	278	30
One parent allergic	1159	40	468	52
Two parents allergic	262	9	168	18
Parental level of education§				
Low	349	12	100	11
Intermediate	1796	63	570	62
High	718	25	244	27
Atopic dermatitis at 3 months	221	8	83	9
Neonatal total IgE ≥0.50 IU/ml	n.a.	n.a.	83	9
Frequent RTI	120	4	42	5
Otitis	1398	49	455	50
LRTI	780	27	261	29
Frequent wheeze**	196	7	60	7

*At 1 year of age; †At 3 months of age; ‡According to validated questionnaire; §Low parental level of education: both parents maximum of 4 years at high-school; high parental level of education: both parents went to university; intermediate parental level of education: the remaining parents; **Defined as ≥4 episodes of wheeze in year 1

Table 2. The association between frequent RTI* and several risk factors (n=2863)

	Crude OR (95% CI)	aOR† (95% CI)
Maternal smoking during pregnancy	1.64 (1.06-2.55)	1.46 (0.85-2.49)
ETS exposure at 3 months	1.32 (0.88-1.97)	0.96 (0.59-1.58)
ETS exposure at 1 year	0.99 (0.66-1.49)	1.53 (0.93-2.50)
Atopic dermatitis at 3 months	2.21 (1.31-3.72)	1.84 (1.07-3.17)
Elevated neonatal total IgE‡	1.38 (0.53-3.60)	1.11 (0.41-3.01)
Parental allergy§	1.61 (1.11-2.34)	1.56 (1.06-2.29)
Frequent wheeze**	4.65 (2.96-7.30)	4.05 (2.55-6.44)
Breastfeeding < 12 weeks	1.76 (1.21-2.56)	1.56 (1.06-2.31)
Low level of parental education††	2.24 (1.31-3.82)	2.06 (1.18-3.57)

*Frequent RTI: 3 or more respiratory tract infections per year in 3 or 4 years during first 4 years of life; †aOR = OR adjusted for other risk factors; ‡Elevated neonatal total IgE: total IgE ≥0.50 IU/ml, available for 914 children; §According to validated questionnaire; **Frequent wheeze: ≥4 episodes of wheeze in year 1; ††Low parental level of education: both parents maximum of 4 years at high-school; high parental level of education: both parents went to university; intermediate parental level of education: the remaining parents

risk of doctor-diagnosed LRTI and otitis was not increased by the presence of elevated neonatal tIgE (results not shown).

The largest effect of AD was seen for frequent RTI (aOR 5.69; 95% CI 2.01-16.04; p-value of the interaction 0.14) (Table 3). However, the effect of prenatal exposure on risk of doctor-diagnosed LRTI (aOR 4.34; 95% CI 1.90-9.92) and otitis (aOR 2.90, 95% CI 1.19-7.06) was also increased by the presence of AD (not shown). In children without AD the associations were not significant (aOR 1.39, 0.78-2.47; 1.26, 0.96-1.66; 1.18, 0.93-1.51) for respectively frequent RTI, LRTI and otitis.

Parental allergy did not show significant effect modification (p-value of the interaction 0.26, results not shown). Postnatal ETS exposure in relation to frequent RTI did not show effect modification by any of the early markers of atopic constitution (results not shown).

Table 3. The association between prenatal ETS exposure and frequent RTI*: potential effect modification by elevated neonatal total IgE (n=914) and atopic dermatitis (n=2863)

Maternal smoking during pregnancy	Elevated neonatal total IgE‡		Crude OR	95% CI	aOR§	95% CI
-	-	718	1		1	
+	-	113	0.76	0.27-2.19	0.50	0.15-1.66
-	+	70	0.30	0.04-2.23	0.24	0.03-1.86
+	+	13	9.23	2.70-31.52	6.18	1.45-26.34

Interaction p-value: 0.006

Maternal smoking during pregnancy	Atopic dermatitis		Crude OR	95% CI	aOR†	95% CI
-	-	2225	1		1	
+	-	417	1.49	0.92-2.42	1.39	0.78-2.47
-	+	197	1.89	1.03-3.47	1.76	0.95-3.24
+	+	24	7.06	2.57-19.37	5.69	2.01-16.04

Interaction p-value: 0.14

*Frequent RTI: 3 or more respiratory tract infections per year in 3 or 4 years during first 4 years of life; †aOR = OR adjusted for breastfeeding (at 12 weeks), level of parental education, parental allergy, postnatal ETS exposure (at 3 months) and frequent wheeze (≥4 episodes of wheeze) in year 1; ‡Elevated neonatal total IgE: total IgE ≥0.50 IU/ml; §aOR = OR adjusted for breastfeeding (at 12 weeks), level of parental education, parental allergy, postnatal ETS exposure (at 3 months), frequent wheeze (≥4 episodes of wheeze) in year 1 and atopic dermatitis at 3 months

DISCUSSION

In this large prospective birth cohort study, we found that elevated neonatal tIgE, as an early marker of atopic status, enhanced the effect of prenatal ETS exposure on the susceptibility to RTI during the first 4 years of life. This was also true for a clinical marker like AD. By contrast, postnatal ETS exposure was not evidently associated with increased risk of RTI, independent of early atopic status.

Several studies also showed an association between ETS exposure and RTI [10-15].

Exposure to tobacco smoke may increase the risk of RTI through suppression or modulation of the immune system, enhancement of bacterial adherence factors, or impairment of the mucociliary apparatus of the respiratory tract [23]. Previously, eczema has been related to increased infection rate as well [24]. To our knowledge, we are the first to report that the association between prenatal ETS exposure and frequent RTI is significantly modified by elevated neonatal tIgE or early AD. The increased vulnerability for RTI in children with early markers of atopy exposed to ETS may be explained by

subclinical atopic inflammation of the airways. Atopic inflammation of the airways can be present at early age even before clinical respiratory symptoms [25]. Moreover, adjusting the analyses for the influence of clinical symptoms such as frequent wheeze, as we have done, did not change the results. This shows that elevated neonatal tIgE is the actual effect modifier, not symptoms of asthma. Future studies are necessary to elucidate a possible effect of subclinical atopic airway inflammation on the relationship between ETS exposure and RTI in early childhood.

The importance of timing of the exposure to tobacco smoke for effect on RTI that we found has been seen in some previous studies, although others reported conflicting results [10-15,26-29]. Methodological characteristics of the studies may explain the reported differences in outcome to a large extent. Studying the independent effect of in utero and postnatal exposure to ETS is difficult because of the strong correlation between the two factors. We tried to tackle this difficulty by adjusting the OR of maternal smoking for postnatal ETS exposure and vice versa and found the strongest effect for prenatal exposure. The absence of associations in the postnatal group may be caused by report bias, e.g. if mothers who smoked postnatally were less prone to report smoking. On the other hand, it may be that prenatal exposure is indeed more harmful [13,27-29]. The results of our sensitivity analyses with different cut-offs for postnatal ETS exposure, e.g. with a more narrow exposure variable smoking by both parents at age 3 months (7%), were in agreement with the other results.

The major strengths of our study are the population-based prospective design, and collection of exposure data at several stages before disease manifestation. Moreover, the large sample size allowed us to control for several confounders in studying the association between ETS exposure and RTI, and above all, to study potential effect modification by early atopic status. These interactions are relevant, since frequent RTI have a multifactorial origin and many different factors may contribute.

Some of our findings deserve further discussion. First, outcomes were based on parental questionnaire information. The use of questionnaire information enabled us to study a large group of children for long period of time, but might have resulted in some misclassification. However, we do believe that our misclassification is non-differential since we do not expect that mothers from children with frequent RTI were more prone to report smoking in the questionnaires collected during pregnancy than mothers from children without frequent infections. Therefore, the effect estimate will be underestimated. Second, due to the relatively rare risk factors and primary outcome measure, the numbers are very low in some of the groups. This may explain lack of significant findings related to ETS exposure. Third, in the early history of the PIAMA cohort there was an intervention. Both the intervention part and the natural history part of the study were included in our analyses. Nevertheless, we separated the children by type of study and found similar results in both groups (data not shown). Fourth, we used elevated

neonatal tIgE and AD as early markers of atopy in the child itself. Different results have been reported for the association between elevated neonatal tIgE and atopic diseases. However, within the PIAMA cohort, is had been shown that elevated neonatal tIgE (≥0.50 IU/ml) is associated with sensitization at 1 year [22]. Moreover, there is disagreement about the definition of AD, but compared with other definitions possible with the available data of the PIAMA study, the present definition seems a fairly good indicator of AD [30]. Finally, response bias may have occurred if the association between ETS exposure and frequent RTI was different for the children who were included in the analyses and those who were excluded because of missing information, leading to an overestimation of the effect.

Overall, we conclude that elevated neonatal tIgE, as an early marker of atopic constitution, seems to enhance the risk of RTI during the first 4 years in children with prenatal ETS exposure. This was also found for the clinical marker of atopy, AD. This suggests that host factors may modify the association between ETS exposure and RTI.

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Chapter 3

Are serum IgE and skin prick tests associated with frequent respiratory tract infections in school-aged children?

Jopje M. Ruskamp
Maarten O. Hoekstra
Maroeska Rovers
Henriette A. Smit
Bert Brunekreef
Alet H. Wijga
Marjan Kerkhof
Johan C. de Jongste
Elisabeth A. Sanders

Manuscript in preparation

ABSTRACT

Background: Although the role of atopy in respiratory tract infections (RTI) is likely from a pathophysiological point of view, conflicting data have been reported on the association between atopy and RTI.

Methods: We investigated the association between atopic markers and frequency of RTI in school-aged children from a large birth cohort. Markers of atopy were measured by determining specific and total serum IgE (sIgE and tIgE) levels at the age of 4 (n=588/598) and 8 years (n=1453/1462). In addition, skin prick tests (SPT) were performed at the age of 8 years (n=848). Frequency of RTI was prospectively assessed by annual parental questionnaires from 5 till 8 years of age. The children were stratified by maternal allergy.

Results: No significant association was found between any of the atopic markers at age 4 years and frequent RTI from 5 till 8 years of age in children from non-allergic mothers (OR 1.57, 95% CI 0.32-7.81; and 0.81, 0.17-3.97). Results were similar in analyses for these atopic markers and positive SPT in children from non-allergic mothers at the age of 8 years. In children from allergic mothers elevated tIgE at the age of 4 years was associated with frequent RTI (2.25, 1.09-4.65), and both positive sIgE and SPT at the age of 8 years were borderline associated with frequent RTI (1.85, 0.95-3.63; and 2.02, 0.99-4.11).

Conclusions: Positive sIgE, elevated tIgE or positive SPT in school-aged children are associated with frequent RTI in children born to allergic mothers, but not in children born to non-allergic mothers.

INTRODUCTION

Respiratory tract infections (RTI) are the most common infectious disease in children and among leading causes of high direct and indirect health care costs [1;2]. In general, most RTI are self-limiting. However, in a subgroup of children symptoms persist or recur frequently with serious impact on health [3]. In young children RTI have been seen as the result of the ineviTable encounter between micro-biological factors and an immature immunological defense system. However, the cause of frequent RTI is multi-factorial, and apart from age and anatomy, symptoms result from complex interactions between immunological (adaptive and innate), atopic, genetic, and environmental factors [4]. Atopy might be one of the factors especially important in older school-aged children.

From a pathophysiological view, a relationship between atopy and RTI may be likely. For instance, atopy may affect the middle ear status by Eustachian tube (ET) dysfunction due to swelling of the ET lining and predispose for otitis media. Also, atopy is reported to up-regulate adhesion factors for respiratory pathogens in atopic inflamed epithelium like increased intracellular adhesion molecule-1 (ICAM-1) expression in the atopic nasal mucosa that serves as receptor for rhinoviruses [5]. Rhinoviruses cause 30% of childhood upper respiratory tract infections [6,7].

So far, the evidence regarding the role of atopy on RTI has been conflicting. Whereas several studies in children and adults suggest a relation between atopy and RTI [8-19], others did not [20-25]. These conflicting findings are mainly caused by methodological limitations. In most studies RTI and/or atopy were measured retrospectively or cross-sectionally and a control group was lacking. Moreover, in some studies atopy was measured by means of a questionnaire rather than by a more objective test suchs as IgE or skin prick test (SPT). Besides, almost all studies were based on small groups of children or adults, and confounders and effect modifiers were often not taken into account. In an earlier study within the Prevention and Incidence of Asthma and Mite Allergy (PIAMA) birth cohort Koopman et al found that crowding (day-care attendance or presence of siblings) increased the risk of RTI in the first year of life to a greater extent in infants with atopic parents compared with infants of non-atopic parents [26]. Our aim was to use the same cohort to study the association between atopy in the child itself and frequent RTI later in life.

METHODS

Study population

Children participated in the PIAMA birth cohort study. Details of the study design have been published previously [27,28]. After birth the baseline study population consisted of

3963 children. The study includes a natural history part and an intervention part, i.e. a double-blind placebo-controlled study with mattress covers. The intervention part consists of 855 children, all from allergic mothers. The natural history part consists of 472 children from allergic mothers and 2819 children from non-allergic mothers. Questionnaires for parental completion, based on the ISAAC core questionnaires were sent to the parents during pregnancy, at the child's age of 3 and 12 months, and yearly thereafter up to the age of 8 years [29]. All children from allergic mothers (n=1181) and a random sample of children from non-allergic mothers (n=634) were selected for extensive investigation at the age of 4 years, including measurement of serum IgE levels. At the age of 8 years 1084 children from allergic mothers and 2580 children from non-allergic mothers were selected for extensive investigation including measurement of serum IgE levels and a skin prick test (SPT).

Complete data on RTI frequency and specific IgE (sIgE) were available for respectively 588 and 1453 children at the age of 4 and 8 years. Complete data on RTI frequency and total IgE (tIgE) were available for respectively 598 and 1462 children at the age of 4 and 8 years. Complete data on RTI frequency and SPT at the age of 8 years was available for 848 children.

The study was approved by the Institutional Review Board and all parents gave written informed consent.

Outcome variables

Information about frequency of RTI was collected from annual questionnaires from 5 till 8 years of age by the following question: "How often did your child have serious respiratory tract and/or ear-nose-throat infections such as flu, infection of the throat, middle ear or sinuses, bronchitis or pneumonia during the last 12 months?" Answers were recorded in 3 classes: 0, 1-2 times, or ≥ 3 times". We defined the following (dichotomous) outcome, frequent RTI year 5-8: ≥ 3 RTI per year reported in 2, 3 or 4 of the four annual questionnaires collected at age 5-8 years.

IgE measurements

Total and specific IgE levels were determined in capillary or venous blood collected at the age of 4 and 8 years (Sanquin Research, Amsterdam). Total IgE (tIgE) levels were measured by radioimmunoassay as described previously and expressed as international units per milliliter (1 IU representing 2.4 ng of IgE) [30,31]. Elevated tIgE was defined as logarithmically transformed tIgE ≥ 75th percentile based on data from our own study population (75th percentile was 101.00 IU/ml at the age of 4 years, and 219.99 IU/ml at the age of 8 years). Specific IgE (sIgE) levels to the aero-allergens mite (*Dermatophagoides pteronyssinus*), cat (*Fel d1*), dog (*Can f1*), grass (*Dactylis glomerata*), and birch

pollen (*Betula verrucosa*) were measured by Radio Allergo Sorbent Test. Positive sIgE was defined as sIgE ≥ 0.35 IU/ml against 1 or more of the 5 aero-allergens.

Skin prick test

Skin prick tests (SPT) were performed at the age of 8 years on the flexor side of the forearm with a prick needle and commercial allergen extract (ALK-Abello, Nieuwegein, the Netherlands; http://www.alk-abello.com/Pages/CorpFrontPage.aspx). Histamine dihydrochloride (10 mg/mL) and the glycerol diluent of the SPT extracts served as positive and negative controls, respectively. Tested allergens were: house dust mite (Dermatophagoides pteronyssinus and Dermatophagoides farinae), cat, dog, mixed grass, mixed trees, and fungus (Alternaria tenuis). SPT reactivity was measured after 15 min and transferred with a transparent adhesive tape onto a record sheet. The diameter of the wheals from the various allergens was measured twice by trained field workers from the record sheet and the mean of both measurements was calculated. A positive SPT was defined as ≥ 3 mm wheal diameter for 1 or more of the tested allergens, under the condition of a correct positive and negative control (≥ 3 mm wheal diameter for the positive histamine control and < 3 mm wheal diameter for the negative control glycerol diluent).

Statistical analysis

Because of the disproportionate representation of subgroups caused by the study design, the children were stratified by maternal allergy to investigate potential effect modification by this factor. Cross-tabulations and logistic regression analyses were used to evaluate the associations between markers of atopic constitution and frequent RTI if necessary adjusted for potential confounders. The following potential confounders were studied: duration of pregnancy, gender, birth weight, maternal smoking during pregnancy (any smoking after the 4th week of pregnancy), environmental tobacco smoke exposure at 1 year (≥ 1 cigarette / cigar / pipe per day in the house and/or when the child stayed with family, neighbors, acquaintances, host parents, day-care center or elsewhere), breastfeeding (< 12 weeks), day care attendance (at 1 year for at least 4 hours/week), presence of siblings (at 1 year) and parental education [31]. To evaluate potential confounding, we compared at baseline whether the groups with and without exposure differed in relation to the potential confounders. If that was the case, we calculated aORs, which were subsequently compared with the crude ORs. A difference of 10% was our cut-off point for actual confounding. All analyses were performed with SPSS statistical software version 15.0 (SPSS Inc, Chicago, IL).

RESULTS

Associations between specific and total IgE at the age of 4 years and frequent RTI from 5 till 8 years of age

Both maternal and paternal allergy were associated with positive sIgE in the child at the age of 4 years (OR 1.74, 95% CI 1.10-2.75; and 1.89, 1.24-2.88, respectively). None of the studied potential confounders was found to be an actual confounder and therefore none was included in the final analyses.

No association was observed between positive sIgE or elevated tIgE at the age of 4 years and frequent RTI from 5 till 8 years of age in children from non-allergic mothers (OR 1.57, 95% CI 0.32-7.81; and 0.81, 0.17-3.97 respectively) (Table 1). In children from

Table 1. Association between markers of atopy at the age of 4 years and frequent RTI* from 5 till 8 years of age

	Frequent RTI			
	Non-allergic mothers		Allergic mothers	
	N	OR 95% CI†	N	OR 95% CI
Positive sIgE‡	207	1.57 0.32-7.81	381	1.15 0.50-2.64
Elevated tIgE§	210	0.81 0.17-3.97	388	2.25 1.09-4.65

*Frequent RTI year 5-8: ≥ 2 years 3 or more respiratory tract infections per year during year 5-8; †95% CI: 95% confidence interval; ‡Elevated specific IgE (sIgE): ≥ 0.35 IU/ml (RAST class 1 or higher) against 1 or more aero-allergen(s): mite, cat, dog, dactylis (grass) and birch; §Elevated total IgE (tIgE): logarithmically transformed tIgE ≥ 75th percentile (101 IU/ml).

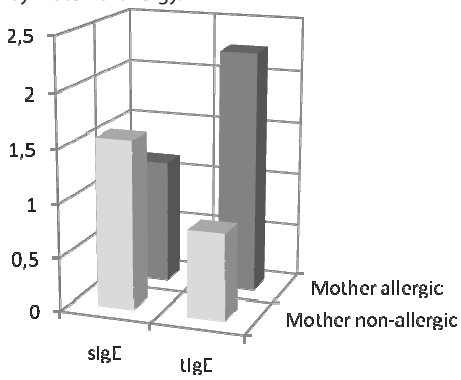
Table 2. Association between markers of atopy at the age of 8 years and frequent RTI* from 5 till 8 years of age

	Frequent RTI			
	Non-allergic mothers		Allergic mothers	
	N	OR 95% CI †	N	OR 95% CI
Positive sIgE‡	942	0.86 0.44-1.69	511	1.85 0.95-3.63
Elevated tIgE§	945	0.73 0.35-1.54	517	1.43 0.71-2.89
Positive SPT**	313	0.50 0.06-4.00	535	2.02 0.99-4.11

*Frequent RTI year 5-8: ≥ 2 years 3 or more respiratory tract infections per year during year 5-8; †95% CI: 95% confidence interval; ‡Elevated specific IgE (sIgE): ≥ 0.35 IU/ml (RAST class 1 or higher) against 1 or more aero-allergen(s): mite, cat, dog, dactylis (grass) and birch; §Elevated total IgE (tIgE): logarithmically transformed tIgE ≥ 75th percentile (220 IU/ml); **Positive skin-prick test (SPT): ≥ 1 allergen positive at the age of 8 years.

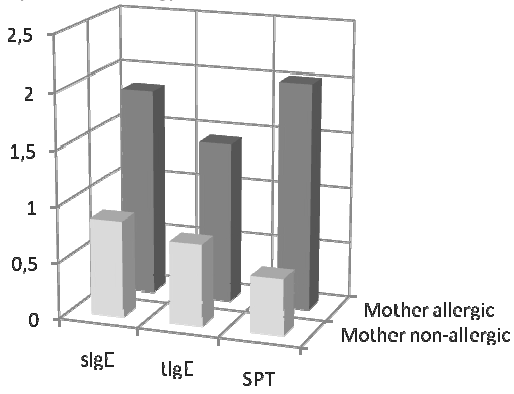
allergic mothers positive sIgE was not associated with frequent RTI from 5 till 8 years of life (OR 1.15, 95% CI 0.50-2.64), whereas elevated tIgE was significantly associated with frequent RTI from 5 till 8 years of life (OR 2.25, 95% CI 1.09-4.65) (Table 1).

Figure 1.a. OR between markers of atopy at age 4 years and frequent RTI at ages 5-8 years: stratification by maternal allergy.



OR: Odds ratio; Frequent RTI: year 5-8: ≥ 2 years 3 or more respiratory tract infections per year during year 5-8; Elevated specific IgE (sIgE): ≥ 0.35 IU/ml (RAST class 1 or higher) against 1 or more aero-allergen(s): mite, cat, dog, dactylis (grass) and birch; Elevated total IgE (tIgE): logarithmically transformed tIgE ≥ 75th percentile (101 IU/ml).

Figure 1.b. OR between markers of atopy at age 8 years and frequent RTI at ages 5-8 years: stratification by maternal allergy.



OR: Odds ratio; Frequent RTI: year 5-8: ≥ 2 years 3 or more respiratory tract infections per year during year 5-8; Elevated specific IgE (sIgE): ≥ 0.35 IU/ml (RAST class 1 or higher) against 1 or more aero-allergen(s): mite, cat, dog, dactylis (grass) and birch; Elevated total IgE (tIgE): logarithmically transformed tIgE ≥ 75th percentile (220 IU/ml); Positive skin-prick test (SPT): ≥ 1 allergen positive at the age of 8 years.

Associations between specific and total IgE and skin prick test at the age of 8 years and frequent RTI from 5 till 8 years of age

Similar to the age of 4 years, positive sIgE, elevated tIgE and positive SPT at the age of 8 years were not associated with frequent RTI from 5 till 8 years of age in children from non-allergic mothers (OR 0.86, 95% CI 0.44-1.69; 0.73, 0.35-1.54; and 0.50, 0.06-4.00) (Table 2).

In children from allergic mothers positive sIgE (OR 1.85; 95% CI 0.95-3.63), and positive SPT (OR 2.02; 95% CI 0.99-4.11) at 8 years of age were borderline associated with frequent RTI from 5 till 8 years of age (Table 2).

The overall tendency for stronger associations between atopic markers and frequent RTI in children born to allergic mothers compared to children born to non–allergic mothers is visualized in Figure 1a and 1.b. Separating the children born to allergic mothers by type of study (intervention study versus natural history study) did not show significant interaction.

Moreover, for sIgE a relatively low cut-off point was chosen (0.35 IU/ml). We also performed the analyses with 0.70 IU/ml as cut-off point for positive sIgE and found similar results.

DISCUSSION

In this large prospective birth cohort study, we found that positive sIgE, elevated tIgE or positive SPT in school-aged children are associated with frequent RTI in children born to allergic mothers, but not in children born to non-allergic mothers.

These results are in line with the study from Koopman et al within the same cohort. In this study it was shown that contacts with other children (day-care attendance or presence of siblings) increased the risk of RTI in the first year of life to a greater extent in infants with atopic parents compared with infants of non-atopic parents (adjusted OR 6.1 for attendance of a large day-care facility in children from allergic parents versus 3.8 in children from non-allergic parents) [26]. A similar pattern is seen for increasing number of allergic parents and having siblings in relation to doctor-diagnosed lower RTI. The present study showed similar results, but in this study the children were studied at a later age and atopic markers in the children itself were included instead of parental atopy as "proxy" for atopy in the child.

The major strengths of our study are its longitudinal population based design using repeated questionnaires. Furthermore, atopy was assessed by different objective markers, i.e. sIgE, tIgE and SPT, all showing reasonably consistent results.

To appreciate our results, some potential limitations should also be considered. First, the PIAMA study was originally designed to study atopy and asthma. Exact measures of numbers of RTI episodes were not recorded. Instead we grouped the children according to frequency of RTI as reported in annual questionnaires. Strictly speaking, RTI in our study means "respiratory symptoms", which the parents considered to be more serious than a common cold. Large cohort studies have to use simple and practical data-collection methods, which may lead to some misclassification of both exposures and outcomes. However, this type of misclassification is most likely non-differential, which will make associations weaker but not change direction.

Finally, our definition of atopy was based on different atopic markers measured at 2 time-points. This led to the possibility that some children were defined as "atopic" at the age of 4 years and "non-atopic" at the age of 8 years or vice-versa. However, we studied the correlation between the different markers of atopy and found that they were positively correlated ($p<0.005$). Largest correlation was found for serum sIgE and SPT at age 8 years (Pearson correlation r 0.70, $p<0.005$, results not shown), as was expected since these markers are considered favorable to delineate subjects with and without allergic disorders in comparison with tIgE [32]. In fact, we studied the same association (between atopy and RTI susceptibility) within the same children in different ways. The consistency between the results for the different atopic markers certainly strengthens our conclusions.

In conclusion, we show that positive sIgE, elevated tIgE or positive SPT in school-aged children are associated with frequent RTI in children born to allergic mothers, but not in children born to non-allergic mothers.

