INNATE AND ADAPTIVE IMMUNITY IN OTITIS MEDIA

SELMA WIERTSEMA

INNATE AND ADAPTIVE IMMUNITY IN OTITIS MEDIA

Cover design Matthijs Smit
Layout Martijn Senden
Print Haveka
ISBN 90-393-4068-4

INNATE AND ADAPTIVE IMMUNITY IN OTITIS MEDIA

Aangeboren en Specifieke Immuniteit in Otitis Media

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht
op gezag van de Rector Magnificus, Prof. Dr. W.H. Gispen,
ingevolge het besluit van het College voor Promoties in het openbaar te verdedigen
op vrijdag 11 november 2005 des namiddags te 12.45 uur

door Selma Paulien Wiertsema
geboren op 31 maart 1977, te Bunnik

Promoter	Prof. Dr. E.A.M. Sanders
Co-promoter	Dr. ir. G.T. Rijkers

The research described in this thesis was financially supported by ZonMw, Organisation for Health Research and Development (grant numbers 002828480 and 90461092), the Netherlands Health Insurance Company Zilveren Kruis-Achmea, an Australian-Europe Scholarship funded by the Australian Government through the Department of Education, Science and Training and the Ter Meulen Fund, Royal Netherlands Academy of Arts and Sciences.

The printing of this thesis was financially supported by Atos Medical BV, Beltone Netherlands BV, Dr. Ir. van de Laar Stichting, Edward Jenner Stichting, EmiD-Audiologische apparatuur, Eijkman Graduate School, Genzyme Nederland, Innogenetics, J.E. Jurriaanse Stichting, Universiteit Utrecht, Nederlandse Vereniging voor Slechthorenden, Pharmacia Diagnostics, Sanofi Pasteur MSD, Sanquin Reagents, Stichting Atze Spoor Fonds, Wyeth Pharmaceuticals Nederland, and ZonMw Organisation for Health Research and Development.

Journeys are the midwives of thought. Few places are more conducive to internal conversations than a moving plane, ship or train. There is an almost quaint correlation between what is in front of our eyes and the thoughts we are able to have in our heads: large thoughts at times requiring large views, new thoughts new places.

Alain de Botton - The art of travel

Voor mijn ouders

Commissie	Prof. Dr. M.J.M. Bonten
	Prof. Dr. J.Th. van Dissel
	Prof. Dr. J.L.L. Kimpen
	Prof. Dr. L. Koenderman
	Prof. Dr. J.G.J. van de Winkel
Paranimfen	Vanessa Walraven
	Emo Wiertsema

Contents

1

General introduction

Based on the review
Immunologic screening of children with recurrent otitis media

Selma P. Wiertsema[1], Reinier H. Veenhoven[2], Elisabeth A.M. Sanders[1], Ger T. Rijkers[1]

1 Department of Pediatric Immunology, University Medical Center Utrecht, the Netherlands
2 Department of Pediatrics, Spaarne Hospital, Hoofddorp, the Netherlands

Curr Allergy Asthma Rep. 2005 Jul;5(4): 302-7.

Otitis media: Epidemiology and background

Otitis media, or middle ear infection, is the most frequent childhood infectious disease and the primary reason for a child under 3 years of age to visit the general practitioner. In many countries, including the Netherlands, it is the most common indication for the prescription of antibiotics.[1,2] In the mid 1990's it was estimated that there were 14 million otitis episodes per annum in the USA, with total medical costs of around $US 3-5 billion for children under the age of 5.[3] The true financial impact however will be much more, because indirect costs, like parents staying at home from work, are not included in these calculations.[4]

Otitis media (OM) is the common denominator for a variety of related middle ear diseases. There are three main categories of OM: acute otitis media (AOM), otitis media with effusion (OME) and chronic suppurative otitis media (CSOM).[5] AOM is generally defined as the presence of middle ear effusion accompanied with one or more signs of acute inflammation of the middle ear, such as otalgia, new onset otorrhea, fever and malaise or irritability of the child.[6] OME is also characterized by the presence of middle ear fluid, but without the symptoms of acute inflammation.[6] OME can develop as a sequel to AOM, when effusion persists after clearance of the infection, but it can also develop *de novo*, especially in the first year of life.[7-10] The exact pathogenesis of *de novo* OME remains poorly understood.[11] CSOM is a persistent, at least 6 weeks to 3 months, inflammation of the middle ear in the presence of a perforated tympanic membrane and discharge.[12]

Approximately 80% of all children have had at least one episode of AOM by 3 years of age, with a peak incidence between 6 and 18 months.[13-15] Recurrent AOM is generally defined as having had 3 or more episodes of AOM within a period of 6 months or 4 or more episodes in 12 months, which around 10-20% of children are prone to develop.[16-18] For OME it has been estimated that more than 50% of children have experienced at least one episode by their first birthday[14] and that 30-40% of children have recurrent episodes of OME .[19]