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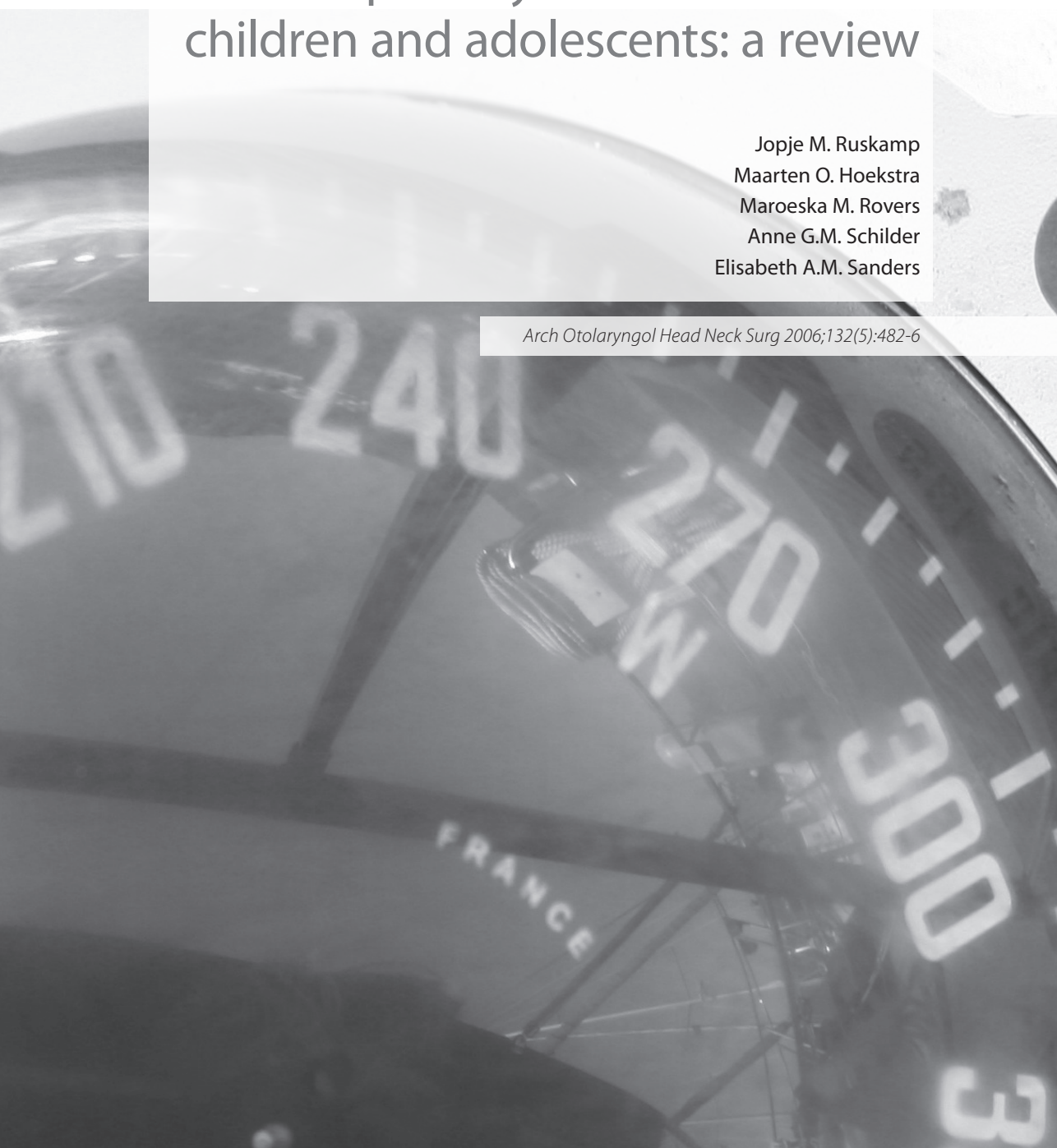
Chapter 4

Mannose-binding lectin and upper respiratory tract infections in children and adolescents: a review

Jopje M. Ruskamp
Maarten O. Hoekstra
Maroeska M. Rovers
Anne G.M. Schilder
Elisabeth A.M. Sanders

Arch Otolaryngol Head Neck Surg 2006;132(5):482-6



ABSTRACT

Objective: To review the literature on mannose-binding lectin (MBL) polymorphisms and susceptibility for upper respiratory tract infection (URTI) in children and adolescents.
Data sources: We searched PubMed from 1966 and EMBASE from 1974 to July 2005, using the terms respiratory tract infection, respiratory infection, upper respiratory infection, MBL, and mannose-binding lectin.
Study selection: Initially, 110 studies were identified. Two reviewers independently screened identified titles and abstracts. Potentially relevant studies were obtained and the full text examined. Inclusion criteria were human subjects, 18 years or younger, URTI, and MBL polymorphisms. Seven of the initially identified studies met the inclusion criteria.
Data extraction: Information was gathered for each study on study design, population, possible confounders, and outcomes measured.
Data synthesis: Because there was significant heterogeneity between the identified studies, we had to describe the identified studies separately. The largest case-control studies (n=3) as well as the cohort study (n=1) suggest an association between MBL polymorphisms and URTI, especially in young children. Results of the smaller studies (n=3) are inconsistent.
Conclusions: The association between MBL polymorphisms and URTI in children remains controversial. Large prospective cohort studies with regular documentation of URTI and possible confounders such as atopy and environmental factors are required to establish the role of MBL polymorphisms in susceptibility for URTI.

INTRODUCTION

Upper respiratory tract infections (URTI) presenting as common cold, rhinosinusitis, pharyngotonsillitis, and otitis media are the most common infectious disease in children. Most URTI are selflimiting, but symptoms persist or recur frequently in about 10% to 20% of the pediatric population [1]. These infections not only affect children's health and wellbeing, but also generate high medical costs and indirect costs for family and society [2,3]. Strategies for early recognition by clinicians of children at high risk for recurrent infections may offer the possibility of preventive measures such as extra vaccinations. To recognize risk factors, a better insight into the pathogenesis and risk factors of URTI is required.

The cause of recurrent URTI is multifactorial and results from interactions between environmental, immunological (adaptive and innate), and genetic factors [1]. Twin studies demonstrate a strong genetic component for recurrent URTI, with an estimated heritability of approximately 60% [4-6]. Which genes are involved remains to be clarified, as well as how they interact with each other and the environment.

Mannose-binding lectin (MBL) is a key molecule in innate immunity with the capacity to bind to a broad range of micro-organisms and subsequently kill them by initiating the lectin pathway of complement activation [7]. As a first-line defense, MBL seems to be particularly important between ages 6 and 18 months, when adaptive immunity is not yet fully developed [8]. Several variations in the gene encoding MBL have been described, mostly single-nucleotide polymorphisms. Three polymorphisms have been found in exon 1 of the MBL gene at codons 52, 54, and 57, respectively, the D, B, and C variant alleles, of which the B variant is particularly prevalent in white patients. These exon 1 polymorphisms compromise assembly of oligomers, thereby reducing biological activity [9,10]. Serum levels between individuals also vary because of 3 major polymorphisms in the promoter region of the MBL gene (HL, PQ, and XY). These promoter polymorphisms influence transcription activity and synthesis of MBL [11].

Polymorphisms in the MBL gene are very common, and low serum levels of MBL are found in 10% to 15% of white populations [11]. This condition has been connected with increased general susceptibility to infectious diseases and to infection by specific pathogens such as *Streptococcus pneumoniae*, which is one of the most important pathogens causing URTI [12-15]. In this narrative review, we focused on the role of MBL polymorphisms in susceptibility for URTI in children and adolescents.

METHODS

Data sources

We searched PubMed from 1966 and EMBASE from 1974 to July 2005, using the terms respiratory tract infection, respiratory infection, upper respiratory infection, MBL, and mannosebinding lectin to identify articles reporting on the association between polymorphisms of MBL and URTI. In addition, a reference and related article search was performed.

Study selection

Two reviewers independently screened identified titles and abstracts without blinding to authorship or journal. Potentially relevant studies were obtained and the full text examined. Discrepancies between reviewers were resolved by discussion. Criteria for inclusion in this survey were human subjects, 18 years or younger, URTI, and MBL polymorphisms.

Data extraction and synthesis

Information was gathered for each study on study design, population, possible confounders, and outcomes measured. Because there was significant heterogeneity between the identified studies, pooling of the major outcomes was not possible. The results of the studies are therefore described separately.

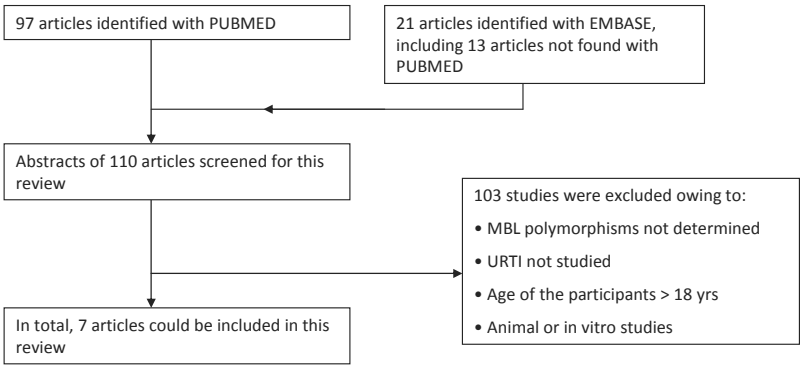
RESULTS

Initially, 97 articles were identified with PubMed, whereas EMBASE revealed 13 studies that were not found on PubMed. Of these 110 studies, only 7 articles met the inclusion criteria (Figure 1).

Of the 7 included studies, 4 demonstrated a positive association between variant MBL alleles and URTI and 3 studies did not find a positive association. The main characteristics of the studies are given in the Table. Various designs were used (ie, case-control (n=5), cross-sectional survey (n=1), and cohort (n=1)). The age of the studied populations varied from birth to 18 years, and 2 studies only included young children (age <3 years). Because the heterogeneity between studies was large, we discuss each study separately.

Summerfield et al. examined children attending a hospital and compared children who were admitted with infections (including URTI) with children who were admitted as having various other diagnoses [12]. The prevalence of variant alleles of the MBL gene in children with infections was twice that in children without infections. Increased sus-

Figure 1. Flowchart of study selection.



MBL indicates mannose-binding lectin; URTI, upper respiratory tract infection.

ceptibility to infections was found in both heterozygotic and homozygotic children, but homozygotic children had more severe infections (including recurrent URTI).

Koch et al. investigated the effect of MBL polymorphisms on risk for acute respiratory tract infection in a population-based cohort of young children [16]. The cohort was followed up weekly during a 2-year period for respiratory morbidity, and MBL genotypes were determined at the end of the study period. A 2-fold risk of acute respiratory tract infections was found in children with variant MBL alleles compared with children with normal alleles, but detailed analyses showed that this association was restricted to children aged 6 to 17 months.

Cedzynski et al. compared the frequency of variant MBL alleles in children attending the hospital with recurrent respiratory tract infections and healthy controls [17]. Children with recurrent respiratory tract infections had a 2 times higher risk of carrying variant alleles compared with healthy control children.

Garred et al. compared the frequency of variant MBL alleles in patients suspected of immunodeficiency with healthy adults [13]. Although the age of the study population exceeded 18 years (range, 2 months to 76.3 years), we decided to include this study because the median age of the study population was 57 months, with an inter-quartile range of 20 to 137 months. The frequency of heterozygotes for the variant alleles did not differ between patients and controls. The frequency of homozygotes, however, was higher in the patient group. They also found an association between homozygosity for MBL variants and severe recurrent otitis media and throat infections. The validity of the side outcome of URTI can be argued because the study population comprised patients suspected of various immunodeficiencies. Nonetheless, it remains noteworthy that 5 of 20 MBL homozygotic patients were reported to have recurrent URTI. With their repertoire of laboratory analyses, the authors could not identify 1 precipitating cofactor (ie, other immunodeficiency), except for a possible association with IgG subclass deficiency.

Table 1. Summary of Studies on MBL Polymorphisms and URTI in Children and Adolescents

Source	Design	Participants (P) and controls (C)	Outcome measure	Conclusion
Summerfield et al [12] (London, England)	Case-control Hospital-based	P: 345 children admitted with infections C: 272 children admitted with other diagnoses Age: 0-18 y	Frequency of MBL genotypes (A,B,C and D allele)	Increased prevalence of B, C and D allele in children with infections, including URTI (42,3 versus 23,5%); OR=2,4 (p<0.0001)
Koch et al [16] (Sisimiut, Greenland)	Cohort Population-based	P: 252 children in cohort Age: <2 y	Risk of acute respiratory infections compared by MBL genotype	MBL insufficiency (XA/O + O/O) associated with a 2,08-fold increased risk for acute respiratory infections (95% CI 1,41-3,06); risk association largely restricted to those aged 6-17 mo
Cedzynski et al[17] (Lodz, Poland)	Case-control Hospital-based	P: 335 children with recurrent RTI C: 78 healthy children Age: 1-16 y	Frequency of MBL genotypes (A, B, C and D allele)	Increased proportion of O/O (p=0,03) or O/A (p=0,01) in children with recurrent respiratory infections
Garred et al[13] (Copenhagen, Denmark)	Case-control Hospital-based	P: 229 patients suspected for immunodeficiency C: 123 healthy adults Age: 0-76 y, mean age almost 5 y	Frequency of MBL genotypes (A, B, C and D allele)	Frequency of homozygosity for variant alleles in patients increased (p=0,0017); variant alleles B and D associated with severe recurrent otitis media occurring with throat infections
Garred et al[18] (Copenhagen, Denmark)	Case-control Hospital-based	P: 89 children with otitis media, age: unknown C: 123 healthy adults	Frequency of MBL genotypes (A and B allele)	Frequency of B allele not different in children with recurrent otitis media (p=0,65)
Homøe et al[19] (Nuuk, Greenland)	Cross-sectional survey Population-based	P: 73 unselected children Age: 3-8 y	Frequency of MBL genotypes (A, B, C and D allele)	Presence of B or D allele not associated with (recurrent) acute otitis media or age at first acute otitis media episode
Ozbas-Gerceker et al[20] (Ankara, Turkey)	Case-control Hospital-based	P: 69 children with recurrent otitis media, age: 0-3 y C: 100 healthy adults	Frequency of MBL genotypes (A, B and C allele)	Frequency of B allele lower in children with recurrent otitis media (OR=2,33, 95% CI 1,06-5,11, p=0,03); no difference in overall distribution of the genotypes (p=0,068)

NOTE: A allele, normal, wild-type; B allele, single base pair substitution at codon 54; C allele, single base pair substitution at codon 57; CI, confidence interval; D allele, single base pair substitution at codon 52; MBL, mannose-binding lectin; OR, odds ratio; RTI, respiratory tract infection; URTI, upper respiratory tract infection.

Three studies did not find an association between MBL polymorphisms and URTI. Garred et al. compared the MBL genotyping of children with recurrent otitis media, who were admitted to the hospital for ventilating tubes and/or adenoidectomy with healthy controls [18]. The frequency of the B allele did not differ between groups, but a trend was shown toward more homozygosity of the B allele in the patient group.

Homøe et al. studied the association between variant MBL alleles and episodes of acute otitis media in a survey of community-based, unselected children [19]. No association was found with respect to the presence of variant MBL alleles and acute otitis media, recurrent acute otitis media, or age at first acute otitis media episode.

Finally, Ozbas-Gerceker et al. compared variant alleles of MBL in children with recurrent otitis media with healthy controls [20]. The frequency of the B allele was found to be lower in children with recurrent otitis media compared with healthy controls, but the overall distribution of genotypes did not significantly differ these 2 groups.

COMMENT

Differences in study design

The 7 studies reviewed showed inconsistent results, which may be because of differences in study design, study population, and outcomes measured. Most of the identified studies were case-control studies, and it is known that measurement of exposure of the determinant (ie, MBL polymorphisms) leading to the disease or outcome (ie, URTI) often is less accurate in case-control studies compared with cohort studies. Another problem with case-control studies is selecting the control group, which might lead to selection bias. Furthermore, only Koch et al. adjusted for potential confounders such as age, sex, ethnicity, and calendar period [16].

Differences in study population such as genetic background, age, and number of subjects are potentially a major source of variation. In the studies by Ozbas-Gerceker et al.,Cedzynski et al., and Garred et al. only white children were included [20,7,13]. However, Summerfield et al., Koch et al., and Homøe et al. studied children with different genetic backgrounds [12,16,19]. Koch et al. and Homøe et al. included Greenlandic Eskimo, mixed-race, and white children [16,19]. Upper respiratory tract infections including acute otitis media are known to occur earlier and more frequently among Eskimo children. In general, frequencies of variant MBL alleles differ among ethnic populations.

Age is another important characteristic of the study population in this setting, as it has been suggested that MBL is particularly important in children between ages 6 and 18 months. In that period, the adaptive system is still immature, and levels of maternally derived immunoglobulins are decreasing [8]. This is confirmed by the study by Koch et al., which showed that MBL polymorphisms particularly influence the risk of acute respira-

tory tract infections in children aged 6 to 17 months, while might explain the different results found by Homøe et al. because in their study children younger than 3 years were excluded [16,19]. The number of children studied also varied between the studies. A positive association between MBL polymorphisms and URTI was found in the larger studies, whereas the smaller studies did not find an effect, which might reflect a power problem.

In our opinion, the study by Koch et al. is most valuable because it is a large prospective cohort study with regular documentation of infections [16]. Nevertheless, it is important to realize that they studied mostly Eskimo children. Their specific genetic background and a priori high risk for URTI make results hard to generalize to white children. Finally, it should be noted that publication bias cannot be precluded because registers for non-experimental studies have not yet been created. Differing outcomes measured by the included studies might be another explanation of the different study results. One study measured severity of URTI, 2 studies measured risk of URTI, and the other studies measured risk of recurrence of infection [13,12,16,17-20].

We addressed URTI instead of the individual diseases like common colds, rhinosinusitis, pharyngotonsillitis, and otitis media. In 4 of the included studies only otitis media was studied, whereas in the other studies respiratory tract infections in general were studied. The effect of MBL polymorphisms may differ regarding different clinical manifestations or various microbial agents. However, the pathogenesis of different URTI is very similar and often associated.

Circumstantial evidence

There is a strong association between MBL polymorphisms and MBL serum levels [9-11]. However, like an acute-phase protein, MBL serum levels may rise under stress [21].

Moreover, genotyping becomes more and more easily accessible, and genotyping costs are declining. Therefore, in our opinion, genotyping is preferable in future studies.

In this review we included only MBL genotyping studies, but studies focusing on MBL levels in serum provide additional results. Aittoniemi et al. determined MBL serum levels in children with increased susceptibility to infections, mostly respiratory tract infections [22]. Levels of MBL serum did not differ between these children and the general Finnish population, but MBL deficiency seemed to be manifested in combination with other immunodeficiencies. The same observation was seen in a recent cohort study by Thorarinsdottir et al., in which MBL serum levels were determined in children with recurrent otitis media and lower respiratory tract infections [23]. No association was seen between MBL levels at birth or age 2 years and these respiratory tract infections, but recurrent otitis media was associated with sustained low levels of both MBL and immunoglobulin type A. Both studies are in line with previous findings, as it has been suggested that MBL plays a major role in the immune system of young children with immature adaptive immunity and decreasing levels of maternal antibodies.

In the present review we included only studies in children and adolescents, but the association between MBL polymorphisms and respiratory tract infections has also been studied in adults. Gomi et al. showed a higher frequency of MBL variants resulting in low serum levels among patients with recurrent respiratory tract infections compared with control subjects, whereas Dahl et al. found no significant association between MBL variants and respiratory tract infections in a large group of adults [24,25].

Clinical implications

Mannose-binding lectin polymorphisms and subsequently MBL insufficiency occur frequently, and many children considered to be healthy will also be MBL deficient. Therefore, MBL insufficiency on its own cannot fully explain proneness to URTI. Recurrent URTI has a multifactorial background. The coexistence of risk factors in the environment (ie, day care attendance, siblings, and smoking), anatomy and other genetically predisposing factors (ie, atopy, low IgG subclasses or IgA deficiency, and low response to polysaccharide vaccination), or potentially additional polymorphisms, for instance in the Toll-like receptor pathway, may together lead to a URTI-prone condition, particularly at an early age. In clinics,

MBL insufficiency can be considered as an additional factor predisposing to recurrent URTI.

CONCLUSIONS

Inconsistent results were found in the literature regarding MBL polymorphisms and susceptibility for URTI in children. Case-control studies including larger numbers of children as well as a cohort study suggest an association between MBL polymorphisms and susceptibility for URTI in children. Other studies, however, did not find an association between MBL polymorphisms and URTI. The ideal study to establish the role of MBL polymorphisms in susceptibility for URTI in children would be a large long-term prospective cohort study with repeated documentation of respiratory tract infections and possible confounders such as atopy and other genetic and environmental factors.

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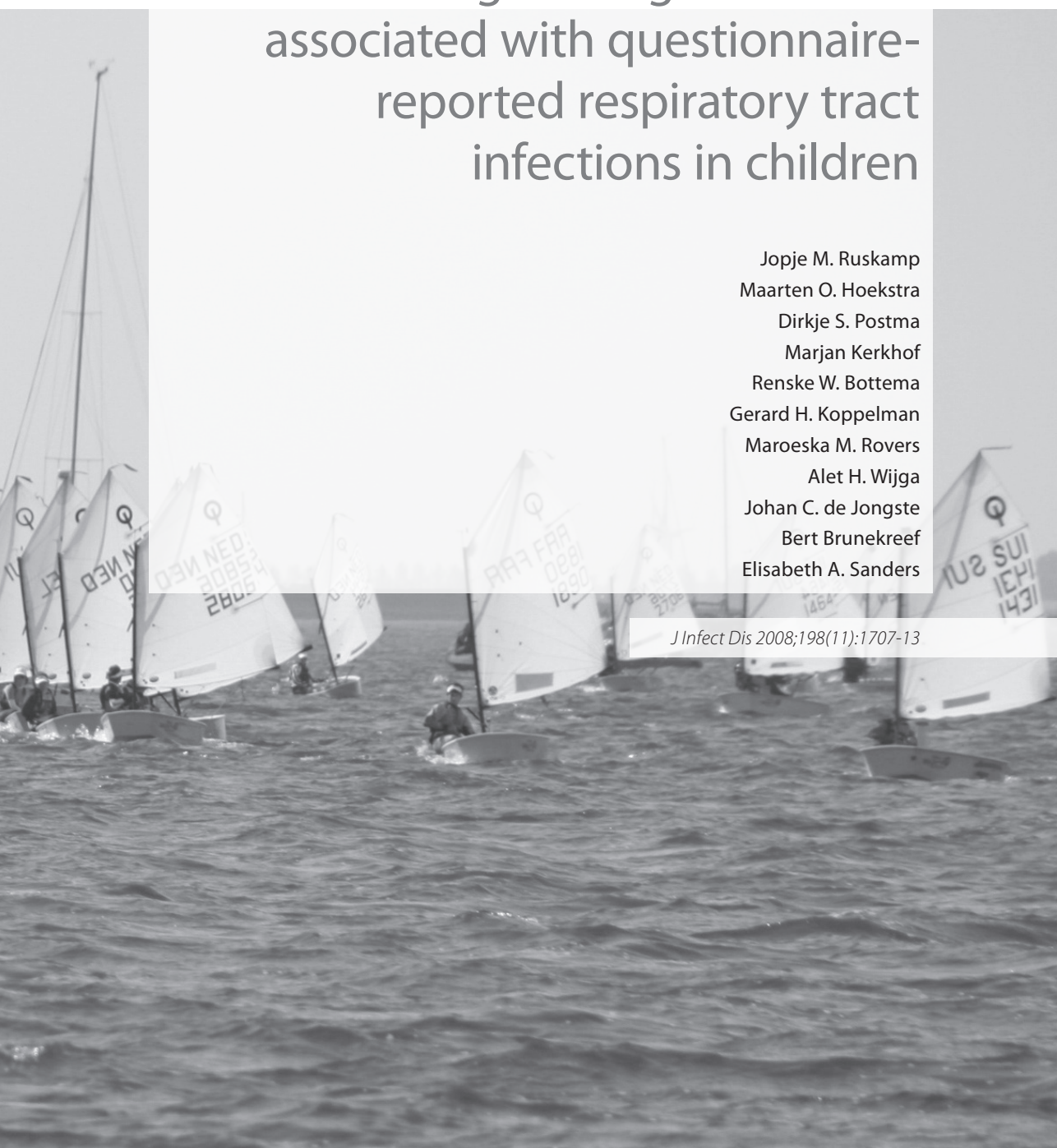
Chapter 5

Polymorphisms in the mannan-binding lectin gene are not associated with questionnaire-reported respiratory tract infections in children

Jopje M. Ruskamp
Maarten O. Hoekstra
Dirkje S. Postma
Marjan Kerkhof
Renske W. Bottema
Gerard H. Koppelman
Maroeska M. Rovers
Alet H. Wijga
Johan C. de Jongste
Bert Brunekreef
Elisabeth A. Sanders

J Infect Dis 2008;198(11):1707-13



ABSTRACT

Background: Low mannan-binding lectin (MBL) levels, caused by *MBL2* polymorphisms, are suggested to contribute to susceptibility to respiratory tract infections (RTI), particularly early in life. Large-scale replication of previous associations is needed, however. We investigated the association between *MBL2* polymorphisms and the frequency of RTI in a large population-based birth cohort of white children.

Methods: The frequency of RTI was prospectively assessed by annual parental questionnaires until children were 4 years of age. Thirteen polymorphisms in *MBL2* were determined in 987 Dutch children. Haplotypes, previously shown to be associated with functional levels of MBL, were constructed, and their associations with the frequency of RTI during year 1, year 2, and the first 4 years of life were assessed. High-producing, intermediate-producing, and deficient *MBL2* genotypes were defined on the basis of exon 1 and Y/X promoter polymorphisms.

Results: No differences were found between investigated polymorphisms and haplotype frequencies in the population as a whole or between the groups with frequent, moderately frequent, or no RTI reported. Deficient *MBL2* genotypes were not associated with an increased risk of RTI (odds ratio, 0.71 [95% confidence interval, 0.25 to 2.05]) during years 1–4 of life. This was also true when year 1 and year 2 were studied separately.

Conclusion: These results suggest that, at the population level, *MBL2* polymorphisms do not contribute to the risk of questionnaire-reported RTI in white children.

INTRODUCTION

Mannan-binding lectin (MBL) is a central player in the innate immune defense. It is suggested to contribute to increased susceptibility to infections in the case of deficiency [1,2]. This protein has the capacity to bind to a broad range of microorganisms and subsequently initiate the lectin pathway of complement activation and immune defense [3]. The *MBL2* gene is located on chromosome 10 and codes for different *MBL2* haplotypes that are functional and are associated with MBL serum levels [4–6]. Three common single-nucleotide polymorphisms (SNPs) in exon 1 of the gene affecting codons 52, 54, and 57 (D, B, and C alleles, respectively, collectively known as the O allele) have been shown to lead to low or absent MBL serum levels, both in homozygous and heterozygous states [7–9]. Later studies identified common polymorphisms in the promoter region of *MBL2* (-619G>C [H/L], -290G>C [Y/X], and -66C>T [P/Q]) that further influence MBL serum levels by affecting transcription [4,8].

The clinical significance of MBL deficiency is still debated, and inconsistent results are found in the literature regarding *MBL2* polymorphisms and susceptibility to respiratory tract infections (RTI) in children [10]. Although the findings of several studies, mostly hospital based, suggest that *MBL2* genotypes associated with low levels of MBL lead to an increased risk of RTI [4, 11–15], other studies have failed to confirm this association [16–19]. The redundancy of the innate immune system, which may compensate sufficiently to prevent infection, could fail to limit the severity of infection when innate immunity is compromised by MBL deficiency. This may be why hospital-based studies tend to show an effect of *MBL2* polymorphisms on susceptibility to infectious disease.

Two prospective population-based cohort studies have been published to date. The first is the study by Koch et al. [13], performed in 252 Eskimo children who were investigated weekly for the number of RTI episodes, from 6 weeks to 2 years of age. In this study, deficient *MBL2* genotypes were found to be associated with a 2-fold increased risk for acute RTI, an observation that was restricted to children aged 6–17 months. That MBL deficiency might be relevant during that particular age period is thought to result from the fact that the adaptive immunity needed to protect against RTI is still immature in children <2 years of age but may compensate for MBL deficiency after that age. The second study is the recently published study by Müller et al. [19], performed in a large German birth cohort selected for the study of allergies. In this study, low-producing *MBL2* genotypes were found not to be associated with an increased frequency of questionnaire-based RTI, which was calculated for the following age periods: 0–1, 0–2, 0–5, and 0–11 years. However, it should be mentioned that this study population did not have a homogenetic background, and haplotypes were not studied. Validation or replication of a study finding is essential to understand its significance and to sort true-positive from false-positive associations. Therefore, previous associations between

MBL2 polymorphisms and RTI need to be replicated in a large independent population. To establish the actual role played by *MBL2* polymorphisms in the susceptibility to RTI in white children, we studied the association between haplotypes of *MBL2* and the occurrence of RTI in a population-based birth cohort of children of Dutch ancestry.

METHODS

Study population

Children were participants in the Prevention and Incidence of Asthma and Mite Allergy (PIAMA) birth cohort study, originally designed to investigate atopy and asthma. Details of the study design have been published elsewhere [20]. Recruitment took place in 1996 and 1997. A screening questionnaire on maternal allergy was completed by 10,232 pregnant women visiting 1 of 52 prenatal clinics in The Netherlands [21]. On the basis of this screening, 7862 women were invited to participate, and 4146 agreed and gave informed consent. After birth, the baseline study population consisted of 3963 children. Questionnaires for parental completion were sent to the parents during pregnancy, when the children reached the ages of 3 and 12 months, and yearly thereafter. DNA was successfully collected from 1037 children. Of these children, 987 were of Dutch ancestry and were used for analysis; the 50 children of non-Dutch origin were excluded. The frequency of RTI among the children was included on the questionnaire, first at 1 year of age and then annually until 4 years of age. Data on genotypes and the frequency of RTI were complete in 896 Dutch children. The general characteristics of the study population were comparable between participants and non-participants in the genetic study, apart from allergy in the mother (66% for participants vs. 20% for non-participants). Other variables were also investigated and found to be approximately equally distributed between participants and non-participants: maternal smoking during pregnancy, duration of pregnancy, birth weight, sex, breast-feeding for ≥12 weeks, day care, environmental tobacco smoke exposure, and existence of siblings (data not shown). The study was approved by the institutional review board, and parents gave informed consent.

Polymorphisms in the MBL2 gene

Genomic DNA was extracted from buccal swabs or blood by performing chloroform–2-propanol extraction. DNA was amplified using REPLI-g UltraFast technology (Qiagen). SNPs of *MBL2* were selected on the basis of known functionality (n=7) combined with haplotype-tagging SNPs that have a minor allele frequency of >0.1 (n=6), selected from the publicly available database of the International HapMap Project [22]. The studied SNPs are listed in Table 1. Genotyping was performed by competitive allele-specific polymerase chain reaction using KASPar genotyping chemistry, performed under contract by

Table 1. MBL2 single-nucleotide polymorphisms (SNPs) studied in the Prevention and Incidence of Asthma and Mite Allergy study cohort

dbSNP identifier	SNP	Allele names	SNP type	MAF
rs11003125	-619G>C	H/L	Proximal promoter, Ht	0.370
rs7096206	-290G>C	Y/X	Proximal promoter	0.232
rs7095891	-66C>T	P/Q	5'-UTR	0.214
rs5030737	154C>T, Arg52Cys	D	Exon 1, codon 52	0.066
rs1800450	161G>A, Gly54Asp	B	Exon1, codon 54, Ht	0.135
rs1800451	170G>A, Gly57Glu	C	Exon 1, codon 57	0.014
rs12246310	297C>T		Ht	0.214
rs10824793	1916A>G		Ht	0.449
rs1838066	2071A>G		Ht	0.374
rs1838065	2139A>G		Ht	0.376
rs930507	3130G>C		Exon 4, Ht	0.185
rs10824792	5190C>T		Ht	0.417
rs2120132	5356C>T		Ht	0.250

NOTE. Ht, haplotype tagging; MAF, minor allele frequency (determined in a study population of Dutch white ethnicity); UTR, untranslated region.

KBiosciences (http://www.kbiosciences.co.uk/). Previous functional analyses by Madsen et al. [5] and others, including Wiertsema et al. [4], demonstrated that the YA/O genotype has intermediate levels of functional MBL multimers. The YA/O genotype has been reported to be functionally different from YA/A, and was recently reported to be beneficial for improved outcomes of severe infections [23]. Thus, exon 1 SNPs were combined with the promoter polymorphism Y/X to define high-producing (A/A), intermediate-producing (YA/O), and deficient (XA/O and O/O) *MBL2* genotypes.

Frequency of RTI

Information on the frequency of RTI in children 1–4 years of age was collected from annual parental questionnaires using the following question: "How often did your child have serious respiratory tract and/or ear-nose-throat infections, such as flu, infection of the throat, middle ear, or sinuses, bronchitis or pneumonia, during the last 12 months?" Four answers were possible: never, 1–2 times, 3–5 times, and ≥6 times. On the basis of the answers given on the 4 consecutive annual questionnaires, 3 groups were defined according to the reported frequency of RTI. We defined frequent RTI as ≥3 RTI per year reported on 3 or 4 annual questionnaires (group 1). Group 2 consisted of children without any reported RTI during the 4 years. Group 3 consisted of the remaining children, who had a low to moderate frequency of RTI (1 or more RTI reported on the 4 annual questionnaires but not frequent RTI according to the study definition). The periods of

0–12 months and 13–24 months of life were also analyzed separately, because an effect of *MBL2* polymorphisms has been shown in these age groups particularly [4,13]. Frequencies of *MBL2* genotypes in children with 3 or more reported RTI during the first year of life were compared with those in children with <3 infections during the same year. The same analyses were done for the second year of life.

Statistical analyses

The genotype data were analyzed for deviations from Hardy-Weinberg equilibrium by means of the χ^2. Haplotypes were estimated using PHASE software (version 2.1; available at http://stephenslab.uchicago .edu/software.html). Differences in haplotype and genotype frequencies (with 95% confidence intervals [CIs]) between children with frequent RTI and those without reported RTI were calculated. *MBL2* haplotypes were finally divided into those associated with high (LYPA), intermediate (HYPA, LYQA, and LXPA-C), or low (LXPA-G, HYPD, LYPB, and LYQC) expression of MBL, to increase power. Crude odds ratios (ORs) with 95% CIs were estimated by means of χ^2 statistics for the association between deficient *MBL2* genotypes and frequent RTI versus no reported RTI. Finally, all children were included in ordinal regression analyses (per year and for years 1– 4 cumulatively), to estimate the association between *MBL2* haplotypes, deficient *MBL2* genotypes, and categories of annual RTI frequency. Analyses were performed with SPSS software (version 12.0.2; SPSS). $P<0.05$ was considered to indicate statistical significance.

RESULTS

Of the 896 children in our final study population with complete information on genotypes and annual RTI rates, 55 (6%) fulfilled the criteria for frequent RTI during the first 4 years of life, according to our definition (group 1). A total of 130 children (15%) did not have any reported RTI in these years (group 2), and 711 children (79%) had a low to moderate frequency of reported RTI (group 3). RTI rates were similar in the 50 children of non-Dutch origin who were excluded (data not shown). Minor allele frequencies of the 13 determined polymorphisms of *MBL2* were similar to those previously reported in white Dutch subjects (Table 1) [4,24]. All SNPs adhered to Hardy-Weinberg expectations ($p<0.05$). Data for both parents were available for 193 children. These data were analyzed for inheritance patterns, using family-based association tests, which showed inheritance errors (supposedly caused by genotyping errors) in <1% of the cases.

MBL2 haplotype associations

PHASE 2.1 software constructed 34 haplotypes from the genotype data. Strong linkage disequilibrium led to 7 well-known haplotypes based on *MBL2* exon 1 (A or B/C/D) and H/L, Y/X, and P/Q promoter SNPs: HYPA, LYPA, LYQA, LXPA, HYPD, LYPB, and LYQC (Table 2). The LXPA haplotype can further be divided on the basis of the 3130G>C polymorphism in exon 4, resulting in 8 haplotypes that have been associated with functional MBL serum levels [4,5]. Two haplotypes that have not been described previously were found at a low prevalence (LXPB, n=2; HYQA, n=2) and were excluded in further analyses. The observed haplotype frequencies with corresponding predicted MBL serum levels are listed in Table 2; they are in agreement with previous findings in Dutch studies [4,24].

No significant difference in haplotype frequencies was observed between the 2 extreme groups, that is, groups 1 and 2 (Table 2). The differences in the percentages varied from -5 (95% CI, -14 to 4) for LYQA to 3 (95% CI, -4 to 10) for LYPB. Finally, analyses of 3 *MBL2* haplotype groups (group 1 associated with high, group 2 with intermediate, and group 3 with low expression of MBL) showed similar results (data not shown).

Table 2. Percentages of children with different haplotypes of MBL2, by frequency of questionnaire-reported respiratory tract infections (RTI) during years 1– 4

		No. (%)								
Haplo Type	Predicted MBL serum level*	All children † (n=896/1792)		No RTI (n=130/260)		Moderate frequency of RTI (n=711/1422)		Frequent RTI‡ (n=55/110)		Difference in percentage§ (95% CI)
LYPA	High	73	4	9	3	58	4	6	5	-2 (-7;3)
HYPA	Intermediate	554	31	90	35	428	30	36	33	2 (-9;13)
LYQA	Intermediate	358	20	47	18	286	20	25	23	-5 (-14;4)
LXPA**										
C allel	Intermediate	325	18	48	18	256	18	21	19	-1 (-10;8)
G allel	Low	96	5	14	5	76	5	6	5	0 (-5;5)
HYPD	Low	114	6	18	7	91	6	5	5	2 (-3;7)
LYPB	Low	249	14	33	13	205	14	11	10	3 (-4;10)
LYQC	Very low	23	1	1	0	22	2	0	0	0 (0;1)

NOTE. Indicated n values are no. of children/no. of haplotypes. CI, confidence interval; MBL, mannan-binding lectin.

*Predicted MBL level according to Madsen et al. [5] and Wiertsema et al. [4]; †Excluded were children with an ethnicity other than Dutch (n=50), children with incorrect haplotypes (n=2), and children without complete RTI information; ‡Frequent RTI were defined as 3 or more RTI per year in 3 or 4 years during the first 4 years of life; §Difference between children with frequent RTI and children with no RTI; **Haplotype LXPA is further divided on the basis of the 3130G3>C polymorphism in exon 4 of *MBL2* [4].

MBL2 genotype associations

Considering genotypes of exon 1, 547 children (61%) were homozygous for the wild-type A allele (genotype A/A), 37 (4%) were homozygous for variant alleles (genotype O/O), and 312 (35%) were heterozygous (genotype A/O) (Table 3). Prevalences of exon 1 genotypes in children with frequent RTI did not differ from those in children with no reported RTI during years 1–4. The differences in the percentages were -10 (95% CI, -24 to 4), 7 (95% CI, 6 to 20), and 1 (95% CI, -5 to 7) for A/A, A/O, and O/O, respectively. We also combined exon 1 polymorphisms with the Y/X promoter polymorphism to define high-producing (A/A), intermediate-producing (YA/O), and deficient (XA/O and O/O) *MBL2* genotypes. One hundred thirty-two children (15%) had deficient *MBL2* genotypes, 216 (24%) had intermediate-producing *MBL2* genotypes, and 548 (61%) had genotypes related to high MBL serum levels (Table 3). Children with deficient *MBL2* genotypes had a risk of frequent RTI during the first 4 years of life (OR, 0.71 [95% CI, 0.25 to 2.05]) similar to that in children with sufficient *MBL2* (high-producing plus intermediate-producing *MBL2*) genotypes. Moreover, no differences existed between the children with frequent infections and those with no reported infections in the frequencies of high-producing, intermediate-producing, or deficient *MBL2* genotypes. In addition, similar analyses were

Table 3. Association between MBL2 genotypes and frequency of reported respiratory tract infections (RTI) during years 1– 4

	No. (%)								
	All children*		No RTI		Moderate frequency of RTI		Frequent RTI†		Difference in percentage‡ (95% CI)
Exon 1 polymorphisms									
A/A	547	61	85	65	421	59	41	75	-10 (-24;4)
A/O	312	35	38	29	262	37	12	22	7 (-6;20)
O/O	37	4	7	5	28	4	2	4	1 (-5;7)
Promoter alleles included§									
High-MBL	548	61	85	65	422	59	41	75	-10 (-24;4)
Intermediate-MBL	216	24	29	22	178	25	9	16	6 (-6:18)
Deficient-MBL	132	15	16	12	111	16	5	9	3 (-6;12)
All	896	100	130	100	711	100	55	100	

NOTE. The odds ratio for frequent RTI comparing deficient *MBL2* genotypes with intermediate-producing plus high-producing *MBL2* genotypes is 0.71 (95% CI, 0.25 to 2.05), estimated on the basis of χ^2 statistics. CI, confidence interval; MBL, mannan-binding lectin.

*Excluded were children with an ethnicity other than Dutch (n=50), children with incorrect haplotypes (n=2), and children without complete RTI information; †Frequent RTI were defined as 3 or more RTI per year in 3 or 4 years during the first 4 years of life; ‡Difference between children with frequent RTI and children with no RTI; §High-producing *MBL2* genotype, A/A; intermediate-producing *MBL2* genotype, YA/O; deficient *MBL2* genotype, XA/O plus O/O.

performed for years 1 and 2 separately (data not shown). Even during this particularly vulnerable period of time, no association was found between deficient *MBL2* genotypes and susceptibility to frequent RTI (OR for year 1, 0.84 [95% CI, 0.50 to 1.41]; OR for year 2, 1.05 [95% CI, 0.61 to 1.80]).

Ordinal regression analyses

Finally, ordinal regression analyses, including all children per year and for years 1–4 cumulatively, showed that *MBL2* haplotypes and deficient *MBL2* genotypes were not associated with categories of the annually reported RTI frequencies (data not shown).

DISCUSSION

In this large, prospective, population-based birth cohort study, we found that Dutch children with deficient *MBL2* polymorphisms did not have an increased risk of questionnaire-reported RTI during their preschool years. In contrast to 2 other studies, we could not confirm an effect of variant *MBL2* genotypes on susceptibility to RTI within year 1 or 2 of life [4,13].

The first contrasting study was performed by Koch et al. [13] in Greenland. This well-designed study (n=252) was a prospective clinical study that estimated the number of days at risk of acute respiratory infections (equivalent to RTI) and recorded the number of acute respiratory infection episodes weekly during a 2-year period, with data gathered from 6 weeks after birth. An increased risk for acute RTI was found in MBL-insufficient children (determined on the basis of exon 1 and promoter polymorphisms) compared with MBL-sufficient children, but the risk association was largely restricted to children aged 6–17 months. There could be several reasons for the conflict in results between this study and ours. First, there are differences in the genetic background of the study populations, because the Greenlandic study included mainly Eskimo children (80%) and our study included only white children. RTI are known to occur earlier and more frequently among Eskimo children [25]. The specific genetic background and an a priori high risk of RTI make the results of Koch et al. hard to generalize to white children. Second, RTI were measured differently in both studies. We used questionnaire data to assess RTI, whereas Koch et al. used exact measures of numbers of RTI episodes assessed by clinical examination. Third, we analyzed haplotypes in relation to 3 *MBL2* genotype groups (high producing, intermediate producing, and deficient) based on exon 1 and promoter SNPs, whereas Koch et al. used 2 groups (MBL sufficient and MBL insufficient) based on the same polymorphisms. Finally, we analyzed years 1 and 2 of life separately, but further determination of the vulnerable period, as done by Koch et al., was impossible with our annual data.

The second study showing an effect in a young age group was performed in The Netherlands by Wiertsema et al. [4]. This study included children (n=383) seen in the hospital with a history of 2 or more previous episodes of acute otitis media (AOM). A positive association was found with recurrent AOM in children 12–24 months of age (n=113) but not in older children (n=131). That the latter study involved a cohort of children with a history of AOM, whereas we studied a birth cohort representing the general population, might explain the discrepancy between the results.

Several other studies have also shown an association between RTI and variant *MBL2* genotypes [11,12,14,15], but this association was not verified by others [16 –19]. All of the studies that have shown a relationship have been hospital-based case-control studies, conducted in children with recurrent RTI [14], children who had undergone adenoidectomy and/or tonsillectomy because of recurrent upper RTI [15], or children with suspected immunodeficiency [11]. This phenomenon might be explained by a possible association between MBL deficiency and the severity of infectious diseases, although the supposition that MBL deficiency affects the severity of RTI has not been accurately tested.

The results of our study are in line with the results of the recent study by Müller et al. [19] in a large German birth cohort, which was also used for studying allergy (Multicenter Atopy Study). This study (n=749) did not find an association between *MBL2* polymorphisms and risk of RTI. In addition to the exon 1 and promoter polymorphisms that were studied by Müller et al, in our study we included analyses with haplotypes of *MBL2* and followed up a larger homogenous study population for a longer period of time (n=896 at the 4-year vs. n=749 at the 1-year follow-up time point).

Finally, unlike some previous investigations, we addressed RTI as a whole. The spectrum of infections in our study was wide: from otitis to pneumonia and bronchitis. The effect of *MBL2* mutations may differ for different clinical manifestations or etiological agents. In a Dutch study, rhinovirus was the most common respiratory tract pathogen (24%), followed by influenza virus type A (11%) and coronavirus (7%) [26]. Binding of MBL to influenza A virus involves recognition of oligosaccharides of the viral proteins hemagglutinin and neuraminidase [27]. MBL does not appear to recognize any specific rhinovirus structure; therefore, MBL deficiency may not have altered the incidence of RTI in our study if this pathogen was present in a large number of subjects.

The major strengths of our study are its large sample size, prospectively repeated questionnaires, and population-based sample. Ordinal regression analyses enabled us to study all children as well as to study only the extreme groups; both analyses showed similar results. *MBL2* genotyping was performed according to recent standards. Exon 1 and promoter polymorphisms were included and were combined to create genotypes related to high, intermediate, and deficient MBL serum levels. Moreover, haplotypes were created on the basis of haplotype-tagging polymorphisms.

To appreciate our results, it is also necessary to consider some potential limitations. First, the PIAMA study was originally designed to study atopy and asthma. Exact numbers of RTI episodes, as analyzed by Koch et al. [13], were not recorded. Instead, we grouped the children according to frequency of RTI, as reported in annual questionnaires. Strictly speaking, RTI in our study indicates the presence of respiratory symptoms that the parents considered more serious than a common cold. This may lead to misclassification in the reported frequency of RTI, but the misclassification can be supposed to be nondifferential (i.e., to be independent of *MBL2* genotype status). To study the validity of our definitions, we compared the questionnaire data on the reported frequency of RTI with data on the prescription of antibiotics and adenoidectomy and tonsillectomy. Antibiotic prescription was highly associated ($p<0.01$) with frequent RTI, as defined by the questionnaires, and a similar association was seen with adenoidectomy and tonsillectomy. Furthermore, as with the frequency of RTI, we observed no association of *MBL2* genotype status with ear, nose, and throat surgery or antibiotic prescription rates.

Second, the overall response rate of the study was moderate (53%), and the study population may not be representative of the general population of newborns in the study areas. In studies of this kind, it is important that losses to follow-up be minimal. In the recruitment phase, it was made explicit to the prospective participants that a long-term commitment was necessary, which may have dampened enthusiasm to participate [20]. Fortunately, loss to follow-up has been relatively small, and we maintain that valid conclusions can still be reached despite the moderate magnitude of response.

Third, selection bias may have occurred if the association between *MBL2* polymorphisms and frequent RTI differed between the children included in this part of the study and those who were excluded from analysis because of missing information. Among the children who were not included, a higher percentage had non-allergic mothers. This difference is caused by the original design of the PIAMA study, not by selective dropout [20]. Nevertheless, we separated the children by maternal allergy and confirmed the lack of association between *MBL2* polymorphisms and frequent RTI in both groups (data not shown). Moreover, selection bias is unlikely, because the *MBL2* genotype distribution we found is similar to that in other studies involving white Dutch subjects and conforms to Hardy-Weinberg equilibrium. Finally, we performed a power analysis to strengthen our negative findings. In this calculation, we assumed an allele frequency of 0.232 for the high-risk allele (Y/X promoter SNP) and a prevalence of 6.1% for frequent RTI, on the basis of on our own data. The Genetic Power Calculator [28] showed that our number of cases (n=55) was sufficient to exclude genotype-relative risks of 2 and 2.5, respectively, for heterozygotes and homozygotes for the high-risk allele (power of >80% to reject the null hypothesis at $\alpha=0.05$). Genotypic risks of this size are in line with the findings of Koch et al. [13], and we consider relative risks of this size to be clinically relevant. Therefore, our study population seems sufficient to exclude clinically relevant effects of *MBL2*

polymorphisms on susceptibility to RTI. We do not rule out the possibility that *MBL2* polymorphisms may contribute to the severity of infections or, in specific circumstances of impaired immunity, to the complex genetic control of protective immunity to infection. Nevertheless, in most circumstances other components of the immune system may compensate for the lack of MBL.

In conclusion, this population-based birth cohort study in white children shows that *MBL2* polymorphisms known to result in low serum levels of MBL do not seem to pose an increased risk of RTI during the first 4 years of life, as indicated by parental questionnaires. The effect of these polymorphisms was also absent in children <2 years of age. Thus, MBL deficiency seems to be of no clinical significance for the risk of RTI in white children at a population level.

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Chapter 6

Exploring the role of polymorphisms in ficolin genes in respiratory tract infections in children

Jopje M. Ruskamp
Maarten O. Hoekstra
Dirkje S. Postma
Marjan Kerkhof
Renske W. Bottema
Gerard H. Koppelman
Maroeska M. Rovers
Alet H. Wijga
Johan C. de Jongste
Bert Brunekreef
Elisabeth A.M. Sanders

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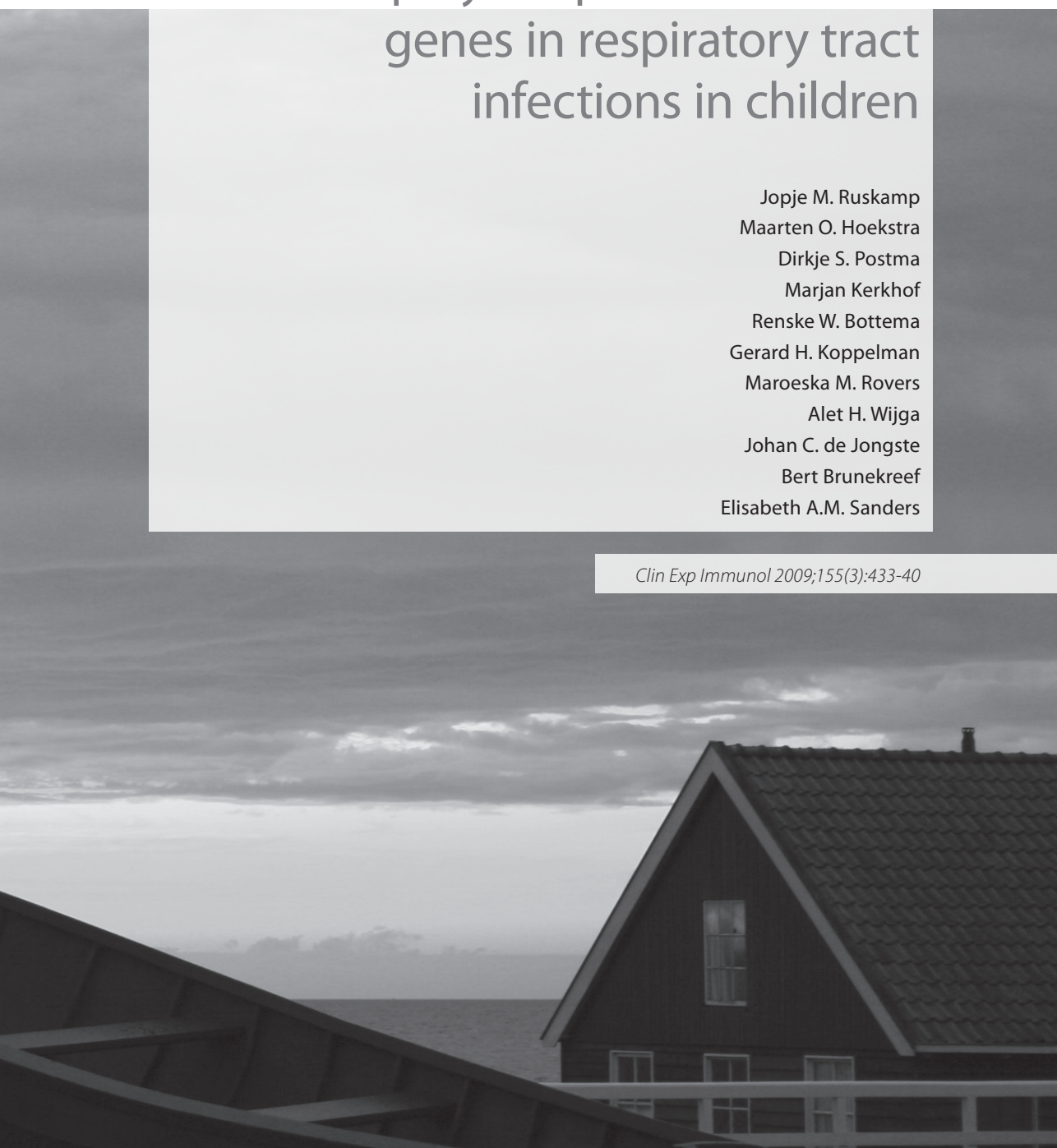


ABSTRACT

Ficolins are pattern-recognition molecules that appear to be relevant for innate immune defence against infections. The ficolin genes in Caucasians are polymorphic and genetic variations may have functional consequences, both in relation to function and concentration. Low levels of Ficolin-2 have been suggested to associate with recurrent respiratory tract infections (RTI), whereas data on Ficolin-3 are still very limited. We investigated the association between variation in genes encoding Ficolin-2 (*FCN2*) and Ficolin-3 (*FCN3*) and frequency of RTI during the first 4 years of life. The study population consisted of 900 children from a large, population-based birth cohort of Dutch children, followed prospectively from birth to 4 years of age. The number of RTI was assessed by annual parental questionnaires. Nine single nucleotide polymorphisms in *FCN2* and two in *FCN3*, all based on functionality or haplotype-tagging characteristics, were determined and haplotypes constructed. We found that single nucleotide polymorphisms in *FCN2* and *FCN3* were not associated with increased risk of RTI during the first 4 years of life. No difference existed between haplotype-frequencies of *FCN2* and *FCN3* in children grouped according to the reported number of RTI. In conclusion, at a population level, genetic variation in ficolin genes *FCN2* and *FCN3* do not seem to contribute to the risk of RTI in Caucasian children.