Approximately 80% of AOM cases resolve spontaneously within 2-14 days and also OME is characterized by a self-liming nature: 60% of newly detected OME resolves within 3 months.[19,20] Antibiotic treatment has a positive but limited effect for both AOM and OME.[21,22] These high rates of spontaneous resolution plus the limited benefits of antibiotic treatment suggest that children with AOM or OME are suitable for initial observation, without instant anti-microbial treatment. Children diagnosed with AOM do need adequate analgesics, especially for the first 24h after diagnosis, and observation is advised only when appropriate follow-up and communication can be assured.[23] If symptoms worsen or do not improve within 48-72 hours, children should receive prompt antibiotic treatment to fight the infection and prevent complications. Medical treatment for OME is appropriate only if clinical and persistent benefits can be achieved beyond spontaneous resolution, and thus otherwise healthy children with OME should be observed for 3 months or longer before intervention is considered.[23] Surgical treatment with tympanostomy tubes does decrease recurrence of AOM and reduces middle ear effusion prevalence in children with a history of recurrent AOM.[24] In OME only short-term improvements of tympanostomy tube insertion have been reported on hearing, language and developmental outcomes.[25-30] Similarly the benefits of adenoidectomy as treatment for OME

and AOM are modest.[25,28,31-34]

The impact of treatment from child to child is likely to be highly variable, and thus identification of children to benefit mostly of antibiotic or surgical treatment is of prime importance.[23] For children with recurrent OM, those at increased risk to develop complications like acute mastoiditis, facial paralysis, and external otitis[35] or those children suffering from associated symptoms like otalgia, hearing loss or a delay in speak and language development,[23] treatment would be required. The controversial results on the treatment of OM have prompted the search for widely useable preventive strategies like vaccination to manage recurrent OM.

Host risk factors in the development of OM

The pathogenesis of AOM is multi-factorial, mainly including factors related to immune responses and environmental microbial load (figure 1). Also the onset of OME, although it has fewer symptoms than AOM, probably involves a similar multi-factorial sequence of events.

Innate immune systems including mucosal defense, pathogen recognition receptors, and complement, and adaptive immune responses, such as local production of secretory IgA, systemically produced IgG and the production of cytokines, together play an important role in the protection against recurrent OM.[36-40] Therefore, an immature immune system as seen in young children or defective immunity are predisposing factors in the aetiology of OM.[41,42]

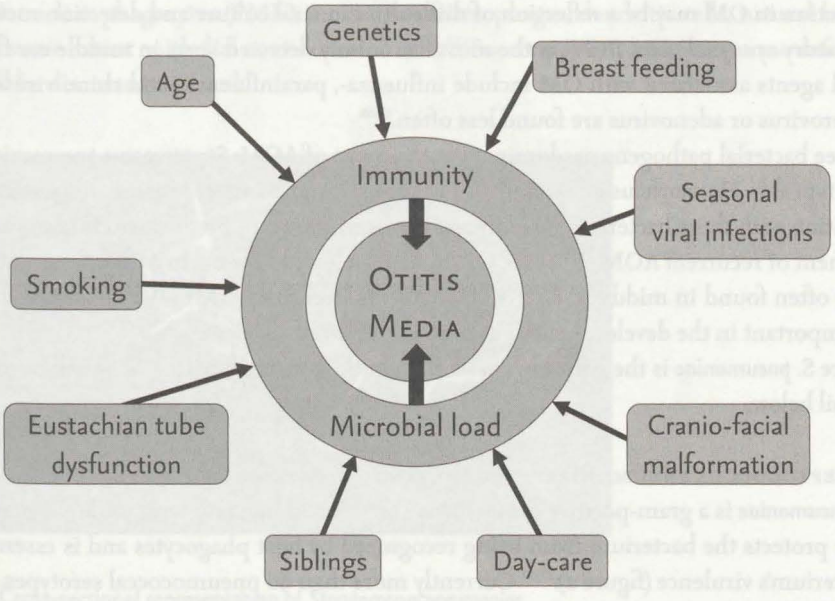


Figure 1 Risk factors, to a certain degree related to immunity and microbial load, which may be involved in the development of OM.

Twin studies suggest a marked genetic component in the development of OM.[43,44] Furthermore, genetic factors, like male gender and HLA type, predispose to get recurrent OM.[45-48] The fact that OM is more common in certain populations like Alaska Inuits, Australian Aborigines, and some Native Americans, also points to a genetic component in OM susceptibility.[49-53] The genetics of OM are, however, complex with many genes probably contributing.
The microbial burden from older siblings or day-care attendance, environmental factors like cigarette smoke exposure, the use of pacifiers and the withdrawal of breast milk with its immunological properties, are all associated with an increased risk for OM.[14,54-59]
Anatomical factors are important in the development of OM and an increased incidence of OM is seen in patients with cranio-facial abnormalities.[60-62] The Eustachian tube (ET) with its ventilatory, protective and clearance function plays a role in maintaining middle-ear health. Infants and young children have a poorly functioning short, floppy, and horizontal ET. These ET characteristics might contribute to the beginning of OM, but whether it is a main cause in the majority of children with OM is doubtful.[42,63,64]