INTRODUCTION

Ficolins are pattern-recognition molecules that bind carbohydrates on the surface of microorganisms. Increasing evidence shows that Ficolin-2 (L-ficolin, encoded by *FCN2*) and Ficolin-3 (H-ficolin, encoded by *FCN3*) act to enhance phagocytosis and activate complement via the lectin pathway, similar to mannose-binding lectin (MBL) [1]. The *FCN2* gene is located on chromosome 9q34, and polymorphisms within this gene have been found to account for inter-individual variation in serum level and function of Ficolin-2 [2–4]. Currently, five common and functionally relevant polymorphisms within this gene have been described in healthy Danish Caucasian populations: three promoter polymorphisms (-986A>G, -602G>A and -4A>G) and two coding polymorphisms in exon 8 (Thr236Met and Ala258Ser) [2,4]. Whereas these five polymorphisms were found to be associated with high or low Ficolin-2 serum levels [3,4], the polymorphisms in exon 8 have also been shown to lead to increased (Ala258Ser) or decreased (Thr236Met) binding of Ficolin-2 to carbohydrates [5]. Ficolin-3 is encoded by the *FCN3* gene located on chromosome 1p36, but further knowledge on this type of ficolin is limited. Large variations in Ficolin-3 serum concentration have been observed in healthy individuals, which might be determined genetically. However, no common polymorphisms causing deficiency or lack of function in *FCN3* have been discovered so far [4,6,7]. Functionally, like Ficolin-2, Ficolin-3 has been shown to interact with the MBL-associated serine proteases enabling activation of the complement system [8,9]. Moreover, a recent comparative study found Ficolin-3 to have the highest complement activating capacity among the lectin pathway initiator molecules [10]. The exact ligands for Ficolin-3 are unknown, although distinct binding to certain strains of bacteria has been demonstrated [11].

The MBL deficiency has been associated with increased respiratory infectious susceptibility [12–15]. Similarly, low Ficolin-2 serum levels have been suggested previously to constitute a risk for recurrent respiratory tract infections (RTI) [16,17]. This might be explained by the fact that Ficolin-2, like MBL, can bind to bacteria such as *Streptococcus pneumoniae*, an important bacterial pathogen causing RTI [18]. To our knowledge, no association studies on the clinical relevance of low Ficolin-3 serum concentrations or any *FCN3* polymorphisms have been performed so far.

Similarities in function between ficolins and MBL and the evidence for a role of Ficolin-2 in the innate immune response to respiratory pathogens led us to hypothesize that polymorphisms and haplotypes of *FCN2* and *FCN3* might be associated with susceptibility to RTI. This was explored in a large population-based birth cohort of children with Dutch ancestry followed prospectively from birth to 4 years. Secondly, this cohort represents the largest (Caucasian) population studied for genetic variations in *FCN2* and *FCN3* so far.

METHODS

Study population

Children participated in the Prevention and Incidence of Asthma and Mite Allergy (PIAMA) birth cohort study, designed originally to investigate atopy and asthma. Details of the study design have been published previously [19]. Recruitment took place in the years 1996 and 1997. A screening questionnaire was completed by 10 232 pregnant women visiting one of 52 prenatal clinics in the Netherlands [20]. Based on this screening, 7862 women were invited, and 4146 agreed and gave informed consent. After birth the baseline study population consisted of 3963 children. Questionnaires for parental completion, based partly on the International Study of Asthma and Allergies in Childhood core questionnaires, were sent to the parents during pregnancy, at the child's ages of 3 and 12 months, and yearly thereafter to the age of 4 years [21]. DNA was collected successfully from 1037 children. From these children, 987 were of Dutch ancestry and used for analysis. Fifty children of non-Dutch origin were excluded from the current analysis to avoid effects resulting from population stratification. Reportage of frequency of RTI of the children was part of the questionnaire first at 1 year of age and continued annually to 4 years of age. Complete data on frequency of RTI from years 1 to 4 and DNA were available in 900 children of Dutch Caucasian origin.

General characteristics of the study population were comparable between participants and non-participants in the genetic study, apart from allergy in the mother (Table 1).

Other variables were also investigated and found to be distributed approximately equally between participants and non-participants: maternal smoking during preg-

Table 1. General characteristics of the study population

	DNA available (n=987)*	
	n	%
Allergic mother†	651	66
Maternal smoking during pregnancy (> 4 weeks from start)	138	14
Breastfeeding ≥ 12 weeks	537	55
Environmental tobacco smoke exposure‡	243	25
Frequency of RTI§		
Frequent**	55	6
Moderate	715	79
No RTI	130	14
Missing	87	
Dutch Caucasian	987	100

*N=987, DNA available and Dutch Caucasian ethnicity; †Allergic mother according to validated questionnaire; ‡At 1 year of age; §RTI: respiratory tract infections, frequency of RTI based on annual parental questionnaires from 1-4 years; **Frequent RTI: 3 or more RTI per year in 3 or 4 years during first 4 years of life.

nancy, duration of pregnancy, birth weight, gender, breastfeeding for 12 weeks or more, day care, environmental tobacco smoke exposure and presence of siblings (not shown).

In line with Atkinson et al. [16], a subgroup analysis was performed in atopic children from the cohort (n=237, atopy defined as specific immunoglobulin E >0.7 IU/ml against at least one inhalation allergen and/or positive skin prick test to at least one allergen).

The study protocol was approved by the institutional review board of each participating institute and informed written consent was obtained from all participants.

Polymorphisms and haplotypes of FCN2 and FCN3

Genomic DNA was extracted from buccal swabs or blood by performing chloroform-2-propanol extraction. DNA was amplified by using REPLI-g UltraFast technology (Qiagen™). Single nucleotide polymorphisms (SNPs) of *FCN2* were selected based on known functionality (n=5), combined with haplotype-tagging SNPs with minor allele frequency (MAF) >0.1 (n=4) selected from the publicly available database of the International HapMap Project (International HapMap Consortium, 2005; www.hapmap. org). Because no functionally relevant SNPs of *FCN3* are currently known, only haplotype-tagging SNPs with a minor allele frequency MAF >0.1 of this gene (n=2) were selected. The studied SNPs are listed in Table 2.

Genotyping was performed by competitive allele-specific polymerase chain reaction using KASPar™ genotyping chemistry, performed under contract by K-Biosciences

Table 2. Single nucleotide polymorphisms (SNPs) in Ficolin-2 (*FCN2*) and Ficolin-3 (*FCN3*) studied in the Prevention and Incidence of Asthma and Mite Allergy cohort

Gene	dbSNP identifier	SNP location	SNP type	Minor allele	MAF*	HWE† P-value
FCN2	rs3124952	-986A>G	Promoter, Ht‡	G	0.485	0.68
	rs3124953	-602G>A	Promoter, Ht	A	0.208	0.60
	rs17514136	-4A>G	Promoter/exon 1	G	0.260	0.79
	rs3128626	888	Intron 1, Ht	T	0.291	0.29
	rs3128625	950	Intron 1, Ht	A	0.351	0.68
	rs7037264	2545	Intron 3, Ht	A	0.410	0.65
	rs7041446	3386	Intron 3, Ht	A	0.479	0.98
	rs17549193	6359C>T	Thr236Met Exon 8, coding	T	0.278	0.82
	rs7851696	6424G>T	Ala258Ser Exon 8, coding	T	0.122	0.91
FCN3	rs10794501	1861A>T	Intron 5, Ht	A	0.241	0.06
	rs4494157	4473A>C	Intron 7, Ht	A	0.309	0.79

* MAF: minor allele frequency (determined in study population of Dutch Caucasian ethnicity); †HWE: Hardy–Weinberg equilibrium; ‡Ht: haplotype-tagging SNP according to HapMap individuals of Northern European ancestry.

(http://www.kbioscience.co.uk/). The quality of genotype data was guaranteed by K-Biosciences standards. We verified the genotyping quality by another two steps: (1) inheritance of alleles between parents and children was checked using family-based association tests (FBAT; http://www.biostat.harvard.edu/~fbat/fbat.html); and (2) genotype data were analysed for deviations from Hardy–Weinberg equilibrium.

Previously, haplotypes of *FCN2* based on four functionally relevant SNPs have been related to serum levels of Ficolin-2 in a population of Danish blood donors [2]. We used one extra functionally relevant SNP to construct haplotypes, and serum levels of Ficolin-2 associated with these haplotypes were estimated based on previous data [2]. Schematic representation of SNPs and haplotypes of the *FCN2* and *FCN3* gene that were studied is visualized in Figures 1 and 2.

Respiratory tract infections

Information about frequency of RTI was collected from annual parental questionnaires from 1 to 4 years of age by the following question: 'How often did your child have serious respiratory tract and/or ear–nose–throat infections such as flu, infection of the throat, middle ear or sinuses, bronchitis or pneumonia during the last 12 months?'. Four answers were possible: 'never', '1–2 times', '3–5 times' or '≥6 times'. Based on the answers in the four annual consecutive questionnaires, three groups were defined according to the reported frequency of RTI. We defined frequent RTI as three or more RTI per year reported in three or four annual questionnaires (group 1). The second group consisted of children with a low to moderate frequency of RTI (one or more RTI reported in the four annual questionnaires but not according to the definition of frequent RTI). The third group consisted of the remaining children without any reported RTI in 4 years.

Statistical analyses

The genotype data were analysed for deviations from Hardy–Weinberg equilibrium with X^2 statistics ($p>0.050$). X^2 statistics were also used to compare genotype frequencies of the SNPs between children with frequent RTI and those without reported RTI. Haplotypes were estimated from unphased genotype data using the Bayasian statistical method in phase version 2.1 (http://www.stat.washington.edu/stephens/software.html). Percentage differences in haplotype and genotype frequencies (with corresponding 95% confidence intervals (CI)) between children with frequent RTI and those without reported RTI or those with moderate frequency of reported RTI were calculated.

All analyses were performed with spss statistical software version 12.0.2 (SPSS Inc., Chicago, IL, USA). A post hoc power calculation was performed using the Genetic Power Calculator (http://pngu.mgh.harvard.edu/~purcell/gpc/) [22].

RESULTS

Among the 900 children of our final study population with complete information on RTI, according to our definition 55 (6%) had frequent RTI during the first 4 years of life (group1), 715 children (79%) had a low to moderate reported frequency of RTI (group 2) and 130 (14%) did not have any reported RTI during this period (group 3).

Single locus analyses

All determined SNPs in *FCN2* and *FCN3* were common with the MAF >12% (Table 2). The MAF of the nine SNPs in *FCN2* were similar to those reported previously in Caucasians [2,4,5,23]. The MAF of the two SNPs in *FCN3* were comparable to reported frequencies in

Figure 1. Schematic representation of single nucleotide polymorphisms (SNPs) and haplotypes studied of the Ficolin-2 (*FCN2*) gene.

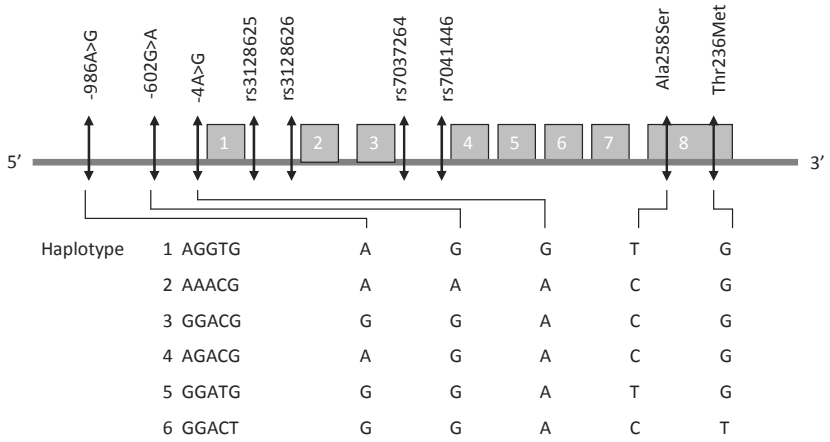
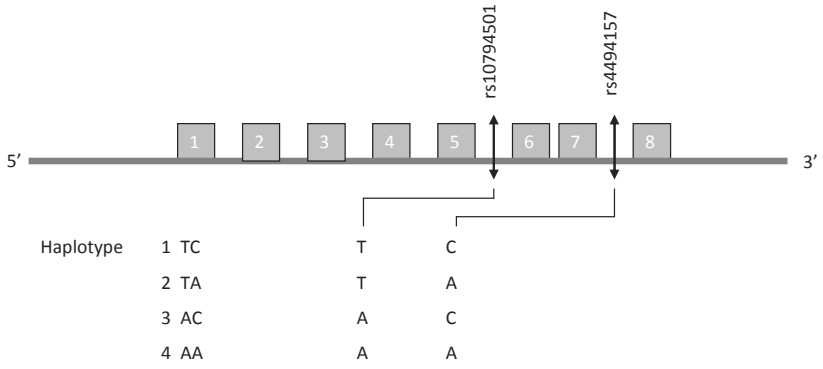


Figure 2. Schematic representation of single nucleotide polymorphisms (SNPs) and haplotypes studied of the Ficolin-3 (*FCN3*) gene.



a population of northern and western European ancestry (http://hapmap.org/hapmap-populations.html). All SNPs adhered to Hardy–Weinberg expectations (p>0.05). Data of both parents were available for 193 children. Analyses for inheritance patterns on these data by FBAT showed inheritance errors (caused supposedly by genotyping errors) in < 1% of the cases.

No significant difference in genotype frequencies of *FCN2* and *FCN3* was observed between the children with frequent RTI and those without RTI reported (p=0.25), nor with the group with intermediate number of infections reported (Table 3).

Table 3. Genotype frequencies of single nucleotide polymorphisms (SNPs) of Ficolin-2 (*FCN2*) and Ficolin-3 (*FCN3*) according to frequency of reported respiratory tract infections (RTI) in 1–4 years

		No RTI (n=130)		Moderate frequency of RTI (n=715)		Frequent RTI* (n=55)		PD†	95% CI‡	P-value**
FCN2 SNP	Geno type	n	%	n	%	n	%			
-986A>G	AA	35	28	157	22	12	23	5	-9;19	0.25
	AG	60	49	347	50	33	62	-13	-28;2	
	GG	28	23	195	28	8	15	8	-4;20	
-602G>A	GG	78	64	437	62	31	61	3	-12;18	0.89
	GA	41	34	229	33	19	37	-3	-18;12	
	AA	3	2	37	5	1	2	0	-4;4	
-4A>G	AA	71	58	371	54	30	57	1	-15;17	0.94
	AG	43	35	266	39	20	38	-3	-18;12	
	GG	8	7	46	7	3	6	1	-2;6	
Thr236Met	CC	54	53	286	52	24	51	2	-14;18	0.81
	CT	39	38	227	41	20	43	-5	-21;11	
	TT	9	9	41	7	3	6	3	-5;11	
Ala258Ser	GG	101	79	537	77	43	81	-2	-15;11	0.92
	GT	25	20	152	22	9	17	3	-9;15	
	TT	2	2	10	1	1	2	0	-4;4	
FCN3 SNP										
1861A>T	AA	72	60	388	56	27	49	11	-5;27	0.30
	AT	42	35	270	39	26	47	-12	-28;4	
	TT	6	5	30	4	2	4	1	-5;7	
4473A>C	AA	58	49	327	48	24	47	2	-14;18	0.98
	AC	48	40	291	43	21	41	-1	-16;14	
	CC	13	11	66	10	6	12	-1	-11;9	

*Frequent RTI: 3 or more RTI per year in 3 or 4 years during first 4 years of life; †PD: percentage difference between children with frequent RTI and children without RTI; ‡95% CI of percentage difference;
**P-values derived from 3 x 2 Chi-squared comparisons

Haplotype analyses

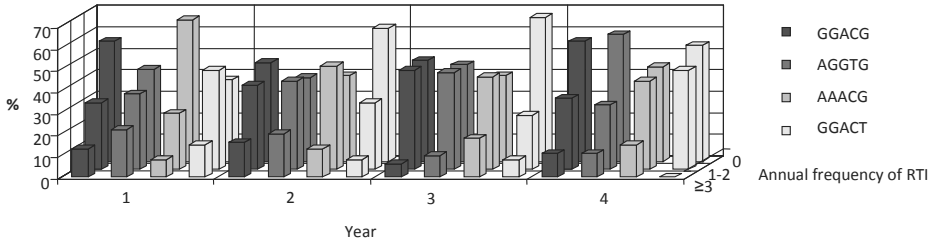
A total of 36 *FCN2* haplotypes was constructed from the genotype data. Linkage disequilibrium led to six haplotypes with a frequency of >1% based on the five well-known functionally relevant SNPs of *FCN2* (-986A>G, -602G>A, -4A>G, Thr236Met and Ala258Ser) (Table 4). Haplotype frequencies are in agreement with previous reported findings in Caucasian studies [2,23]. No significant difference in haplotype frequencies was observed between the groups, i.e. children with frequent RTI and those without RTI reported or the intermediate group. Percentage differences in prevalence of specific

Table 4. Estimated percentage of children with different haplotypes of Ficolin-2 (*FCN2*) based on five functionally relevant single nucleotide polymorphisms (SNPs)* within the groups of questionnaire reported frequency of respiratory tract infections (RTI) in 1–4 years

Haplotype	Ficolin-2 serum level pre diction†	No RTI (n=130/260) ‡		Moderate frequency of RTI (n=715/1430)		Frequent RTI** (n=55/110)		PD††	95% CI‡‡
		n	%	n	%	n	%		
AGGTG	High	59	22.7	370	25.9	27	24.5	-1.8	-11;8
AAACG	High	51	19.6	306	21.4	23	20.9	-1.3	-10;8
GGACG	Medium	98	37.7	473	33.1	44	40.0	-2.3	-13;9
AGACG	Medium	8	3.1	59	4.1	1	0.9	2.2	-1;5
GGATG	Medium	8	3.1	21	1.5	3	2.7	0.4	-3;4
GGACT	Low	30	11.5	177	12.4	12	10.9	0.6	-6;8
Total***		254	97.7	1406	98.3	110	100		

*Functionally relevant SNPs, -986A>G, -602G>A, -4A>G, Thr236Met and Ala258Ser; †Ficolin-2 serum concentration estimated on the basis of the article by Munthe-Fog [2]; high ≈ >6 µg/ml, medium ≈ 3-6 µg/ml, low ≈ <3 µg/ml; ‡Number of patients / number of haplotypes; **Frequent RTI: 3 or more RTI per year in 3 or 4 years during first 4 years of life; ††PD: percentage difference between children with frequent RTI and children without RTI; ‡‡95% CI: 95 % confidence interval of percentage difference; ***Total: number of haplotypes, only haplotypes with estimated frequency >1% are shown.

Figure 3. Percentage of children according to annual reported frequency of respiratory tract infections (RTI) (0, 1–2, ≥3) as derived from questionnaires in years 1, 2, 3 and 4 and stratified for estimated Ficolin-2 (*FCN2*) haplotype.



Only children homozygous for haplotypes with estimated frequency >5% are included in this analysis. The haplotypes are constructed from the five functionally relevant polymorphisms in *FCN2*: -986A > G, -602G>A, -4A>G, Thr236Met and Ala258Ser.

haplotypes between children with frequent RTI and without RTI varied between -2 (95% CI: -13; 9) for GGACG and 2 (95% CI: -1; 5) for AGACG (Table 4). The absence of an association between haplotypes of *FCN2* and percentages of children in all categories of frequencies of RTI reported in the annual questionnaires at age 1–4 years is visualized in Figure 3. The presence of the GGACT haplotype, known to result in low serum levels of Ficolin-2, was not related to a larger percentage of children in the higher categories of reported frequency of RTI (Figure 3).

Four *FCN3* haplotypes were found; the frequency of the homozygous wild-type haplotype was 47·5% in contrast to the homozygous variant haplotype, which was rare (1·8%) (Table 5). No significant difference in frequencies of haplotypes was observed between the children with frequent RTI and those without RTI reported or the intermediate group.

Percentage differences in the prevalence of the specific haplotypes varied between -7 (95% CI: -17; 2) for the heterozygous haplotype AC and 8 (95% CI: -3;19) for the homozygous wild-type haplotype TC (Table 5).

Atopic children

We performed a subgroup analysis in the 237 atopic children from our cohort. Results of these analyses were similar to those obtained in the group as a whole, i.e. *FCN2* and *FCN3* genotypes and haplotypes were not associated with susceptibility to RTI (not shown).

Table 5. Estimated percentage of children with different haplotypes of Ficolin-3 (*FCN3*) based on two haplotype tagging single nucleotide polymorphisms (SNPs) within the groups of questionnaire reported frequency of respiratory tract infections (RTI) in 1–4 years

Haplotype	No RTI (n=130/260)*		Moderate frequency of RTI (n=715/1430)		Frequent RTI† (n=55/110)		PD‡	95% CI**
	n	%	n	%	n	%		
TC	126	48.5	686	48.0	45	40.9	7.6	-3;19
TA	80	30.8	414	29.0	35	31.8	-1.0	-11;9
AC	50	19.2	300	21.0	29	26.4	-7.2	-17;2
AA	4	1.5	30	2.1	1	0.9	0.6	-2;3
Total	260	100	1430	100	110	100		

*Number of patients / number of haplotypes; †Frequent RTI: 3 or more RTI per year in 3 or 4 years during first 4 years of life; ‡PD: percentage difference between children with frequent RTI and children with no RTI; ** 95% CI of percentage difference.

DISCUSSION

This is the first large prospective population-based birth cohort study on the clinical significance of *FCN2* and *FCN3* polymorphisms with respect to susceptibility to RTI. We found that neither *FCN2* nor *FCN3* polymorphisms or their haplotypes were associated with reported frequency of RTI in Dutch Caucasian children during preschool years in the general population.

A previous study by Atkinson et al. suggested that Ficolin-2 levels might be lower in children with recurrent RTI compared with control children attending the hospital for reasons unconnected with infections or respiratory disease [16]. The association appeared to be most outspoken in a relatively small group of 90 children with allergic signs or symptoms (mainly asthma or allergic rhinitis and a few with elevated immunoglobulin E not further specified).

Based on the results from Atkinson et al., we performed a subgroup analysis in the atopic children from our cohort. In these analyses *FCN2* and *FCN3* genotypes and haplotypes were also not associated with susceptibility to RTI. This contrasts with the findings by Atkinson et al., but reinforces our results.

More recently, Chapman et al. showed that functionally relevant polymorphisms in the *FCN2* gene are not associated with invasive pneumococcal disease [23]. The results of this study are in line with ours, as *Streptococcus pneumoniae* is one of the most important bacterial pathogens in infection of the respiratory tract. The role of ficolins in innate immune defence against viral infections is still unknown, and the relevance of polymorphisms of *FCN2* or *FCN3* in susceptibility to other diseases has not yet been studied.

To our knowledge, we are the first to analyse SNPs in *FCN3* and find no association with RTI in childhood. These findings need replication in other cohorts, but our *FCN3* results point in the same direction as our observations on *FCN2*. Recently, we showed in the same birth cohort that *MBL2* polymorphisms, known to result in low serum levels of MBL, do not seem to pose an increased risk of RTI during the first 4 years of life as based on parental questionnaires [24].

The major strengths of our study are its large sample size, prospective follow-up, population-based character and homogeneous background of the study population. We studied all children next to studying only the extreme groups; both analyses showed similar results. Moreover, *FCN2* and *FCN3* genotyping was performed according to the highest standards and haplotypes were created based on functionally relevant or haplotype-tagging polymorphisms.

To appreciate our results, some potential limitations should also be considered. First, the PIAMA study was designed originally to study atopy and asthma. Exact measures of numbers of RTI episodes, as was conducted for the MBL study by Koch et al. [15], were

not recorded. Instead, we grouped the children according to the frequency of RTI, as reported in annual questionnaires. Moreover, we addressed RTI as a whole. The spectrum of infections in our study was wide: from otitis to pneumonia and bronchitis. The effect of *FCN* mutations may differ when observing different clinical manifestations or aetiological agents. Strictly speaking, in our study RTI means 'respiratory symptoms' which the parents considered to be more serious than a common cold. This may lead to misclassification of reported frequency of RTI, but the misclassification can be supposed to be non-differential (independent of FCN genotype status). To study the validity of our definitions, we compared the questionnaire data on reported frequency of RTI with data on prescription of antibiotics and adenoidectomy and tonsillectomy. Antibiotic prescription was associated highly ($p<0.01$) with frequent RTI as defined by the questionnaires, and a similar association was seen with adenoidectomy and tonsillectomy ($p<0.01$). Furthermore, similar to the frequency of RTI, no association of FCN genotypes status with ear, nose and throat surgery or antibiotic prescription rates was observed in our study. Secondly, we did not determine serum levels of Ficolin-2 and Ficolin-3 but used instead polymorphisms considered to be associated with serum levels [2]. This approach is used commonly in association studies, especially in studies with relatively large study populations [23–25]. Thirdly, selection bias may have occurred if the association between *FCN2* and *FCN3* polymorphisms and frequent RTI was different for the included children and those who were excluded from the analyses because of missing information. Among the children who were not included, there were higher percentages of children from non-allergic mothers (Table 1). This difference is caused by the original design of the PIAMA study, not by selective dropout [19]. Nevertheless, we separated the children by allergy and confirmed the lack of association between *FCN2* and *FCN3* polymorphisms and frequent RTI in both groups (results not shown). Moreover, selection bias is unlikely, as the genotype distribution of the ficolin genes we found is similar to other studies involving Dutch Caucasians and conforms to the Hardy–Weinberg equilibrium.

Finally, we performed a post hoc power analysis to strengthen our negative findings. In this calculation we assumed an allele frequency of 0.208 for the high-risk allele (-602G>A) and a prevalence of 6.1% for frequent RTI based on our own data. The Genetic Power Calculator showed that the number of cases (n=55) is sufficient to detect genotype relative risks of, respectively, 2 and 2.5 for heterozygotes and homozygotes for the high-risk allele (power >80% to reject the null hypothesis with an alpha of 0.050) [22]. Genotypic risks of this size were found for MBL by Koch et al. [15]. Moreover, we consider relative risks of this size to be clinically relevant. Therefore, our study population seems to be sufficient to elucidate clinically relevant effects of ficolin polymorphisms on susceptibility to RTI.

To our knowledge, we have explored for the first time the impact of *FCN2* and *FCN3* polymorphisms on susceptibility to RTI in a large birth cohort of Caucasian children.

Knowledge on the genetic control of serum levels and functionality of the ficolins is still emerging, and we expect more disease association studies to appear in the future [10]. We do not exclude that *FCN2* or *FCN3* polymorphisms may contribute, in specific circumstances of impaired immunity, to the complex genetic control of protective immunity to infection.

Nevertheless, our results suggest that in healthy children susceptibility to RTI is not influenced by polymorphisms in the ficolin genes.

In conclusion, this population-based birth cohort study shows that Caucasian children with *FCN2* polymorphisms, presumed to result in low serum levels of Ficolin-2, do not seem to have an increased risk of RTI during the first 4 years of life. Moreover, *FCN3* polymorphisms were also not associated with risk of RTI during these years. The results suggest that Ficolin-2 and Ficolin-3 may not be critical for host defence against pathogens causing RTI during preschool years in Caucasian children at a population level.

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Chapter 7

Polymorphisms in the TLR pathway and respiratory tract infections in children

Jopje M. Ruskamp
Maarten O. Hoekstra
Dirkje S. Postma
Marjan Kerkhof
Naomi E. Reijmerink
Gerard H. Koppelman
Charlotte Onland-Moret
Alet H. Wijga
Johan C. de Jongste
Bert Brunekreef
Elisabeth A. Sanders

Submitted

ABSTRACT

Variation in Toll-like receptors (TLR) genes, which play an important part in both innate and adaptive immunity, has been shown to be associated with various infectious diseases. The association between single nucleotide polymorphisms (SNPs) in genes of the TLR pathway and frequency of respiratory tract infections (RTI) was investigated in a large population-based birth cohort.

Frequency of RTI was prospectively assessed by annual parental questionnaires. Sixty three functionally relevant or haplotype-tagging SNPs in TLR genes (*TLR1-6, TLR9, TLR10, CD14, MYD88, TIRAP, IRAK1,* and *IRAK4*) were determined in 897 Dutch children and associated with frequency of RTI during the first 4 years of life.

Minor alleles of rs11536878 (*TLR4*) and rs851186 (*TLR5*) were associated with an increased risk of frequent RTI: relative risk (RR) respectively 2.69 (95% CI 1.31-5.50); and 3.31 (1.44-7.61). These associations showed a clear dose-response effect and they remained significant after correction for multiple testing.

SNPs in *TLR4* and *TLR5* of the TLR pathway were associated with questionnaire reported RTI during the first 4 years of life in this large population-based cohort of Dutch children.

INTRODUCTION

Toll-like receptors (TLRs) are key regulators of both innate and adaptive immunity [1]. They recognize a broad range of microbial molecular patterns, which triggers intracellular signals for activation of the immune response to infection [2]. Some TLRs are expressed on the surface of cells of the immune system (TLR1, TLR2, TLR4, TLR5, TLR6 and TLR10), whereas others are expressed on the membranes of intracellular organelles of phagocytes such as macrophages (TLR3, TLR7, TLR8 and TLR9)[1,2]. Various genetic variations in parts of the TLR pathway have been associated with altered susceptibility to infectious diseases [3,4]. In studies focusing on respiratory tract infections (RTI), associations have been described for polymorphisms in *TLR2* (tuberculosis and recurrent febrile bacterial respiratory infections) [5,6], *TLR4* (recurrent acute otitis media and severe RSV infection)[7-9] and *CD14* (recurrent acute otitis media)[10]. However, evidence for these associations has not been robust or consistent, most likely due to the fact that studies were small and mostly cross-sectional. Furthermore validation or replication of results is essential to understand its significance and to sort true-positive from false-positive associations. Therefore, replication of previous associations between TLR polymorphisms and RTI in a large independent population is needed.

We hypothesize that variations in genes encoding components of the TLR pathway are associated with susceptibility to RTI. This was explored in a large population-based birth cohort of children with Dutch ancestry prospectively followed from birth till the age of 4 years.

MATERIAL AND METHODS

Study population

Children participated in the PIAMA birth cohort study, originally designed to investigate atopy and asthma. Details of the study design have been published previously [11]. Recruitment took place in the years 1996 and 1997. A screening questionnaire on maternal allergy was filled in by 10 232 pregnant women visiting one of 52 prenatal clinics in The Netherlands [12]. Based on this screening, 7862 women were invited, and 4146 agreed and gave informed consent. The PIAMA study includes an 'intervention part', studying the effect of impermeable mattress covers in children from allergic women (n=855), and a 'natural history part', which consists of children from allergic (n=472) and non-allergic women (n=2819). After birth the baseline study population consisted of 3963 children. Questionnaires for parental completion were sent to the parents during pregnancy, at the child's ages of 3 and 12 months, and yearly thereafter. All children from allergic

mothers were asked for genetic research (n=1173), combined with a random sample of children from non-allergic mothers from the natural history part of the study (n=635). Permission for genetic research was given for 1060 (59%) children from the 1808 that were asked. Parents of nonparticipating children refused for various reasons (e.g. no permission for collecting blood sample, or lost to follow up).

General characteristics of the children that participated in the genetic studies (n=1060) were compared to the characteristics of the other children from the PIAMA cohort. Children that participated in the genetic studies were more likely to have an allergic mother based on the original design of the study (66 vs. 20%). Variables which were also investigated and found to be approximately equally distributed between participants and non-participants of the genetic study: breastfeeding for 12 weeks or more, presence of older siblings at 1 year of age, parental level of educational, exposure to environmental tobacco smoke both pre-and postnatal, birth weight, gender and day-care attendance (not shown). DNA was successfully collected from 1037 children. From these children, 987 were of Dutch ancestry and used for analysis. Questions of RTI frequency in the children were at first part of the questionnaire at the age of 1 year and continued annually till 4 years of age. Data on genotypes and frequency of RTI were available for 897 Dutch children (91%). From 90 of the 987 genotyped children of Dutch ancestry complete information on RTI frequency was lacking (9%).

The study was approved by the Institutional Review Board and parents gave written informed consent.

Outcome measure

Information about frequency of RTI was collected from annual parental questionnaires from 1 till 4 years of age by the following question: "How often did your child have serious respiratory tract and/or ear-nose-throat infections such as flu, infection of the throat, middle ear or sinuses, bronchitis or pneumonia during the last 12 months?" Four answers were possible: never, 1-2 times, 3-5 times or ≥ 6 times". Based on the answers in the four annual subsequent questionnaires, three groups were defined according to reported frequency of RTI. We defined frequent RTI as three or more RTI considered serious per year reported in three or four annual questionnaires (group 1). The second group consisted of children with a low to moderate frequency of RTI (one or more RTI reported in the four annual questionnaires but not according to the definition of frequent RTI). The third group consisted of the remaining children without any reported RTI in four years.

Genotyping

Genomic DNA was extracted from buccal swabs or blood using standard methods [13]. DNA was amplified by using REPLI-g UltraFast technology (Qiagen™). Thirteen candidate

genes in the TLR pathway were selected based on known biological activity: *TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR9, TLR10, CD14, MyD88, TIRAP, IRAK1* and *IRAK4* [2]. Within these genes haplotype-tagging (ht) single nucleotide polymorphisms (SNPs) with minor allele frequency (MAF) >0.10 and r2 <0.80 were selected with Haploview (Haploview 4.1; http://www.broad.mit.edu/mpg/haploview/) from the publicly available database of the International HapMap Project [14] or from the Innate Immunity website (http://.innateimmunity.net) [15]. SNPs with known functional relevance of previous associations were also included for genotyping, irrespective of their concordance with the described selection process (n=8). The 63 studied SNPs are listed in Table 1.

Genotyping was performed by Competitive Allele-Specific PCR using KASPar™ genotyping chemistry, performed under contract by K-Biosciences (http://www.kbiosciences.co.uk/) with extensive quality control measures, including check for HWE and inheritance checks [16]. Genotype data of both parents were available for 193 children. Analyses for inheritance patterns on these data by family-based association tests (FBAT) showed inheritance errors (supposedly caused by genotyping errors) in <1% of the cases. Only 4 boys were heterozygous for 2 SNPs of the X-chromosomal *IRAK1*. These boys were excluded from further analyses.

Statistical analyses

The genotype data were analyzed for deviations from Hardy-Weinberg equilibrium (p>0.05) with chi-square statistics. Three SNPs (rs3804099, rs10759931 and rs1461567) deviated from HWE (p<0.05); these SNPs were excluded from further analyses.

Genotype frequencies of the 60 remaining SNPs were compared between children with frequent versus no RTI and frequent versus moderate frequency of RTI by using chi-square statistics. Relative risks (RRs) with 95% confidence intervals (CI) were estimated for both a dominant and a recessive model. Adjustment for environmental confounders is not necessarily required in a genetic study since there is no plausible relationship of the TLR SNPs with those variables. In addition, because of the disproportionate representation of subgroups caused by the study design, the children were stratified by maternal allergy to investigate potential effect modification by this factor.

Pairwise linkage disequilibrium (LD) was assessed between single SNPs from our own data by using the r2 statistic as implemented in Haploview.

To account for multiple testing, we used the false discovery rate (FDR) according to Benjamini and Hochberg [17]. FDR q values were calculated as follows: the p values are modified by multiplying them by the number of tests performed and then dividing them by the rank order of each p value (where rank order 1 is assigned to the smallest p value). Rather than expressing the probability of a single false-positive result among all tests, the q value estimates the proportion of results declared interesting that are actually false. Although the use of FDR is well known in genetic association studies, no

Table 1. SNPs in TLR pathway studied in the PIAMA cohort

Gene	Location	dbSNP identifier	SNP type	HWE p value	MA	MAF
TLR1	4p14	rs5743557	Ht, promoter	0.38	T	0.20
		rs5743594	Ht, intron	0.91	T	0.17
		rs5743604	Ht, intron	0.85	C	0.26
		rs5743618	Ht, exon, nonsynonymous, Ser602Ile	0.59	T	0.31
TLR2	4q32	rs4696480	Ht, promoter	0.06	A	0.47
		rs1898830	Ht, promoter	0.67	G	0.36
		rs3804099	Ht, exon, synonymous, 199Asn	0.02	C	0.45
		rs3804100	Ht, exon, synonymous, 450Ser	0.58	C	0.07
TLR3	4q35	rs11721827	Ht	0.96	C	0.16
		rs13126816	Ht, intron	0.84	A	0.23
		rs3775291	Ht, exon, nonsynonymous, Leu412Phe	0.24	A	0.28
		rs3775292	Ht, intron	0.28	G	0.22
		rs3775296	Ht	0.68	T	0.19
		rs7657186	Ht, intron	0.96	A	0.23
		rs7668666	Ht, intron	0.89	A	0.26
TLR4	9q32-q33	rs10759931	Ht, promoter	0.004	A	0.41
		rs10759932	Ht, promoter	0.40	C	0.12
		rs2770150	Ht, promoter	0.46	C	0.29
		rs6478317	Ht, promoter	0.46	G	0.32
		rs11536878	Ht, intron	0.26	A	0.12
		rs1927911	Ht, intron	0.43	T	0.24
		rs4986790	Associated with severe RSV [9], exon, nonsynonymous, Asp299Gly	0.31	G	0.06
		rs4986791	Ht, associated with severe RSV [9], exon, nonsynonymous, Thr399Ile	0.59	T	0.06
		rs11536889	Ht, 3'UTR	0.55	C	0.14
TLR5	1q41-q42	rs2072493	Ht, exon, Asn592Ser	0.84	G	0.15
		rs5744105	Ht, promoter	0.93	G	0.49
		rs2241096	Ht, promoter	0.23	T	0.09
		rs5744109	Ht, promoter	0.91	C	0.09
		rs5744157	Ht, intron	0.28	G	0.11
		rs851186	Ht, exon	0.49	T	0.44
		rs5744168	Functional [22], exon, Arg392Stop	0.29	T	0.06
TLR6	4p14	rs5743810	Ht, exon, nonsynonymous, Ser249Pro	0.92	T	0.41
		rs1039559	Ht	0.33	C	0.47

Gene	Location	dbSNP identifier	SNP type	HWE p value	MA	MAF
		rs6531666	Ht, exon	0.61	C	0.30
		rs5743788	Ht, promoter	0.55	G	0.49
		rs5743798	Ht, promoter	0.58	T	0.25
TLR9	3p21.3	rs187084	Ht, promoter	0.67	C	0.43
		rs5743836	Ht, promoter, T-1237C, [24]	0.59	C	0.14
		rs352140	Ht, promoter, G2848A	0.63	C	0.44
TLR10	4p14	rs10856839	Ht, promoter	0.42	C	0.17
		rs11096956	Ht, exon	0.73	T	0.22
		rs11096957	Ht, exon Asn241His	0.86	C	0.38
		rs11466652	Ht, exon	0.35	G	0.15
		rs4129009	Ht, exon, Ile2322Val	0.50	G	0.19
		rs4274855	Ht, intron	0.48	A	0.20
CD14	5q31.1	rs2915863	Ht, promoter, A-1619G	0.46	C	0.40
		rs2569191	Ht, promoter	0.17	C	0.47
		rs5744455	Ht, promoter	0.23	T	0.23
		rs2569190	Ht, functional, associated with acute otitis media [10], promoter, C-159T	0.12	A	0.47
		rs2563298	Ht, 3'UTR	0.55	A	0.28
MYD88	3p22	rs7744	Ht	0.65	G	0.14
		rs6853	Ht	0.67	G	0.12
TIRAP	11q24.2	rs1893352	Ht	0.89	G	0.15
		rs1786704	Ht, promoter	0.50	C	0.20
		rs625413	Ht, promoter	0.33	T	0.21
		rs8177375	Ht, intron	0.12	G	0.13
		rs8177376	Ht, 3' UTR	0.10	G	0.25
IRAK1	Xq28	rs1059701	Ht, exon, synonymous	0.46	C	0.17
		rs1059703	Ht, exon, 453Ser	0.74	C	0.12
		rs2239673	Ht, exon	0.31	C	0.17
IRAK4	12q12	rs4251520	Ht	0.43	C	0.11
		rs4251513	Ht	0.30	G	0.50
		rs1461567	Ht, intron	0.03	T	0.26

NOTE: SNPs: Single nucleotide polymorphisms; HWE: Hardy-Weinberg equilibrium; MA: minor allele; MAF: minor allele frequency in study population of Dutch ethnicity; Ht: haplotype-tagging; rs3804099, rs10759931 and rs1461567 deviated from HWE and were excluded from further analyses; HWE tested in females only, since IRAK1 lies on X chromosome.

conventional q value threshold has been established to separate false discoveries from true discoveries. A low threshold of 0.05 is often used in genome-wide association studies, which do not use prior information to select candidate genes. In our candidate gene

study, we used a FDR threshold of 0.20, similar to a previous candidate gene study, and so should expect up to only 20% of the declared discoveries to be false [18].

Analyses were performed with SPSS 15.0 (SPSS Inc, Chicago, IL) or Episheet (spreadsheets for the analysis of epidemiologic data by Rothman, 2002; http://members.aol.com/krothman/episheet.xls).

RESULTS

Respiratory tract infections

Among the 897 children of our final study population with complete information on genotypes and annual RTI, 55 (6%) fulfilled the criteria of frequent RTI during the first four years of life according to our definition (group 1). A total of 712 children (79%) had a low to moderate frequency of reported RTI (group 2) and 130 children (14%) did not have any reported RTI in these years (group 3).

TLR genotypes and frequent RTI

Genotype frequencies and associations based on both a dominant and a recessive model of all SNPs in children with frequent, moderate frequency and no reported RTI are listed in supplementary Table 1, which can be found in the online version of this manuscript. In supplementary Table 1 it is shown that analyses based on a recessive model are not available for a substantial number of the SNPs, since small numbers lead to n=0 in one of the cells. Therefore, a dominant model was used in further analyses.

At first, genotype frequencies in children with most distinctive phenotypes were compared: the children with frequent versus no RTI reported. In these analyses, minor alleles of five SNPs were found to be associated with frequent RTI (dominant model, $p<0.05$, Table 2 and Table 3). Minor alleles of rs11536878 in *TLR4* and rs851186 in *TLR5* were associated with increased risk of frequent RTI: respectively relative risk (RR) 2.69 (95% CI 1.31-5.50); and 3.31 (1.44-7.61). Minor alleles of the known functional SNPs in *TLR4* (rs4986790 and rs4986791) and rs5744168 in *TLR5* were not associated with frequent RTI. Minor alleles of rs5743810 and rs1039559 in *TLR6* and rs5743836 in *TLR9* were associated with a decreased risk of frequent RTI: respectively RR 0.47 (0.24-0.91); 0.46 (0.24-0.91); and 0.44 (0.20-0.96). The first two results (rs11536878 (*TLR4*) and rs851186 (*TLR5*)) remained significant after correction for multiple testing (respectively FDR q values 0.19 and 0.19).

Furthermore, we compared genotype frequencies between children with frequent and moderate frequency of RTI. In these analyses the same direction of effects was found, although the RR were somewhat closer to 1 (Table 3 and supplementary Table 1), as would be expected with these less distinguished phenotypes.

Table 2. Genotype frequencies of SNPs associated with frequency of reported RTI in year 1-4 in children with frequent, moderate frequency and no reported RTI

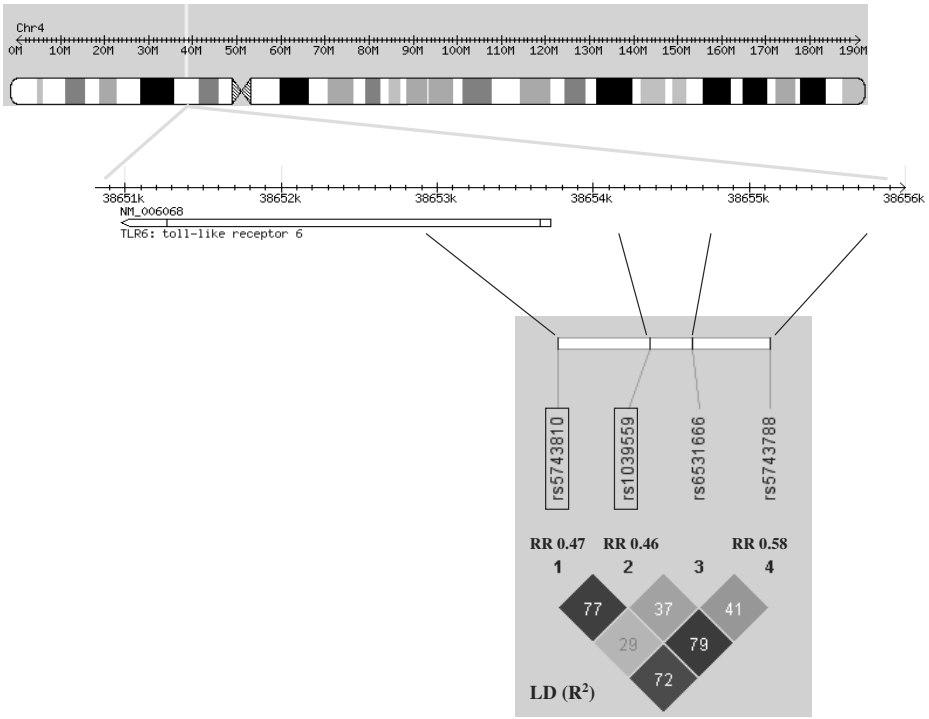
		Frequent RTI (n=55)	Moderate frequency of RTI (n=712)	No RTI (n=130)
		n (%)	n (%)	n (%)
TLR4				
rs11536878	CC	33 (62)	550 (79)	102 (82)
	CA	20 (38)	139 (20)	21 (17)
	AA	0 (0)	11 (2)	2 (2)
TLR5				
rs851186	GG	8 (15)	220 (32)	46 (37)
	GT	36 (67)	322 (47)	60 (48)
	TT	10 (19)	142 (21)	20 (16)
TLR6				
rs5743810	CC	26 (49)	236 (34)	38 (31)
	CT	19 (36)	342 (49)	68 (56)
	TT	8 (15)	115 (17)	16 (13)
rs1039559	TT	23 (43)	198 (29)	31 (26)
	TC	20 (37)	337 (49)	65 (54)
	CC	11 (20)	148 (22)	25 (21)
TLR9				
rs5743836	TT	44 (81)	524 (75)	85 (66)
	TC	10 (19)	161 (23)	41 (32)
	CC	0 (0)	10 (1)	3 (2)

Finally, we constructed LD plots based on our own data of the genes with SNPs that were associated with frequency of RTI (*TLR4, TLR5, TLR6* and *TLR9*). As can be seen in the LD plot of *TLR6*, rs5743810 and rs1039559 show LD with an r^2 of 0.77 (Figure 1). Minor alleles of these SNPs were both associated with a decreased risk of frequent RTI with comparable RRs (respectively 0.47 and 0.46), as would be expected with this LD. Similarly, r^2 between rs1039559 and rs5743788 was 0.79, with a RR for the minor allele of the latter of 0.58 (0.29-1.16). In the other genes (*TLR4, TLR5* and *TLR9*) the associated SNPs did not show LD with r^2>0.75 with other SNPs in these genes (not shown).

Maternal allergy

Among the 897 children of our final study population, 583 (65%) were born from allergic mothers and 314 (35%) from non-allergic mothers. In the analyses stratified for maternal allergy, minor alleles of four SNPs were found to be associated with frequent RTI only in the children from allergic mothers (dominant model, p<0.05, Table 4). In these children, the minor allele of rs11536878 in *TLR4* was associated with increased risk of frequent RTI: RR 3.80 (95% CI 1.62-8.88), whereas minor alleles of rs5743810 and rs1039559 in *TLR6* and

Figure 1. Genetic lay-out of *TLR6* with LD plot based on studied SNPs.



The shade of grey indicates the level of LD between the SNPs; the darkest blocks indicate highest LD, light grey blocks indicate low LD; RR: risk ratio based on dominant model (AB+BB versus AA) for frequent versus no RTI.

rs5743836 in *TLR9* were associated with a decreased risk of frequent RTI: respectively RR 0.37 (0.16-0.83); 0.38 (0.17-0.88); and 0.35 (0.13-0.93). However, interaction by maternal allergy as tested with logistic regression analyses in the complete study population, was only borderline significant for rs11536878 in *TLR4* (p interaction term=0.09) (Table 4).

DISCUSSION

In this large prospective population-based birth cohort study, we found that frequency of questionnaire reported RTI in Dutch children was associated with genetic variation within the TLR pathway. We found associations with a clear dose-response effect in *TLR4, TLR5, TLR6* and *TLR9*, the first two withstanding correction for multiple testing. Interestingly, these effects were specifically observed in children from allergic mothers.

Table 3. Associations between minor alleles of SNPs in TLR pathway and frequency of reported RTI* in year 1-4 (frequent versus no and frequent versus moderate frequency of RTI)

	RR† frequent versus no RTI	95% CI	p value	RR‡ frequent versus moderate frequency of RTI	95% CI	p value
TLR4						
rs11536878	2.69	1.31-5.50	0.01	2.22	1.24-3.98	0.01
TLR5						
rs851186	3.31	1.44-7.61	0.004	2.73	1.27-5.87	0.01
TLR6						
rs5743810	0.47	0.24-0.91	0.02	0.54	0.31-0.94	0.03
rs1039559	0.46	0.24-0.91	0.03	0.55	0.31-0.97	0.04
TLR9						
rs5743836	0.44	0.20-0.96	0.04	0.70	0.34-1.41	0.31

*RTI: respiratory tract infections; †RR: risk ratio based on dominant model (AB+BB versus AA) for frequent versus no RTI; ‡RR: risk ratio based on dominant model (AB+BB versus AA) for frequent versus moderate frequency of RTI.

Table 4. Associations between minor alleles of SNPs in TLR pathway and frequency of reported RTI in year 1-4 stratified by maternal allergy*

	Maternal allergy + (n=583) RR†	95% CI	p value	Maternal allergy – (n=314) RR‡	95% CI	p value	Interaction p value**
TLR4							
rs11536878	3.80	1.62-8.88	0.002	0.75	0.14-3.98	0.09	0.09
TLR5							
rs851186	2.20	0.89-5.41	0.08	n.a.	n.a.	n.a.	n.a.
TLR6							
rs5743810	0.37	0.16-0.83	0.01	0.67	0.20-2.23	0.51	0.43
rs1039559	0.38	0.17-0.88	0.02	0.62	0.18-2.14	0.45	0.52
TLR9							
rs5743836	0.35	0.13-0.93	0.03	0.75	0.21-2.76	0.67	0.35

*Maternal allergy: based on validated questionnaire according [12]; †RR: risk ratio based on dominant model (AB+BB versus AA) for frequent versus no RTI; ‡RR: risk ratio based on dominant model (AB+BB versus AA) for frequent versus moderate frequency of RTI; **Interaction term tested with logistic regression based on dominant model; ††N.a.: not available, caused by n=0 in one cell.

TLR genotypes and frequent RTI

Each TLR detects specific microbial components, although many TLR-ligand interactions still have to be discovered. We found a strong association for haplotype-tagging rs11536878 in *TLR4*. TLR4 has been implicated in signal transduction events induced by lipopolysaccharide (LPS) found in most gram-negative bacteria, including nontypeable

Haemophilus influenzae which is an important respiratory pathogen [19]. Other studies showed the importance of TLR4 in RSV infection and acute otitis media, although the effect of *TLR4* polymorphisms on the latter has to be confirmed by an independent study [7-9]. RSV is another important respiratory pathogen, with the capacity to cause severe RTI in young children. Our results seem to be in line with previous studies, although these studies focused on different infectious phenotypes and showed associations for the functional SNPs rs4986790 (Asp299Gly) and rs4986791 (Thr399Ile). These two functional SNPs were not associated with frequent RTI in our study. Moreover, they showed LD with r^2=0.89 in our data (not shown), but were not in LD with the associated SNP (rs11536878) we found in our study (r^2=0). Therefore, our TLR4 results can at the most be considered as loose replication of previous findings, both on the level of phenotype and genotype [20].

TLR5 recognizes bacterial flagellin, a potent stimulus present in the flagellar structure of many bacteria [21]. Interestingly, we found the minor allele of one haplotype-tagging SNP in *TLR5* (rs851186) to be associated with a strongly increased risk of frequent RTI. Previously, TLR5 has been implicated in the immune response to flagellated pathogens like *Legionella pneumophila* and *Pseudomonas aeruginosa* [22,23]. However, the functional SNP in *TLR5* (rs5744168) was not associated with frequent RTI in our study, which makes interpretation of the meaning of the observed association complex. Nevertheless, the observed association with the haplotype-tagging SNP showed a clear dose-response effect, which ensures us that this association should be considered valuable.

Furthermore, we found minor alleles of two linked haplotype-tagging SNPs in *TLR6* (rs5743810 and rs1039559) to be associated with a decreased risk of frequent RTI. The same was found for the promoter SNP rs5743836 in *TLR9*. This SNP was previously associated with asthma, although this association was observed with the other allele [24]. TLR6 functionally interacts with TLR2 to mediate the immune response to bacterial lipoproteins from mycoplasma, zymosan from fungi and lipoteichoic acid from group B streptococcus and staphylococcus [25,26]. TLR9 responds to unmethylated CpG dinucleotides in bacterial and viral DNA [27]. All these microorganisms are capable of inducing severe RTI. However, the associations with *TLR6* and *TLR9* were not significant after correction for multiple testing.

Previous reported associations for polymorphisms in *CD14* with recurrent acute otitis media and recurrent febrile bacterial respiratory infections in *TLR2* were not confirmed in our study [6,10]. These inconsistencies can be explained by the fact that we addressed RTI as a whole and the spectrum of infections in our study was wide: from otitis to pneumonia and bronchitis without a specific pathogen. The effect of a specific TLR mutation may differ when observing different clinical manifestations or etiological agents. However, our findings advocate the importance of the complex TLR pathway in the overall susceptibility to RTI. Since frequent RTI in our study are thought to be mostly

caused by viral pathogens, our results could suggest that TLR4, and possibly TLR5, TLR6 and TLR9, are part of the immune response to respiratory viruses, or that they interact with other TLR in their viral response. Another explanation may be that the associations we found are based on the influence on susceptibility to bacterial infections, which can be considered to be less frequent but possibly more severe than viral infections in our population [28]. This can specifically be true for the association in *TLR5*, since TLR5 is the receptor for bacterial flagellin and viruses do not contain flagellin.

Overall, the major findings of our study are with SNPs with unknown functional consequences. This makes interpretation of the meaning of the data complex. Further studies to assess the functional significance of the SNPs found to be associated with RTI frequency in our study would be the next logical step, but beyond the scope of this paper.

Maternal allergy

Maternal allergy, which is a well-recognized risk factor for allergic diseases, seems to modify the effect of variation in TLR genes on reported RTI. Effect modification by this factor, as "a proxy" for atopy in the child itself, was strongest for rs11536878 in *TLR4*. Interestingly, variation within this gene has also been associated with risk of asthma [29]. A possible explanation is that the respiratory symptoms in our study, which the parents considered to be caused by infection, are in fact symptoms of asthma, and that part of the effect of the associated SNPs on the risk of RTI is caused by their effect on the risk of asthma. Another possibility is that children from allergic mothers have airways that are more vulnerable to infections than airways of children from non-allergic mothers. Clearly, replication of all these findings in future studies is needed.

The major strengths of our study are its large sample size, prospectively repeated questionnaires and population-based character. We were able to study all children next to studying only the extreme groups; both analyses showed similar results.

To appreciate our results, some potential limitations should also be considered. First of all, the PIAMA study was originally designed to study atopy and asthma. Exact measures of numbers of RTI episodes were not recorded, nor were they restricted to physician diagnosed RTI. Instead we grouped the children according to frequency of RTI as reported in annual questionnaires. Strictly speaking, RTI in our study means "respiratory symptoms", which the parents considered to be more serious than a common cold. This may lead to misclassification of reported frequency of RTI, but the misclassification can be supposed to be non-differential (independent of genotype status). To study the validity of our definitions, we compared the questionnaire data on reported frequency of RTI with 4-year data on surgical interventions, adenoidectomy and/or tonsillectomy, and prescription of antibiotics. Adenoidectomy and/or tonsillectomy during the first 4

years of life was highly associated with frequent RTI as defined by the questionnaires (OR 15.04 with 95% CI 8.22-27.52), and a strong association was seen with antibiotic prescription in those years as well (OR 10.54 with 95% CI 5.14-21.59). Second, our genetic study population is enriched with maternal allergy, breastfeeding ≥ 12 weeks, high educated parents, absence of siblings and lack of exposure to tobacco smoke as compared to the general population and the original PIAMA birth cohort, which is important for generalization of the results [11]. Third, we studied mostly haplotype-tagging SNPs. Association analyses can typically identify disease-risk variants only when such causative variants are strongly associated with a tag SNP. Additionally, we tried to tackle the problem of multiple testing with the FDR method. Minor alleles of two SNPs remain significantly associated after this correction (rs11536878 in *TLR4* and rs851186 in *TLR5*) [18]. Nevertheless, in our opinion all reported associations are biologically plausible and should not be neglected. Moreover, the fact that effects in the same direction were seen when comparing less discriminating phenotypes ensures us that these associations should be considered interesting and valuable for further research.

Finally, we performed a power analysis to strengthen our findings with the Genetic Power Calculator [30]. In this calculation we assumed an allele frequency of 0.20 for the high-risk allele and a prevalence of 6.1% for frequent RTI based on our own data. The Genetic Power Calculator showed that our number of cases (n=55) is sufficient to detect genotype relative risks of 2.2 for the high risk allele based on a dominant model (power >80% to reject the null hypothesis with an alpha of 0.05). Genotypic risks of this size are in line with the RRs we found in our study and the reported results for rs4986790 (*TLR4*) and recurrent acute otitis media in the article by Emonts et al [7].

Taken together, our findings provide novel insights into the genetic basis for inter-subject differences in susceptibility to RTI in children. We conclude that at population level, polymorphisms in *TLR4* and *TLR5* of the TLR pathway are associated in a clear dose-response manner with questionnaire reported RTI in children during the first 4 years of life.

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Supplementary Table 1. Genotype frequencies and associations of SNPs in genes of TLR pathway in children with frequent, moderately frequent and no reported RTI.

		Frequent RTI (n=55)	Moderate frequency of RTI (n=712)	No RTI (n=130)	RR* frequent vs no RTI	95% CI		*p* value	RR† frequent vs no RTI	95% CI		*p* value	RR* frequent vs moderate frequency of RTI	95% CI		*p* value	RR† frequent vs moderate frequency of RTI	95% CI		*p* value
		n	n	n																
TLR1																				
rs5743557	CC	35	454	73	0.79	0.41	1.51	0.47	1.15	0.20	6.48	0.87	1.06	0.60	1.87	0.84	0.87	0.20	3.75	0.85
	CT	18	216	49																
	TT	2	29	4																
rs5743594	CC	37	468	96	1.46	0.73	2.91	0.28	2.38	0.33	17.32	0.38	0.96	0.54	1.72	0.89	0.99	0.23	4.27	0.98
	CT	16	211	30																
	TT	2	26	2																
rs5743604	TT	30	382	69	1.00	0.53	1.91	0.99	1.34	0.37	4.77	0.65	0.97	0.56	1.69	0.92	1.19	0.41	3.44	0.75
	CT	20	271	48																
	CC	4	44	7																
rs5743618	GG	24	334	60	1.12	0.59	2.12	0.73	1.67	0.64	4.34	0.29	1.15	0.66	2.00	0.63	2.07	0.93	4.62	0.07
	GT	22	310	55																
	TT	8	54	12																
TLR2																				
rs4696480	TT	22	196	37	0.61	0.31	1.19	0.15	0.58	0.26	1.32	0.19	0.57	0.32	1.00	0.05	0.65	0.31	1.36	0.25
	TA	23	338	56																
	AA	9	164	32																
rs1898830	AA	17	290	47	1.31	0.67	2.59	0.43	0.81	0.34	1.96	0.65	1.61	0.89	2.92	0.11	1.35	0.61	2.96	0.46
	AG	29	314	56																
	GG	8	78	22																
rs3804100	TT	50	598	108	0.48	0.15	1.49	0.20	n.a.	n.a.	n.a.	n.a.	0.54	0.19	1.52	0.24	n.a.	n.a.	n.a.	n.a.

		Frequent RTI (n=55)	Moderate frequency of RTI (n=712)	No RTI (n=130)	RR* frequent vs no RTI	95% CI		*p* value	RR† frequent vs no RTI	95% CI		*p* value	RR* frequent vs moderate frequency of RTI	95% CI		*p* value	RR† frequent vs moderate frequency of RTI	95% CI		*p* value
		n	n	n																
	TC	4	86	18																
	CC	0	3	0																
TLR3																				
rs11721827	TT	40	498	91	0.91	0.44	1.87	0.80	n.a.	n.a.	n.a.	n.a.	0.88	0.47	1.65	0.68	n.a.	n.a.	n.a.	n.a.
	TC	14	183	32																
	CC	0	16	3																
rs13126816	GG	34	414	68	0.75	0.39	1.46	0.40	0.63	0.13	3.12	0.57	0.85	0.48	1.51	0.59	0.67	0.16	2.85	0.58
	GA	18	247	46																
	AA	2	38	7																
rs3775291	GG	28	351	68	1.07	0.56	2.03	0.85	0.20	0.03	1.58	0.09	0.92	0.53	1.61	0.78	0.26	0.03	1.90	0.15
	GA	24	292	46																
	AA	1	48	11																
rs3775292	CC	30	421	78	1.33	0.69	2.54	0.39	2.37	0.32	17.25	0.38	1.24	0.71	2.16	0.45	0.79	0.19	3.41	0.76
	CG	22	240	45																
	GG	2	32	2																
rs3775296	GG	37	445	78	0.86	0.43	1.74	0.69	1.34	0.31	5.84	0.69	0.80	0.44	1.47	0.48	1.52	0.44	5.20	0.50
	GT	13	214	34																
	TT	3	26	5																
rs7657186	GG	32	410	78	1.14	0.59	2.19	0.69	1.16	0.21	6.55	0.86	1.02	0.58	1.79	0.94	0.69	0.16	2.97	0.62
	GA	20	240	43																
	AA	2	36	4																
rs7668666	CC	27	372	58	0.83	0.43	1.60	0.58	0.88	0.22	3.44	0.85	1.07	0.61	1.89	0.82	0.79	0.24	2.62	0.70

		Frequent RTI (n=55)	Moderate frequency of RTI (n=712)	No RTI (n=130)	RR* frequent vs no RTI	95% CI		*p* value	RR† frequent vs no RTI	95% CI		*p* value	RR* frequent vs moderate frequency of RTI	95% CI		*p* value	RR† frequent vs moderate frequency of RTI	95% CI		*p* value
		n	n	n																
	CA	21	259	54																
	AA	3	50	8																
TLR4																				
rs10759931	GG	25	248	45	0.69	0.36	1.32	0.26	0.60	0.24	1.50	0.27	0.67	0.38	1.17	0.16	0.63	0.28	1.42	0.26
	GA	22	300	52																
	AA	7	130	24																
rs10759932	TT	49	526	101	0.38	0.14	1.05	0.06	n.a.	n.a.	n.a.	n.a.	0.32	0.13	0.82	0.01	n.a.	n.a.	n.a.	n.a.
	TC	5	158	27																
	CC	0	9	0																
rs2770150	TT	21	351	56	1.16	0.60	2.24	0.66	1.65	0.60	4.52	0.33	1.49	0.84	2.66	0.17	1.98	0.85	4.63	0.11
	TC	23	285	58																
	CC	7	51	11																
rs6478317	AA	24	320	68	1.42	0.75	2.68	0.29	0.41	0.09	1.91	0.24	1.04	0.60	1.82	0.88	0.30	0.07	1.25	0.08
	AG	28	303	49																
	GG	2	80	11																
rs11536878	CC	33	550	102	2.69	1.31	5.50	0.01	n.a.	n.a.	n.a.	n.a.	2.22	1.24	3.98	0.01	n.a.	n.a.	n.a.	n.a.
	CA	20	139	21																
	AA	0	11	2																
rs1927911	CC	26	401	81	1.91	1.00	3.67	0.05	0.94	0.18	5.01	0.94	1.43	0.82	2.49	0.21	0.59	0.14	2.52	0.47
	CT	25	249	39																
	TT	2	43	5																
rs4986790	AA	47	609	111	0.84	0.29	2.48	0.76	n.a.	n.a.	n.a.	n.a.	0.72	0.28	1.86	0.50	n.a.	n.a.	n.a.	n.a.

		Frequent RTI (n=55)	Moderate frequency of RTI (n=712)	No RTI (n=130)	RR* frequent vs no RTI	95% CI		*p* value	RR† frequent vs no RTI	95% CI		*p* value	RR* frequent vs moderate frequency of RTI	95% CI		*p* value	RR† frequent vs moderate frequency of RTI	95% CI		*p* value
		n	n	n																
Asp299Gly	AG	5	89	14																
	GG	0	1	0																
rs4986791	CC	50	608	112	0.48	0.13	1.75	0.26	n.a.	n.a.	n.a.	n.a.	0.40	0.12	1.30	0.11	n.a.	n.a.	n.a.	n.a.
Thr399Ile	CT	3	89	13																
	TT	0	3	1																
rs11536889	GG	43	507	93	0.72	0.33	1.56	0.41	0.38	0.04	3.21	0.36	0.70	0.35	1.39	0.31	1.43	0.18	11.52	0.73
	GC	10	176	27																
	CC	1	9	6																
TLR5																				
rs2072493	AA	40	505	84	0.75	0.37	1.54	0.44	0.75	0.15	3.84	0.73	0.96	0.51	1.81	0.90	2.37	0.51	10.98	0.26
	GA	12	173	33																
	GG	2	11	6																
rs5744105	CC	15	181	27	0.69	0.33	1.44	0.32	0.74	0.34	1.60	0.44	0.89	0.48	1.66	0.72	0.84	0.42	1.67	0.62
	CG	27	348	66																
	GG	11	165	33																
rs2241096	CC	44	583	102	0.97	0.43	2.19	0.93	n.a.	n.a.	n.a.	n.a.	1.24	0.60	2.54	0.56	n.a.	n.a.	n.a.	n.a.
	CT	10	100	22																
	TT	0	7	2																
rs5744109	TT	44	584	102	0.93	0.41	2.09	0.86	n.a.	n.a.	n.a.	n.a.	1.23	0.60	2.52	0.57	n.a.	n.a.	n.a.	n.a.
	TC	10	103	23																
	CC	0	5	2																
rs5744157	CC	43	541	96	0.83	0.37	1.86	0.65	1.16	0.10	13.12	0.90	0.85	0.42	1.73	0.65	1.00	0.13	7.79	1.00

		Frequent RTI (n=55)	Moderate frequency of RTI (n=712)	No RTI (n=130)	RR* frequent vs no RTI	95% CI		*p* value	RR† frequent vs no RTI	95% CI		*p* value	RR* frequent vs moderate frequency of RTI	95% CI		*p* value	RR† frequent vs moderate frequency of RTI	95% CI		*p* value
		n	n	n																
	CG	9	135	25																
	GG	1	13	2																
rs851186	GG	8	220	46	3.31	1.44	7.61	0.00	1.20	0.52	2.78	0.66	2.73	1.27	5.87	0.01	0.87	0.43	1.77	0.70
	GT	36	322	60																
	TT	10	142	20																
rs5744168	CC	48	608	112	1.00	0.36	2.76	1.00	2.36	0.14	38.41	0.54	0.88	0.37	2.13	0.78	4.35	0.44	42.51	0.17
	CT	5	83	13																
	TT	1	3	1																
TLR6																				
rs5743788	CC	19	178	30	0.58	0.29	1.16	0.12	1.34	0.61	2.95	0.47	0.63	0.35	1.12	0.11	0.86	0.44	1.67	0.65
	CG	23	348	73																
	GG	12	175	22																
rs5743810	CC	26	236	38	0.47	0.24	0.91	0.02	1.18	0.47	2.95	0.73	0.54	0.31	0.94	0.03	0.89	0.41	1.95	0.78
	CT	19	342	68																
	TT	8	115	16																
rs6531666	TT	19	268	49	1.37	0.67	2.79	0.39	6.85	1.72	27.22	0.00	1.29	0.70	2.39	0.41	2.12	0.94	4.81	0.07
	TC	18	233	46																
	CC	8	51	3																
rs1039559	TT	23	198	31	0.46	0.24	0.91	0.03	0.98	0.44	2.18	0.97	0.55	0.31	0.97	0.04	0.92	0.47	1.84	0.82
	TC	20	337	65																
	CC	11	148	25																
rs5743798	CC	26	371	69	1.35	0.71	2.58	0.36	2.47	0.76	8.04	0.12	1.24	0.71	2.17	0.45	1.77	0.72	4.35	0.21

		Frequent RTI (n=55)	Moderate frequency of RTI (n=712)	No RTI (n=130)	RR* frequent vs no RTI	95% CI		*p* value	RR† frequent vs no RTI	95% CI		*p* value	RR* frequent vs moderate frequency of RTI	95% CI		*p* value	RR† frequent vs moderate frequency of RTI	95% CI		*p* value
		n	n	n																
	CT	21	265	47																
	TT	6	46	6																
TLR9																				
rs187084	TT	17	218	38	1.00	0.50	1.99	0.99	0.95	0.44	2.05	0.89	1.01	0.55	1.83	0.98	1.40	0.71	2.73	0.33
	TC	25	354	55																
	CC	12	117	28																
rs5743836	TT	44	524	85	0.44	0.20	0.96	0.04	n.a.	n.a.	n.a.	n.a.	0.70	0.34	1.41	0.31	n.a.	n.a.	n.a.	n.a.
	TC	10	161	41																
	CC	0	10	3																
rs352140	TT	16	207	49	1.53	0.77	3.04	0.22	1.73	0.80	3.74	0.16	1.02	0.56	1.87	0.95	1.41	0.75	2.67	0.29
	TC	24	346	55																
	CC	14	137	21																
TLR10																				
rs10856839	AA	29	477	89	1.91	0.98	3.72	0.06	2.54	0.61	10.57	0.19	1.83	1.03	3.24	0.04	2.77	0.91	8.42	0.06
	AC	19	187	33																
	CC	4	20	4																
rs11096956	GG	28	419	64	0.96	0.50	1.85	0.91	1.17	0.21	6.60	0.86	1.44	0.82	2.54	0.20	0.95	0.22	4.11	0.94
	GT	22	222	53																
	TT	2	27	4																
rs11096957	AA	15	266	38	1.15	0.56	2.33	0.71	2.09	0.90	4.84	0.08	1.65	0.89	3.05	0.11	1.81	0.92	3.57	0.08
	CA	26	315	69																
	CC	12	94	15																

		Frequent RTI (n=55)	Moderate frequency of RTI (n=712)	No RTI (n=130)	RR* frequent vs no RTI	95% CI		p value	RR† frequent vs no RTI	95% CI		p value	RR* frequent vs moderate frequency of RTI	95% CI		p value	RR† frequent vs moderate frequency of RTI	95% CI		p value
		n	n	n																
rs11466652	AA	34	491	91	1.45	0.73	2.88	0.28	1.19	0.11	13.44	0.89	1.37	0.76	2.45	0.30	0.93	0.12	7.22	0.95
	GA	18	187	33																
	GG	1	14	2																
rs4129009	AA	35	464	71	0.69	0.35	1.35	0.28	1.18	0.21	6.63	0.85	1.02	0.57	1.85	0.94	1.01	0.23	4.38	0.99
	AG	16	207	49																
	GG	2	26	4																
rs4274855	GG	33	459	72	0.81	0.42	1.56	0.53	1.20	0.21	6.74	0.84	1.19	0.67	2.12	0.56	0.97	0.26	4.18	0.96
	GA	18	207	50																
	AA	2	27	4																
CD14																				
rs2915863	TT	17	231	47	1.20	0.60	2.39	0.61	0.57	0.20	1.63	0.29	1.03	0.56	1.89	0.94	0.64	0.25	1.65	0.35
	TC	27	325	54																
	CC	5	99	20																
rs2569191	TT	17	187	33	0.78	0.39	1.56	0.48	0.82	0.38	1.78	0.61	0.81	0.44	1.46	0.48	1.00	0.50	1.99	1.00
	TC	27	378	65																
	CC	11	141	30																
rs5744455	CC	29	394	80	1.47	0.77	2.80	0.24	0.66	0.13	3.28	0.61	1.12	0.65	1.96	0.68	0.77	0.18	3.31	0.73
	CT	23	269	40																
	TT	2	33	7																
rs2569190	GG	17	187	33	0.79	0.40	1.59	0.51	0.88	0.40	1.91	0.74	0.82	0.45	1.49	0.51	1.03	0.52	2.05	0.93
	GA	27	374	65																
	AA	11	136	28																

		Frequent RTI (n=55)	Moderate frequency of RTI (n=712)	No RTI (n=130)	RR* frequent vs no RTI	95% CI		*p* value	RR† frequent vs no RTI	95% CI		*p* value	RR* frequent vs moderate frequency of RTI	95% CI		*p* value	RR† frequent vs moderate frequency of RTI	95% CI		*p* value
		n	n	n																
rs2563298	CC	30	362	63	0.83	0.44	1.57	0.57	2.08	0.67	6.51	0.20	0.89	0.51	1.55	0.69	1.41	0.58	3.43	0.45
	CA	19	282	56																
	AA	6	56	7																
MYD88																				
rs7744	AA	35	514	99	1.92	0.95	3.86	0.07	2.40	0.33	17.52	0.37	1.49	0.83	2.67	0.18	2.04	0.45	9.26	0.35
	AG	17	174	26																
	GG	2	13	2																
rs6853	AA	44	546	94	0.71	0.32	1.59	0.41	n.a.	n.a.	n.a.	n.a.	0.80	0.39	1.62	0.53	n.a.	n.a.	n.a.	n.a.
	AG	10	145	30																
	GG	0	11	0																
TIRAP																				
rs1893352	AA	41	504	90	0.82	0.39	1.70	0.59	1.16	0.10	13.08	0.90	0.79	0.41	1.50	0.47	0.77	0.10	5.87	0.80
	AG	12	186	33																
	GG	1	17	2																
rs1786704	TT	34	449	92	1.47	0.74	2.91	0.27	1.62	0.26	9.99	0.60	1.00	0.56	1.79	1.00	0.91	0.21	3.91	0.90
	CT	17	222	32																
	CC	2	29	3																
rs625413	CC	29	439	67	0.96	0.50	1.82	0.89	0.94	0.26	9.99	0.94	1.45	0.82	2.54	0.20	1.09	0.21	3.91	0.91
	CT	22	227	53																
	TT	2	24	5																
rs8177375	AA	45	500	97	0.62	0.26	1.46	0.27	n.a.	n.a.	n.a.	n.a.	0.49	0.22	1.05	0.06	n.a.	n.a.	n.a.	n.a.
	GA	8	174	27																

		Frequent RTI (n=55)	Moderate frequency of RTI (n=712)	No RTI (n=130)	RR* frequent vs no RTI	95% CI		p value	RR† frequent vs no RTI	95% CI		p value	RR* frequent vs moderate frequency of RTI	95% CI		p value	RR† frequent vs moderate frequency of RTI	95% CI		p value
		n	n	n																
	GG	0	9	1																
rs8177376	TT	30	388	71	0.94	0.49	1.79	0.85	0.71	0.19	2.70	0.62	0.96	0.55	1.69	0.89	1.17	0.35	3.94	0.80
	TG	20	275	48																
	GG	3	34	10																
IRAK1																				
Girls																				
rs1059701	TT	12	244	41	1.78	0.69	4.61	0.23	5.73	0.49	66.31	0.12	2.52	1.09	5.79	0.03	2.49	0.52	11.84	0.24
	TC	10	85	22																
	CC	2	12	1																
rs1059703	TT	14	271	43	1.62	0.61	4.28	0.33	n.a.	n.a.	n.a.	n.a.	2.89	1.23	6.79	0.01	n.a.	n.a.	n.a.	n.a.
	TC	8	62	19																
	CC	2	5	0																
rs2239673	TT	12	237	41	1.86	0.72	4.83	0.20	5.64	0.49	65.27	0.13	2.47	1.07	5.69	0.03	2.24	0.47	10.54	0.30
	TC	10	83	21																
	CC	2	13	1																
Boys																				
rs1059701	T	28	300	52	0.31	0.06	1.48	0.13	0.31	0.06	1.48	0.13	0.35	0.08	1.51	0.14	0.35	0.08	1.51	0.14
	C	2	61	12																
rs1059703	T	28	309	52	0.37	0.08	1.81	0.21	0.37	0.08	1.81	0.21	0.57	0.13	2.47	0.44	0.57	0.13	2.47	0.44
	C	2	39	10																
rs2239673	T	28	292	52	0.31	0.06	1.48	0.13	0.31	0.06	1.48	0.13	0.36	0.08	1.55	0.15	0.36	0.08	1.55	0.15
	C	2	58	12																

		Frequent RTI (n=55)	Moderate frequency of RTI (n=712)	No RTI (n=130)	RR* frequent vs no RTI	95% CI		*p* value	RR† frequent vs no RTI	95% CI		*p* value	RR* frequent vs moderate frequency of RTI	95% CI		*p* value	RR† frequent vs moderate frequency of RTI	95% CI		*p* value
		n	n	n																
IRAK4																				
rs4251520	TT	41	555	95	0.87	0.41	1.85	0.72	n.a.	n.a.	n.a.	n.a.	1.09	0.56	2.13	0.80	n.a.	n.a.	n.a.	n.a.
	TC	12	143	29																
	CC	0	6	3																
rs4251513	CC	15	172	36	1.02	0.50	2.09	0.95	0.94	0.45	1.99	0.88	0.84	0.45	1.56	0.57	0.93	0.49	1.78	0.83
	CG	25	342	57																
	GG	13	179	32																
rs146156	CC	26	383	81	1.87	0.49	1.79	0.06	1.06	0.19	2.70	0.92	1.28	0.55	1.69	0.38	0.85	0.35	3.94	0.75
	CT	23	249	36																
	TT	4	61	9																

*RR based on a dominant model (AB + BB versus AA)
†RR based on a recessive model (BB versus AA + AB)
N.a.: not available, caused by n=0 in one cell.

Chapter 8

The value of genetic factors in the prediction of frequent respiratory tract infections in childhood

Jopje M. Ruskamp
Maroeska Rovers
Maarten O. Hoekstra
Henriette A. Smit
Bert Brunekreef
Alet H. Wijga
Marjan Kerkhof
Johan C. de Jongste
Elisabeth A. Sanders

Manuscript in preparation

ABSTRACT

Objective: To study the predictive value of non-genetic and genetic factors for frequent respiratory tract infections (RTI) in children aged 1-4 years.

Methods: Data were collected from the PIAMA birth cohort (n=3963). Frequency of RTI was prospectively assessed by annual parental questionnaires from age 1 to 4 years. Potential predictors (gender, breastfeeding, maternal smoking during pregnancy, birth weight, prematurity, parental education, older siblings, environmental tobacco smoke, daycare, parental allergy and asthma) were based on questionnaires during pregnancy and at age 1 year. Genetic variation in important innate immunity genes, encoding mannose binding lectin (MBL) and the toll-like receptor (TLR) pathway were determined in 987 children. Univariate and multivariate logistic regression models were used to evaluate the predictive value of questionnaire and genetic variables for frequent RTI. The discriminating ability of the prognostic functions was assessed by using receiver operating characteristics (ROC) curves.

Results: Of the 3273 children with complete data on RTI frequency, 143 (4.4%) had frequent RTI according to our definition. Of the 897 children from the population with genetic data, 55 (6%) had frequent RTI. Independent questionnaire predictors for frequent RTI in both the total population and the population for which genetic data were available were birth weight <2500 gram, breastfeeding <12 weeks and presence of older siblings (area under the curve (AUC) 0.65, 95% CI 0.60-0.69; and 0.71, 0.64-0.78). After inclusion of genetic polymorphisms, independent predictors were breastfeeding <12 weeks, presence of older siblings and high-risk alleles of *TLR4* and *TLR5* (AUC 0.73, 0.66-0.79). Prediction based on genetic information only (high-risk alleles of *TLR4, TLR5* and *TLR6*) showed similar results (AUC 0.67, 0.59-0.74) as prediction based on questionnaire variables only.

Conclusions: Prediction of frequent RTI in 1-4 year old children was not improved by adding information of TLR alleles to questionnaire-derived prediction variables. However, genetic information on its own has similar predictive value as questionnaire-derived variables.

INTRODUCTION

Respiratory tract infections (RTI) presenting as the common cold, rhinosinusitis, pharyngitis, tonsillitis, otitis media, bronchitis or pneumonia are the most frequent causes of morbidity, especially in preschool children. In the Netherlands, every year 466 out of 1000 children aged 0 to 4 years are diagnosed with a RTI by their general practitioner [1]. Most RTI are self-limiting, but up to 10-20 % of the children may suffer from frequent recurrence of these infections [2;3]. Furthermore, RTI are the leading indication for antibiotic prescription and ear-nose-throat surgery, although these infections are known to be often predominantly primarily of viral origin [4-6]. Unfortunately, there are no tools available to discriminate between children who might benefit from antibiotic prophylaxis or treatment or ENT interventions and those for whom these are not required. Therefore, early identification of children at risk of frequent RTI is of great value, since (preventive) interventions, i.e. vaccinations, ENT surgery, prophylactic antibiotics, could then be targeted at those children to reduce both over and under treatment. An established approach to estimate the individual risk of a condition is the use of a prediction rule [7;8].

Currently, there are very few prognostic studies available with regard to occurrence of frequent RTI [9-16]. These studies are not suiTable to guide (preventive) strategies for frequent RTI due to flaws in the study design and analysis including 1) incomplete health outcomes 2) missing predictors, 3) small sample sizes and selection of study populations. Firstly, the outcomes predicted so far have included a single outcome, e.g. only otitis media, whereas it is known that the common cold, rhinosinusitis, pharyngotonsillitis, otitis media, bronchitis and pneumonia are all closely related entities of RTI. Secondly, only a limited set of possible predictors have been studied, i.e. environmental factors like attending day-care, number of siblings, etc. Other factors known to play an important role in the pathogenesis of RTI such as the genetic predisposition have never been included in the prediction models for frequent RTI. Thirdly, sample sizes required to derive a valid and generally applicable prediction rule are large. However, the sample sizes of the studies performed so far have been small, and often populations studied have been selected rather than random.

Recently, various studies showed the influence of genetic variations in the immune system on infectious susceptibility [16-19]. Therefore, it might be essential to include genetics to come to an improved prediction rule for frequent RTI. However, to be useful in daily practice, a prediction rule should be based on easily obtainable individual risk factors measured early in life, which of course does not account for data on genetic risk factors. On the other hand, declining costs of genetic research and the expanding field of genome wide association analysis will definitely lead to more and more applications of genetics in health care.

The aim of the present study was to study the added value of currently investigated genetic factors in the prediction of frequent RTI during preschool years.

METHODS

Study population

Children participated in the PIAMA birth cohort study. Details of the study design have been published previously [20]. Recruitment took place in the years 1996 and 1997. A screening questionnaire on maternal allergy was filled in by 10 232 pregnant women visiting one of 52 prenatal clinics in The Netherlands [21]. Based on this screening, 7862 women were invited, and 4146 agreed and gave informed consent. After birth the baseline study population consisted of 3963 children. Questionnaires for parental completion were sent to the parents during pregnancy, at the child's ages of 3 and 12 months, and yearly thereafter. All children from allergic mothers were asked for genetic research (n=1173), combined with a random sample of children from non-allergic mothers from the natural history part of the study (n=635). Permission for genetic research was given for 1060 (59%) children from the 1808 that were asked. Parents of nonparticipating children refused for various reasons (e.g. no permission for collecting blood sample or lost to follow up). DNA was successfully collected from 1037 children. From these children, 987 were of Dutch ancestry and used in our prognostic analyses. The study was approved by the Institutional Review Board and all parents gave written informed consent.

Outcome variable

Information about frequency of RTI was collected from annual parental questionnaires from 1 till 4 years of age by the following question: "How often did your child have serious respiratory tract and/or ear-nose-throat infections such as flu, infection of the throat, middle ear or sinuses, bronchitis or pneumonia during the last 12 months?" Four answers were possible: never, 1-2 times, 3-5 times or ≥ 6 times". Based on the answers in the four annual consecutive questionnaires, frequent RTI were defined as three or more RTI per year reported in three or four annual questionnaires.

Potential predictor variables

We considered male gender, breastfeeding (<12 weeks), maternal smoking during pregnancy, birth weight <2500 gram, prematurity (duration of pregnancy <37 weeks) and low level of parental education as potential perinatal predictors of frequent RTI during year 1-4. Presence of older siblings, exposure to environmental tobacco smoke (ETS) at age 1 year and daycare attendance at 1 year of age were considered as potential environmental predictors of frequent RTI during year 1-4. Maternal and paternal allergy

and asthma were considered as potential predictors based on the family history. Finally, mannose-binding lectin (MBL)-deficiency and high-risk alleles of genes of the Toll-like receptor (TLR) pathway (*TLR4, TLR5, TLR6* and *TLR9*), which were genotyped from buccal swabs or blood using standard methods, were considered as potential genetic predictors [16-19].

Statistical analysis

Univariate and multivariate logistic regression models were used to evaluate the predictive value of potential predictors on frequent RTI. Predictors that were univariably associated with the outcome ($p \leq 0.15$) were used in multivariate logistic regression analyses. The predictive accuracy of the models has been estimated by their reliability (goodness-of-fit) using Hosmer-Lemeshow statistics, which evaluate the correspondence between a model's predicted probabilities and the observed frequencies over groups. The discriminating ability of the prognostic functions was assessed by using receiver operating characteristics (ROC) analysis. The area under the ROC curve provides a quantitative summary of the discriminating ability of a predictive model and has a range of 0.5 (no discrimination, like a coin flip) to 1.0 (perfect discrimination). In our analysis a value of 0.8 or higher was considered as satisfactory.

To develop an easy applicable prediction rule, in first instance only the easily applicable patient characteristics were used. Subsequently, we studied the added value of previously investigated relevant genetic factors. The adjusted regression coefficients of the model were multiplied by a factor 10 and rounded to the nearest integer. Scores for each individual child were obtained by assigning points for each variable and adding the results. Children were classified according to their risk score. To simplify the interpretation of the model, the number of children developing or not developing frequent RTI was shown at various cut-off points and corresponding sensitivity, specificity, positive and negative predictive values were calculated.

RESULTS

Study population

Complete data on RTI frequency were obtained from 3273 children (83%) aged 0 to 4 years. From these children 143 (4.4%) fulfilled the criteria for frequent RTI during the first 4 years of life, according to our definition.

Of the 987 children with Dutch ancestry included in the genetic analyses, 897 (91%) had complete data on RTI frequency. From these children 55 (6%) fulfilled the criteria of frequent RTI during the first four years of life according to our definition.

Univariate regression

Table 1 shows the odds ratios (OR) of univariate associations with frequent RTI. Birthweight < 2500 gram (OR 2.16; 95% CI 1.07-4.38), breastfeeding < 12 weeks (OR 1.80;

Table 1. General characteristics of the study population and univariate associations with frequent RTI year 1-4

	Complete data on RTI frequency (n=3273)		DNA available and complete data on RTI frequency (n=897)	
	OR	95% CI	OR	95% CI
Perinatal factors				
Male gender	1.13	0.81-1.59	1.14	0.66-1.97
Birthweight < 2500 g	2.16	1.07-4.38	3.42	1.25-9.33
Breastfeeding < 12 weeks	1.80	1.26-2.58	2.54	1.41-4.57
Maternal smoking during pregnancy (> 4 weeks)	1.32	0.86-2.02	1.32	0.63-2.78
Prematurity	1.05	0.48-2.28	1.76	0.60-5.13
Low parental level of education	1.44	0.90-2.31	2.86	1.37-5.97
Environmental factors				
Presence of older siblings	1.67	1.18-2.37	2.17	1.21-3.88
ETS exposure in year 1	0.92	0.63-1.35	0.91	0.47-1.75
Daycare attendance year 1	1.17	0.80-1.71	0.74	0.39-1.41
Family history				
Maternal allergy and/or asthma	1.69	1.20-2.39	1.62	0.87-3.02
Paternal allergy and/or asthma	1.17	0.82-1.67	0.93	0.51-1.69
Maternal asthma	1.93	0.83-4.53	3.02	1.21-7.55
Paternal asthma	2.54	1.14-5.66	2.72	0.78-9.56
Genetic factors				
MBL-deficiency	-	-	0.56	0.22-1.42
High risk TLR4	-	-	2.29	1.28-4.10
High risk TLR5	-	-	2.81	1.31-6.04
High risk TLR6.1	-	-	1.91	1.09-3.34
High risk TLR6.2	-	-	1.88	1.07-3.29
High risk TLR9	-	-	1.54	0.76-3.12

NOTE: Frequent RTI year 1-4: ≥ 3 years 3 or more respiratory tract infections per year during year 1-4; N=987, DNA available and Dutch Caucasian ethnicity; 95% CI: 95% confidence interval; Prematurity: duration of pregnancy < 37 weeks; Low parental level of education: both parents maximum of 4 years at high-school; Parental allergy and/or asthma based on validated questionnaire during pregnancy; Parental asthma based on questionnaire at age 3 months; High risk *TLR4*: A allele at rs11536878; High risk *TLR5*: T allele at rs851186; High risk *TLR6.1*: C allele at rs5743810; High risk *TLR6.2*: T allele at rs1039559; High risk *TLR9*: T allele at rs5743836.

95% confidence interval (CI) 1.26-2.58), presence of older siblings (OR 1.67; 95% CI 1.18-2.37), maternal allergy (OR 1.69; 95% CI 1.20-2.39) and paternal asthma (OR 2.54; 95% CI 1.14-5.66) were associated with an increased risk of frequent RTI in the complete study population. Within the subgroup of children of whom DNA was collected, the high-risk alleles of *TLR4* (rs11536878), *TLR5* (rs851186) and *TLR6* (rs5743810 and rs1039559) were associated with an increased risk of frequent RTI (ORs and 95% CI: 2.29, 1.28-4.10; 2.81, 1.31-6.04; 1.91, 1.09-3.34; and 1.88, 1.07-3.29, respectively).

Multivariate regression

In the multivariate regression analyses in the total population without genetic polymorphisms, shown in Table 2, birth weight < 2500 gram (OR 2.28; 95% CI 1.11-4.69),

Table 2. Multivariate prediction model for frequent RTI year 1-4

	Total population (n=3273)		Population for which DNA available (n=897)					
	Without genetic factors		Without genetic factors		Genetic and non-genetic factors		Only genetic factors	
	OR	95% CI	OR	95% CI	OR	95% CI	OR	95% CI
Non-genetic predictors								
Birthweight < 2500 g	2.28	1.11-4.69	3.60	1.26-10.28	-	-	-	-
Breastfeeding < 12 weeks	1.86	1.29-2.68	2.93	1.57-5.47	3.03	1.59-5.77	-	-
Presence of older siblings	1.83	1.28-2.61	2.45	1.34-4.50	2.40	1.28-4.47	-	-
Maternal allergy and/or asthma	1.71	1.21-2.44	-	-	-	-	-	-
Maternal asthma	-	-	3.66	1.41-9.50	2.75	0.97-7.80	-	-
Paternal asthma	2.32	1.03-5.27	-	-	-	-	-	-
Genetic predictors								
High risk *TLR4*	-	-	-	-	2.09	1.13-3.86	2.35	1.30-4.24
High risk *TLR5*	-	-	-	-	2.79	1.23-6.35	2.57	1.19-5.55
High risk *TLR6.1*					-	-	1.75	0.99-3.10
Hosmer-Lemeshow (p-value)	0.48		0.51		0.51		0.87	

NOTE: Frequent RTI year 1-4: ≥ 3 years 3 or more respiratory tract infections per year during year 1-4; N=897, DNA available, Dutch Caucasian ethnicity and complete data on RTI frequency; 95% CI: 95% confidence interval; Parental allergy and/or asthma based on validated questionnaire during pregnancy; Parental asthma based on questionnaire at age 3 months.

breastfeeding < 12 weeks (OR 1.86; 95% CI 1.29-2.68), presence of older siblings (OR 1.83; 95% CI 1.28-2.61), maternal allergy (OR 1.71; 95% CI 1.21-2.44) and paternal asthma (OR 2.32; 95% CI 1.03-5.27) were independent predictors for frequent RTI. In the population for which genetic material was available birth weight < 2500 gram (OR 3.60; 95% CI 1.26-10.28), breastfeeding < 12 weeks (OR 2.93; 95% CI 1.57-5.47), presence of older siblings (OR 2.45; 95% CI 1.34-4.50) and maternal asthma (OR 3.66; 95% CI 1.41-9.50) were independent predictors for frequent RTI. The goodness-of-fit test for these models indicated a reasonable fit (p=0.48 and 0.51). The AUCs of these models were 0.65 (95% CI 0.60-0.69) and 0.71 (95% CI 0.64-0.78), respectively.

In the multivariate regression analyses including genetic polymorphisms, also shown in Table 2, breastfeeding < 12 weeks (OR 3.03; 95% CI 1.59-5.77), presence of older siblings (OR 2.40; 95% CI 1.28-4.47), the high-risk allele of *TLR4* (OR 2.09; 95% CI 1.13-3.86) and the high-risk allele of *TLR5* (OR 2.79; 95% CI 1.23-6.35) were independent predictors of frequent RTI. The goodness-of-fit test for this model indicated a reasonable fit (p=0.51). The AUC of this model was 0.73 (95% CI 0.66-0.79).

In the multivariate regression analyses including only genetic polymorphisms, also shown in Table 2, the high-risk allele of *TLR4* (OR 2.35; 95% CI 1.30-4.24), the high-risk allele of *TLR5* (OR 2.57; 95% CI 1.19-5.55) and the high-risk allele of *TLR6* (OR 1.75; 95% CI 0.99-3.10) were independent predictors of frequent RTI. The goodness-of-fit test for this model indicated a reasonable fit (p=0.87). The AUC of this model was 0.67 (95% CI 0.59-0.74).

Using the regression coefficients of the final multivariable predictive model in the total population with genetic factors, the probability of developing frequent RTI can be estimated for each child using the formula given in Table 3. For example, a child who was formula fed (11 points), who has an older sibling (9 points), whose mother is non-allergic (0 points) and whose TLR alleles were low-risk (2 x 0 points), has a total score of 11 + 9 + 0 + 0 + 0 = 20 points, corresponding with a probability of frequent RTI of 9%.

Table 3. Test characteristics at various cut-off points of prediction score (with genetic factors) in the population for which genetic material was available

Cut-off value	N positive test (%)	Sensitivity	Specificity	Positive predictive value	Negative predictive value
> 10	45 (5)	94%	29%	7%	99%
> 19	35 (4)	73%	55%	9%	97%
> 26	23 (3)	48%	79%	12%	96%

NOTE: Positive test: an individual score equal to or above the chosen cut-off value.

Score = 11 x breastfeeding < 12 weeks + 9 x presence of older siblings + 10 x maternal asthma + 7 x high risk *TLR4* + 10 x high risk *TLR5*

For all score thresholds, both for the model without and the model with genetic factors, the number of false-positive (those identified at high-risk of developing frequent RTI, while this outcome did not occur) and false-negatives was too high to be of any value in clinical practice.

DISCUSSION

Independent questionnaire predictors for frequent RTI in 1 to 4 year old children in both the total population and the population for which DNA was available were birth weight <2500 gram, breastfeeding <12 weeks and presence of older siblings. Breastfeeding < 12 weeks and presence of older siblings were also independent predictors for frequent RTI in the model including genetic polymorphisms of innate immunity genes, in addition to the high-risk alleles of *TLR4* and *TLR5*. Independent predictors for frequent RTI in the prognostic model based on genetic information only were high-risk alleles of *TLR4*, *TLR5* and *TLR6.1*.

The performance of all prognostic models for frequent RTI in 1-4 year old children regarding the AUC and the positive predictive value was low. The prediction was not improved by adding genetic information to questionnaire-derived prediction variables. However, genetic information on its own showed similar predictive value as questionnaire-derived variables (AUC 0.67; 95% CI 0.59-0.74; versus AUC 0.65; 95% 0.60-0.69).

The major strengths of our study are its large sample size, prospectively repeated questionnaires and population-based character. Furthermore, to our knowledge we are the first to attempt to develop a prediction rule for frequent RTI on the basis of genetic data next to perinatal, environmental and family characteristics.

To appreciate our results, some potential limitations should also be considered. First, exact measures of numbers of RTI episodes were not recorded, since the PIAMA study was originally designed to study atopy and asthma. Instead we grouped the children according to frequency of RTI as reported in annual questionnaires. Strictly speaking, RTI in our study means "respiratory symptoms", which the parents considered to be more serious than a common cold.

Second, in prognostic studies validation of the model is generally recommended [23]. We did not carry out an external validation study, as the performance of our prediction was poor. For the same reason we did not use random bootstrapping or penalized maximum likelihood techniques to adjust for over-fitting (i.e. over-optimistic estimates of the regression coefficients of the prediction model) [24].

Third, in this study we only included limited data on genetic polymorphisms in MBL and TLRs, previously shown to be positively associated with occurrence of frequent

RTI by us or other investigators [16-19;22]. This certainly will not present the full picture of genetic influence on RTI. Many genes and polymorphisms which influence infectious susceptibility still have to be discovered. Incorporation of this new information on genetic variables into prognostic studies might ultimately improve the prediction of frequent RTI.

Finally, in our earlier studies maternal allergy appeared to modify the effect of variation in TLR genes on RTI, especially for rs11536878 in *TLR4* (unpublished data). We compared general characteristics of the children that participated in the genetic studies (n=1060) with the characteristics of the other children from the PIAMA cohort. Children that participated in the genetic studies were more likely to have an allergic mother based on the original design of the study (66 vs. 20%). Therefore, we tested the interaction between maternal allergy and the genetic factors in the present study, but in this study no significant interaction was detected. However, these analyses are extremely complex, multiple gene-gene and gene-environment interactions are possible and very large study population are needed since in general single genetic loci make modest contributions to occurrence of disease [25]. Complex interactions between different polymorphisms in either different genes (gene-gene interaction) or environmental factors (gene-environment interaction) should be further evaluated in future research. These genetic data and interactions which are unknown at this moment, might possibly improve future prediction rules for frequent RTI.

In conclusion, on the basis of history and currently known genetic data it was not possible to predict which children would develop frequent RTI during the first 4 years of life. Nevertheless, we suggest that this method of combining epidemiologic and genetic data should be developed further as a tool to predict complex diseases such as frequent RTI in the future.

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Chapter 9

Summarizing discussion

SUMMARIZING DISCUSSION

In this thesis common environmental and host factors, as well as plausible genetic factors were evaluated in a large birth cohort study in the Netherlands for their effect on the occurrence of frequent respiratory tract infections (RTI) in children. In this chapter the main conclusions of this thesis will be summarized and discussed in the light of actual clinical practice. In addition the research questions raised in the introduction are addressed and some recommendations are made for future research.
The main research questions addressed in this theses are:

1. What is the role of selected environmental and host factors in the occurrence of frequent RTI?
2. Can we identify genetic factors in the innate immunity which are associated with frequent RTI?
3. Can we predict frequent RTI with a combination of environmental, genetic and other host factors?

1. What is the role of selected environmental and host factors in the occurrence of frequent RTI?

In **Chapter 2** we explored the relationship between frequent RTI, atopy, exposure to environmental tobacco smoke (ETS), and the interaction between the later two in relation to susceptibility to RTI. We showed that children with intrauterine tobacco smoke exposure who also exhibited early markers of atopy, like high neonatal total IgE or symptoms of atopic dermatitis at the age of 3 months, had an increased risk for frequent RTI in the first 4 years of life (aOR 6.18, 95 % CI 1.45-26.34; and 5.69, CI 95% 2.01-16.04; interaction p-values 0.006 and 0.14, respectively). By contrast, intrauterine tobacco smoke exposure was unrelated to frequent RTI in children without these early markers of atopy (aOR 0.50, 95 % CI 0.15-1.66; and 1.39, 95% CI 0.78-2.47, respectively). Odds ratios were adjusted for frequent wheeze, breastfeeding (< 12 weeks), level of parental education and parental allergy. Although the relation with intrauterine exposure to tobacco smoke and RTI was recognized before, we are the first to describe that the increased risk appears to occur mainly in children with an atopic constitution [1-4]. So far, the pathofysiologic mechanism of the modifying effect of atopy, is not yet unraveled. A stronger effect of ETS in atopic children might be explained by the fact that their increased susceptibility results from biological interactions between endogenous and environmental factors [5]. Moreover, it can be hypothesized that subclinical atopic inflammation of the airways, which may be

present at early age even before the occurrence of clinical respiratory symptoms, may lead to a demolished protective function of the airway mucosa in atopic children [6].

The potential association between atopy on its own and frequent RTI was explored in **Chapter 3**. As far as we are aware, we are the first to study the association between different objective markers of atopy and frequent RTI in a population-based cohort of children aged 5-8 years. Others that looked at the relationship between atopy and RTI used retrospective or cross-sectional data, and in most studies atopy was based on questionnaires instead of objective markers [7-15]. This may have resulted in bias. We found that atopy at the age of 4 years, as defined by either the presence of positive serum specific IgE (sIgE) or elevated serum total IgE (tIgE), was not associated with the occurrence of frequent RTI at age 5-8 years in children from non-allergic mothers (OR 1.57, 95% CI 0.32-7.81; and 0.81, 95% CI 0.17-3.97). Neither did we find an association between these atopic markers or a positive skin prick test (SPT) in children from non-allergic mothers at the age of 8 years. However, in children with an allergic mother, elevated tIgE at the age of 4 years and both positive sIgE and SPT at the age of 8 years were associated with frequent RTI (OR 2.25, 95% CI 1.09-4.65; 1.85, 95% CI 0.95-3.63; and 2.02, 95% CI 0.99-4.11, respectively). In these analyses the following potential confounders were studied: duration of pregnancy, gender, birth weight, maternal smoking during pregnancy, environmental tobacco smoke exposure, breastfeeding (< 12 weeks), day-care attendance, presence of siblings and parental education, but none of these factors was found to influence the study results. These findings suggest that atopy might affect susceptibility to RTI in some school-aged children, but the effect seems to be limited to high-risk children with a family predisposition for atopy. A possible explanation for this finding might be an overlap in the genetic predisposition for atopy and RTI, which is visualized in Figure 1. Candidate genes related to both atopy and RTI can be found in innate immunity pathways, such as the Toll-like receptors (TLRs), including CD14. Evidence in this direction can be found in genetic association studies that showed that polymorphisms in TLR4 and CD14 are similarly associated with atopy/asthma and RTI [16-19]. This is further explored in **Chapter 7**.

The results of **Chapter 2** and **Chapter 3** are in line with the study from Koopman et al., who showed that contacts with other children (day-care attendance or presence of siblings) increased the risk of RTI in the first year of life to a greater extent in infants with atopic parents compared with infants of non-atopic parents [20]. Using the same birth cohort, we found similar results at a later age. Furthermore, we studied atopic markers in the children instead of parental atopy as "proxy" for atopy in the child. If a causal relationship between atopy and RTI indeed exists, this could have important therapeutic implications, e.g. RTI in children with proven atopy might be better managed with

anti-allergic or anti-inflammatory interventions than with antibiotics [21-23]. The routine use of antihistamines for treating RTI in children cannot be recommended, as Cochrane reviews suggested [24;25].

2. Can we identify genetic factors in the innate immunity which are associated with frequent RTI?

The second part of this thesis focused on the influence of genetic variation in innate immunity pathways on susceptibility to RTI. A central player in the innate immune defense is mannose–binding lectin (MBL). This protein has the capacity to bind to a broad range of micro-organisms and subsequently initiate the lectin pathway of complement activation [26;27]. MBL is suggested to contribute to increased infectious susceptibility in case of deficiency, but the results regarding MBL polymorphisms and susceptibility for RTI in children are inconsistent (**Chapter 4**). The PIAMA cohort offered the opportunity to study the effect of MBL polymorphisms prospectively in a large population-based sample of children with Dutch ancestry.

In **Chapter 5** we showed that at population level polymorphisms or haplotypes of *MBL2*, the MBL gene, are not associated with frequent RTI during the first 4 years of life (OR for frequent RTI comparing deficient *MBL2* genotypes with intermediate- plus high-producing *MBL2* genotypes 0.71; 95% CI 0.25-2.05). New in this study was that *MBL2* genotyping was performed according to recent standards, which included an update of recently described functional MBL polymorphisms that might contribute to RTI [28]. To our knowledge we were the first to combine exon 1 and promoter polymorphisms with haplotype-tagging polymorphisms to create haplotypes related to high, intermediate, and deficient MBL serum levels and to study the association between these haplotypes and clinical outcomes. In contrast to two previous studies, we could not confirm an effect of variant *MBL2* genotypes on susceptibility to RTI within year 1 or 2 of life (OR year 1 0.84, 95% CI 0.50-1.41; and OR year 2 1.05, 0.61-1.80) [28;29]. MBL deficiency was previously suggested to be relevant during that particular age period because of the immature adaptive immunity in children below the age of 2 years and consequently larger role of innate immunity. Our results were in line with a recent publication on the absence of associated risk of MBL deficiency and RTI based on a German birth cohort [30], although they did not look at haplotypes as we did. Our results were not in agreement with a study in Eskimo children [29], and another study that found a relation between MBL and susceptibility to recurrent acute otitis media in children 12–24 months of age, but not in older children. The discrepancy in results might be explained by the fact that we studied a large birth cohort representing the general population, whereas the other studies involved Eskimos who have a specific genetic background and a priori high risk of RTI,

and a hospital-based cohort of children with a history of recurrent physician-diagnosed acute otitis media.

Other innate immunity factors like ficolins might also be hypothesized to contribute to susceptibility to RTI. Ficolins represent another part of the lectin pathway of complement activation [27;31]. In **Chapter 6** we studied the association between currently recognized variation in genes encoding Ficolin-2 (*FCN2*) and Ficolin-3 (*FCN3*) and frequency of RTI during the first 4 years of life. In this study only a limited number of polymorphisms were explored because the knowledge on genetic variation within these genes is still emerging and especially *FCN3* is a new area for research. Like MBL, known functional polymorphisms in *FCN2* were included and combined with haplotype-tagging polymorphisms in this gene and in *FCN3*. Neither the studied polymorphisms, nor the haplotypes in the ficolin genes (percentage differences in prevalence of specific haplotypes comparing children with frequent RTI and children without RTI varied between -2, 95% CI -13-9; and 2, -1-5) were associated with frequent RTI during the first 4 years of life. We therefore concluded that at a population level, currently known genetic variations in ficolin genes *FCN2* and *FCN3* do not seem to contribute to the susceptibility to RTI in children.

The *FCN2* and *FCN3* results presented in **Chapter 6** are in line with the MBL results in **Chapter 5**. In our opinion, screening for genetic variation within these genes should at present not be incorporated in the care for children with frequent RTI.

Another important pathway in innate immunity is the Toll-like receptor (TLR) pathway and several studies have reported variation in TLR genes to be associated with respiratory infectious diseases, but all studies explored only a limited set of polymorphisms [32;33]. An extensive range of polymorphisms in the TLR pathway was studied in relation to RTI in **Chapter 7**. In this study, various polymorphisms in 13 different TLR genes, which represent the majority of the TLR pathway, were associated with frequency of RTI during the first 4 years of life. Minor alleles of rs11536878 (*TLR4*) and rs851186 (*TLR5*) were shown to be associated, in a dose-responsive matter, with an increased risk of frequent RTI (relative risk 2.69, 95% CI 1.31-5.50; and 3.31, 1.44-7.61, respectively). TLR4 has been implicated in signal transduction events induced by lipopolysaccharide (LPS) found in most gram-negative bacteria, including nontypeable *Haemophilus influenzae* which is an important respiratory pathogen [34]. Other studies showed the importance of TLR4 in RSV infection and acute otitis media, although the effect of *TLR4* polymorphisms on the latter has to be confirmed by an independent study [18;35;36]. TLR5 recognizes bacterial flagellin, a potent stimulus present in the flagellar structure of many bacteria [37]. Previously, TLR5 has been implicated in the immune response to flagellated pathogens like *Legionella pneumophila* and *Pseudomonas aeruginosa* [38;39]. However, the functional SNPs in *TLR4* and *TLR5* were not associated with frequent RTI in our study, which hampers

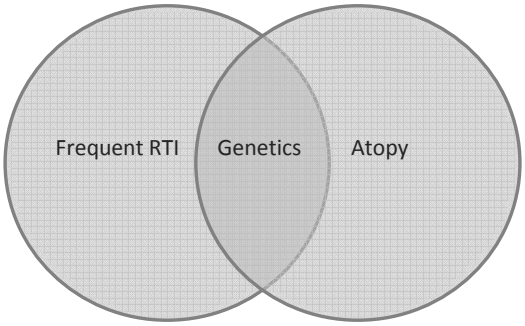
interpretation of the meaning of the observed associations. In our study no pathogen identification was obtained. It should be noted that the actual causal relationship between a specific microbe and a disease is difficult to establish. It has been shown that in 35% of the children with a positive PCR for respiratory viruses on a throat swab, no symptoms of respiratory infection are present [40]. Moreover, in a recent study respiratory pathogens, predominantly rhinovirus, enterovirus and coronaviruses, were found in 40% of the samples of children without respiratory symptoms [41]. This study also showed multiple pathogens in 17% of the symptomatic episodes, compared with 3% of the asymptomatic episodes (an episode was defined as "asymptomatic" if there were no respiratory symptoms during a complete period of 1 week before to 1 week after sampling). These examples illustrate that pathogen information is of limited value if clinical symptoms of respiratory infection are not taken into account. However, especially for genetic studies well defined phenotypes are required, in which case pathogen recognition does have additional value to clinical information [42].

Interestingly, maternal allergy, which is one of the features most commonly used as risk factor for atopy in the child itself, seemed to modify the effect of variation in TLR genes on reported RTI frequency [43;44]. Effect modification by this factor was strongest for rs11536878 in TLR4. Interestingly, variation within this gene has also been associated with risk of asthma [45]. Possibly, the respiratory symptoms in our study, which the parents considered to be a RTI, may in fact have been symptoms of asthma. This would implicate that part of the effect of the associated polymorphisms on the risk of RTI is in fact caused by their effect on the risk of asthma, which on the other hand may have been evoked by an infection [46]. Another possibility is that children from allergic mothers have airways that are more vulnerable to infections than airways of children from non-allergic mothers. Dezateux et al. found a smaller airway caliber and higher airway resistance in healthy infants with a first degree relative with asthma, compared to infants without a positive family history of asthma [47]. The Tucson Children's Respiratory Health Study has shown that small airway caliber and decreased lung function in early life predispose for the development of lower RTI in infancy [48]. We therefore hypothesize that the interaction between TLR polymorphisms and maternal allergy in their effect on frequent RTI can be explained by an intrinsic abnormality of the airways in children with allergic mothers, resulting in an increased vulnerability to RTI. It can also be hypothesized that frequent RTI and atopy have a partly overlapping genetic background (Figure 1). All three hypotheses are in line with previous results in **Chapter 2** and **Chapter 3**.

Within the PIAMA birth cohort associations between innate immunity genes and atopic phenotypes (specific IgE and total IgE) were studied (unpublished data by Reijmerink N. et al.). This study showed different polymorphisms in the TLR-pathway to be important in atopy as compared to our findings for frequent RTI. This shows that overlapping genes

are not found in these genes of the TLR-pathway in our study population, but overlap in other genetic pathways might still be possible, although so far no studies in this direc-

Figure 1. Common genetic predisposition for frequent RTI and atopy?Chapter 2



tion have been published.

Although replication of these findings in future studies is needed, based on our findings we assume that TLR polymorphisms have an impact on infectious susceptibility and symptoms. Moreover, interaction between factors in the TLR genes, infectious susceptibility and factors like atopy need to be evaluated in future research.

Our genetic studies are based on a candidate-gene approach. Candidate-gene studies use information on animal data, clinical data and biological plausibility. Genome-wide studies, in which the whole genome is screened, using hundreds of thousands of single nucleotide polymorphisms (SNPs), have the advantage that a priori no assumption is made about the genes involved. Moreover, with this approach potentially novel or unconsidered genes may be identified. To date only one genetic study with a genome-wide approach is published on infectious diseases. This is a genome-wide association study of major determinants for host control of HIV [49]. Declining costs of genetic research and the expanding field of genotyping technology will definitely lead to more and more applications of genome-wide association analysis in health care in general. However, these studies face a number of challenges, including problems with multiple testing and study design, definition of phenotypes and gene-gene and gene-environment interactions [50]. The problem of multiple testing in genome-wide association studies is major because of the large number of SNPs tested, but it becomes intracTable when gene-gene or gene-environment interactions are allowed. Still, it will be interesting to see how new pathways might be elucidated and how candidate gene studies undertaken till this moment may or may not be supported by genome-wide association studies in the future.

3. Can we predict frequent RTI with a combination of environmental, genetic and other host factors?

For effective preventive measures it is relevant for clinicians to discriminate between children with self-limiting episodes versus those with frequent episodes of RTI which significantly affect their health and quality of life. A prediction rule might help to identify these groups. The coexistence of risk factors in the environment (e.g. day-care attendance, presence of siblings, and ETS exposure), host factors (e.g. age), and genetically predisposing factors (e.g. atopy, polymorphisms in innate immunity genes especially in the TLR pathway), may together lead to a more RTI-prone condition. In **Chapter 8** we used the findings of **Chapter 5** and **Chapter 7** in combination with well-known environmental risk factors in order to develop such a prediction rule for frequent RTI. New in this prognostic study was that we studied the predictive value of genetic variables next to the predictive value of well-known questionnaire-derived variables for frequent RTI. We found that adding genetic information to the model did not result in an improvement of the performance of the potential prediction rule for frequent RTI during the first 4 years of life. However, a prediction rule based on only genetic information has a similar performance as a prediction rule based on environmental and host factors (performance measured as area under the ROC curve). In this study only the MBL and TLR genes were taken into account. Many genes and polymorphisms which influence infectious susceptibility still have to be discovered. Previously, we found maternal allergy, which is a well-recognized risk factor for allergic diseases, to modify the effect of variation in TLR genes on RTI, especially for rs11536878 in *TLR4* (**Chapter 7**). Therefore, we tested the interaction between maternal allergy and the genetic factors in the present study, but found no significant interaction by this factor. However, these analyses are extremely complex, multiple gene-gene and gene-environment interactions are possible and very large study population are needed since in general single genetic loci make only modest contributions to occurrence of disease [42].

Overall, we have to conclude that adequate prediction of frequent RTI is not possible based on the variables collected in our cohort. Therefore, our prediction rule has no value in clinical practice. Previous prognostic studies showed similar results [51-54]. Future research should try to tackle this problem, since prognostic data are pivotal for early indication of which child may need (preventive) treatment.

Conclusions and future research

Our main findings are:

- Intra-uterine ETS exposure is related to frequent RTI during preschool-years in children with early signs of atopy

- Serum IgE and skin prick test are related to frequent RTI in school-aged children from allergic mothers
- Review of the literature shows that the association between variation in the *MBL2* gene and RTI in children remains controversial; therefore, large prospective cohort studies with regular documentation of RTI and possible confounders are required
- Genetic variation in *MBL2, FCN2* and *FCN3* is not related to RTI in our large prospective cohort of Dutch children
- Variation in the genes *TLR4* and *TLR5* is associated with frequent RTI during preschool-years and this association seems to be influenced by maternal allergy
- Adequate prediction of the occurrence of frequent RTI in preschool years in our population is accomplished neither by using questionnaire-derived variables, genetic variables nor by a combination of both

Future epidemiological research into the background of frequent RTI in childhood years should focus on interactions between known environmental risk factors for RTI with each other and host factors such as atopy. Future genetic studies should be aimed at replication of our findings in *TLR4* and *TLR5* to confirm the association between genetic variation in these genes of the TLR-pathway and the occurrence of frequent RTI. Furthermore, functional studies have to investigate the exact biological mechanisms underlying this association. Moreover, focus should be on gene-gene interactions between innate immunity genes related to both infectious and atopic phenotypes, since the influence of allergy or an allergic predisposition on risk factors for frequent RTI has been shown in this thesis. In addition, interactions between genes and environmental factors associated with RTI such as exposure to environmental tobacco smoke (gene-environment interaction) should be taken into account in future studies. To clarify these interactions, large population-based cohort studies are required with a well defined RTI phenotype based on the combination of questionnaire information on symptoms and doctor's diagnosis of RTI, next to pathogen identification. Finally, we should make an effort to combine epidemiological and genetic data in upcoming prognostic studies. Focus should be on translation of genetic data into preventive or therapeutic interventions, which at this moment is not realized in clinical practice.

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Nederlandse samenvatting

NEDERLANDSE SAMENVATTING

Luchtweginfecties (LWI) komen erg veel voor op de kinderleeftijd. Dit is te zien aan het feit dat LWI de meest voorkomende reden zijn waarvoor ouders met een nog jong kind een arts bezoeken. Meestal betreffen het infecties van de bovenste luchtwegen, zoals verkoudheid, keelontsteking en middenoorontsteking, maar infecties van de lagere luchtwegen komen ook frequent voor. Helaas zijn wij momenteel niet in staat om een goed onderscheid te maken tussen kinderen die zeer frequente LWI ontwikkelen en mogelijk baat zullen hebben bij behandeling met antibiotica of verwijzing naar keel-neus-oor-arts voor trommelvliesbuisjes, adenotomie of tonsillectomie, en kinderen bij wie we beter afwachtend kunnen zijn aangezien het spontaan beter zal gaan en deze interventies geen gunstig effect zullen laten zien. Onderzoek naar risicofactoren van het ontwikkelen van frequente LWI is dan ook noodzakelijk.

In dit proefschrift werd het effect van zowel algemene omgevings- en gastheer factoren, als plausibele genetische factoren in het optreden van frequente luchtweginfecties (LWI) bestudeerd in een grote Nederlandse geboortecohort-studie, namelijk de Preventie en Incidentie van Astma en Mijt Allergie (PIAMA) studie.
Hierbij richtten we ons op 3 hoofdvragen over frequente LWI:

1. Wat is de rol van diverse omgevings- en gastheer factoren in het optreden van frequente LWI?
2. Kunnen we erfelijke factoren identificeren in de aangeboren afweer die geassocieerd zijn met frequente LWI?
3. Kunnen we frequente LWI voorspellen met een combinatie van omgevings-, erfelijke en andere gastheer factoren?

Ad vraag 1: Wat is de rol van diverse omgevings- en gastheer factoren in het optreden van frequente LWI?

In **Hoofdstuk 2** bestudeerden we de relatie tussen atopie, blootstelling aan tabaksrook en de interactie tussen de laatste twee in het optreden van frequente LWI. We lieten zien dat kinderen met vroege tekenen van atopie (hoog IgE in de eerste week na geboorte of eczeem op de leeftijd van 3 maanden) die intra-uterien blootgesteld werden aan tabaksrook een verhoogd risico hadden op frequente LWI in de eerste 4 levensjaren. Daarentegen was intra-uteriene blootstelling aan tabaksrook niet gerelateerd aan frequente LWI in kinderen zonder deze vroege markers van atopie. Alhoewel de relatie tussen intra-uteriene blootstelling aan tabaksrook en LWI eerder reeds werd

aangetoond, zijn wij de eersten die beschrijven dat het verhoogde risico van expositie aan tabaksrook vooral lijkt op te treden in kinderen met een atopische constitutie.

Vervolgens werd in **Hoofdstuk 3** de mogelijke associatie tussen atopie op zich zelf en frequente LWI onderzocht. We lieten zien dat atopie op de leeftijd van 4 jaar, gedefinieerd als positief specifiek IgE of verhoogd totaal IgE in serum, niet geassocieerd was met frequente LWI in 5 tot en met 8 jaar oude kinderen van moeders zonder bekende allergie. Ook vonden we geen associatie in kinderen van niet-allergische moeders tussen deze atopische markers of een positieve huidpriktest op de leeftijd van 8 jaar en frequente LWI. Echter, bij kinderen met een allergische moeder bleken een verhoogd totaal IgE op de leeftijd van 4 jaar en een positief specifiek IgE of huidpriktest op de leeftijd van 8 jaar wel geassocieerd te zijn met frequente LWI. Deze resultaten laten zien dat atopie de vatbaarheid voor LWI in sommige kinderen beïnvloedt, maar dat het effect beperkt lijkt te blijven tot hoog-risico kinderen met een genetische predispositie voor atopie. Een mogelijk verklaring voor dit gegeven kan zijn dat er een overlap bestaat in de genetische achtergrond van atopie en LWI. Kandidaat genen voor atopie en LWI kunnen worden gevonden in de aangeboren afweer, zoals Toll-like receptoren (TLR). Dit werd verder bestudeerd in **Hoofdstuk 7**.

Als er inderdaad een oorzakelijke relatie bestaat tussen atopie en LWI, dan kan dit belangrijke therapeutische consequenties hebben. Preventie van LWI in atopische kinderen met anti-allergische of anti-inflammatoire middelen zou dan de voorkeur hebben boven antibiotica.

Ad vraag 2: Kunnen we erfelijke factoren identificeren in de aangeboren afweer die geassocieerd zijn met frequente LWI?

Het tweede deel van dit proefschrift betreft de invloed van variatie in genen van de aangeboren afweer. Een belangrijk onderdeel van de aangeboren afweer is mannose-bindend lectine (MBL). Dit eiwit kan binden aan vele micro-organismen, waarna het de lectine route van complement activatie initieert. Eerdere studies suggereren dat MBL deficiëntie kan leiden tot een verhoogde gevoeligheid voor infecties, maar de relatie tussen genetische variaties leidend tot MBL deficiëntie en een associatie met LWI werd niet eenduidig gevonden. Een overzicht van eerdere studies naar deze associatie is beschreven in **Hoofdstuk 4**.

In **Hoofdstuk 5** toonden we aan dat genetische variatie in *MBL2*, het gen dat codeert voor MBL, niet geassocieerd was met frequente LWI tijdens de eerste 4 levensjaren. Nieuw in deze studie was dat we MBL haplotypen hebben bepaald met behulp van exon 1 en promoter variaties, die gerelateerd zijn aan hoge, gemiddelde en lage MBL serum niveaus. In tegenstelling tot 2 eerdere studies vonden wij geen relatie tussen *MBL2*

variaties en LWI tijdens jaar 1 of jaar 2. MBL deficiëntie werd gesuggereerd juist in deze tijdsperiode belangrijk te zijn in verband met de onrijpe specifieke afweer in kinderen jonger dan 2 jaar.

Een ander onderdeel van de aangeboren afweer dat een rol zou kunnen spelen bij het ontwikkelen van frequente LWI bestaat uit de ficolines. Deze maken net als MBL deel uit van de lectine route van complement activatie. In **Hoofdstuk 6** bestudeerden wij de associatie tussen de thans bekende genetische variaties in de genen coderend voor Ficolin-2 (*FCN2*) en Ficolin-3 (*FCN3*) en frequente LWI. Samenvattend lieten we zien dat de bestudeerde genetische variaties in de ficoline genen niet geassocieerd waren met frequente LWI gedurende de eerste 4 levensjaren. Naar onze mening moet screening naar genetische variatie binnen deze genen momenteel dan ook niet opgenomen worden in de diagnostiek bij kinderen met frequente LWI.

Een ander belangrijk onderdeel van de aangeboren afweer wordt gevormd door de Toll-like receptoren (TLR). Meerdere studies rapporteerden dat variatie in TLR genen geassocieerd is met infecties van de luchtwegen. Echter, al deze studies bekeken slechts een klein gedeelte van de bekende variaties in deze groep genen. Wij onderzochten een uitgebreidere selectie van variaties in deze genen (13 in totaal) in relatie tot LWI in **Hoofdstuk 7**. We vonden dat een specifieke variatie in *TLR4* (namelijk rs11536878) en een in *TLR5* (rs85186) beide geassocieerd waren met het voorkomen van frequente LWI gedurende de eerste 4 levensjaren. TLR4 is betrokken bij de signaaltransductie van LPS, dat wordt gevonden in de meeste gram-negatieve bacteriën, zoals het belangrijke luchtweg pathogeen *Haemophilus influenzae*. TLR5 herkent flagelline, dat kan worden gevonden in pathogenen als *Legionella pneumophila* en *Pseudomonas aeruginosa*.

Opvallend was dat een bekende allergie bij de moeder het effect van de variatie in TLR genen op LWI leek te beïnvloeden. Dit werd vooral gevonden voor rs11536878 in *TLR4*. Variatie binnen dit gen werd eerder geassocieerd met risico op astma. Een mogelijke verklaring zou kunnen zijn dat kinderen van allergische moeders luchtwegen hebben die meer kwetsbaar zijn voor LWI dan luchtwegen van kinderen van niet-allergische moeders. Een andere verklaring zou kunnen zijn dat de genetische achtergrond van allergie/astma en LWI gedeeltelijk met elkaar overlappen. Beide hypotheses zijn in lijn met **Hoofdstuk 2** en 3. Alhoewel replicatie van deze resultaten in toekomstige studies noodzakelijk is, suggereren onze bevindingen dat variatie in TLR genen invloed heeft op individuele vatbaarheid voor LWI. De interactie tussen verschillende factoren in deze genen en andere factoren zoals atopie moet verder worden uitgezocht in toekomstig onderzoek.

Ad vraag 3: Kunnen we frequente LWI voorspellen met een combinatie van omgevings-, erfelijke en andere gastheer factoren?

Voor effectieve preventie moet men een onderscheid kunnen maken tussen kinderen met spontaan voorbijgaande infecties waar ze niet heel veel last van hebben en kinderen met frequente LWI die op belangrijke wijze de gezondheid en kwaliteit van leven beïnvloeden. Een predictie-regel kan helpen deze groepen te identificeren.

In **Hoofdstuk 8** hebben we de eerdere bevindingen uit **Hoofdstuk 5** en 7 gebruikt in combinatie met bekende risicofactoren vanuit de omgeving om te trachten een predictie-regel te vormen waarmee frequente LWI kunnen worden voorspeld. Nieuw in deze studie was dat we de voorspellende waarde van genetische variabelen hebben gebruikt naast de voorspellende waarde van algemeen bekende risicofactoren voor LWI waarvan we de gegevens haalden uit de vragenlijsten. We vonden dat het toevoegen van genetische informatie de werking van de mogelijke predictie-regel voor frequente LWI tijdens de eerste 4 levensjaren niet duidelijk verbeterde. Echter, een predictie-regel gebaseerd op alleen genetische informatie heeft wel een vergelijkbare werking als een regel gebaseerd op omgevings- en gastheer factoren. Samengevat concludeerden wij dat het niet mogelijk is om frequente LWI adequaat te voorspellen o.b.v. variabelen die werden verzameld binnen ons cohort. Het is mogelijk dat toekomstige studies met meerdere tot op heden nog onbekende genetische factoren wel in staat zijn frequente LWI op een betere manier te voorspellen.

Conclusies en toekomstig onderzoek

De belangrijkste bevindingen van dit proefschrift zijn:

- Intra-uteriene blootstelling aan tabaksrook is gerelateerd aan frequente LWI gedurende de eerste vier levensjaren bij kinderen met vroege tekenen van atopie
- Verhoogd serum IgE en positieve huidpriktest zijn geassocieerd met het optreden van frequente LWI gedurende het vijfde tot en met het achtste levensjaar bij kinderen van moeders met een bekende allergie
- Een overzicht van de literatuur laat zien dat de associatie tussen variatie in het *MBL2* gen en LWI controversieel blijft
- Variatie in de genen *MBL2, FCN2* en *FCN3* is niet geassocieerd met frequente LWI in jaar 1 t/m 4 in ons grote cohort van Nederlandse kinderen
- Variatie in de genen *TLR4* en *TLR5* is geassocieerd met frequente LWI in jaar 1 t/m 4 en deze relatie lijkt te worden beïnvloed door een bekende allergie bij de moeder
- Adequaat voorspellen van frequente LWI in jaar 1 t/m 4 in onze populatie kon niet worden bereikt, zowel niet m.b.v. vragenlijst gegevens als met genetische gegevens of een combinatie van deze twee

Het lijkt zinvol om toekomstig onderzoek naar de achtergrond van frequente LWI te concentreren op interacties tussen risicofactoren uit de omgeving en gastheer factoren zoals genetische variatie. De nadruk zou kunnen liggen op gen-gen interacties tussen genen van de aangeboren afweer met betrokkenheid bij infecties en allergie, aangezien de invloed van allergie of een allergische predispositie op het effect van risicofactoren voor frequente LWI is aangetoond in dit proefschrift. Om deze interacties te verduidelijken zijn grote cohort studies noodzakelijk met een duidelijke definitie van LWI gebaseerd op vragenlijstgegevens in combinatie met een dokter's diagnose en identificatie van de oorzakelijke pathogenen. Tot slot zouden we moeten trachten om genetische data te vertalen in preventieve of therapeutische interventies.

List of publications

LIST OF PUBLICATIONS

This thesis

1. Ruskamp JM, Hoekstra MO, Postma DS, Kerkhof M, Bottema RW, Koppelman GH, Rovers MM, Wijga AH, de Jongste JC, Brunekreef B, Sanders EAM. Exploring the role of polymorphisms in ficolin genes in respiratory tract infections. Clin Exp Immunol 2009;155(3):433-40.
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Other

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