Microbiology of OM: viruses and bacteria

Respiratory viruses play a role in the aetio-pathogenesis of AOM. Viruses may cause Eustachian tube dysfunction, increase adherence of bacteria to epithelial cells resulting in a rise in bacterial colonization of the nasopharynx, and may modulate the host's immune function.[65] Respiratory viruses alone account for approximately 30% of OM cases. This apparent low incidence of viral infection in OM may be a reflection of difficulties in viral culture and detection methods.[66] Respiratory syncytial virus (RSV) is the most commonly detected virus in middle ear fluid. Other viral agents associated with OM include influenza-, parainfluenza- and rhino-viruses, whereas enterovirus or adenovirus are found less often.[67,68]
Three bacterial pathogens predominate as the cause of AOM: *Streptococcus pneumoniae* (25-50%), nontypeable *Haemophilus influenzae* (NTHi) (15-30%) and *Moraxella catarrhalis* (3-20%).[69-72] Colonization with these bacteria at an early age has been shown to predispose children to the development of recurrent AOM.[73,74] Since OME can develop as a sequel to AOM, these pathogens are also often found in middle ear effusions from children with OME. Whether these bacteria are as important in the development of *de novo* OME, is not yet clear.[11,42]
Since *S. pneumoniae* is the pathogen most frequently associated with OM, it will be discussed in detail below.

Streptococcus pneumoniae: nasopharyngeal carriage and disease

S. pneumoniae is a gram-positive diplococcus surrounded by a polysaccharide capsule. This capsule protects the bacterium from being recognized by host phagocytes and is essential for the bacterium's virulence (figure 2).[75-77] Currently more than 90 pneumococcal serotypes, which differ in the chemical composition of their polysaccharide capsule, have been identified.[78]
S. pneumoniae is a common component of the nasopharyngeal flora in healthy individuals. The dynamic process of periodical colonization of the upper respiratory tract with *S. pneumoniae*

begins soon after birth, with an increase in carriage during the first year of life. At the age of one year, about 50% of children have been colonized with S. *pneumoniae* at least once. From age 3-5 years carriage declines to a rate of 20-30% in adults.[74,79] The duration of pneumococcal colonization depends on the pneumococcal serotype and age of the host. In children younger than 1 year median nasopharyngeal carriage persists for around 30 days, whereas in adults this is approximately two weeks.[80] When mucociliary clearance is impaired, colonization is followed by rapid replication and clinical infection. The bacterium then vigorously activates inflammatory mediators, which further impairs clearance mechanisms, leading to local and systemic symptoms.[77] In case the bacteria reach the middle ear cavity via the Eustachian tube, S. *pneumoniae* can cause AOM.

Innate immune defense in OM

The innate immune system is an evolutionary ancient form of immunity and offers the main resistance to microbial pathogens within the first minutes, hours or days of an infection.[81] Epithelial surfaces with mechanical barriers and digestive enzymes form the first barrier against pathogens causing OM. However, when microbes do penetrate the body, defensive systems capable of distinguishing pathogens from self-structures are required. This recognition of pathogens is mediated by a set of pattern-recognition receptors (PRRs) that bind conserved pathogen-associated molecular patterns (PAMPs), which are shared by broad classes of microorganisms.[82] The PRRs are expressed on a wide variety of cells of the innate immune system, including polymorphonuclear phagocytes (PMN), monocytes/macrophages, dendritic cells, natural killer cells and to some extent epithelial or endothelial cells.[83] Two important innate immune defense systems will be discussed in detail.

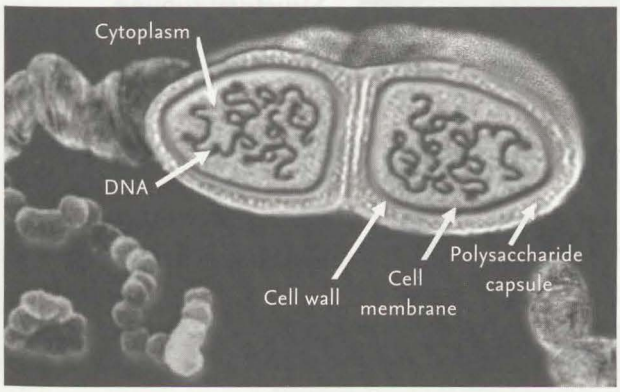


Figure 2 Cross-sectional representation of Streptococcus pneumoniae.
The bacterium is surrounded by a cell membrane, a cell wall and a polysaccharide capsule. The polysaccharide capsule is the main virulence factor of the bacterium, and protects it from phagocytosis by the host. Courtesy United States National Foundation for Infectious Diseases (www.nfid.org).

Mannan-binding lectin

The collectins and ficolins are important PRRs that mediate phagocytic uptake.[84] An important constituent of the collectin family is mannan-binding lectin (MBL). An MBL polypeptide is made up of a collagenous structure and a C-type lectin carbohydrate-recognition domain (CRD).[85] Three polypeptides fold together to form a structural subunit.[86] Oligomers of 3-6 of these structural subunits produce functional human MBL (figure 3).[87]

Upon recognition of an infectious agent, MBL together with MBL-associated serine proteases (MASPs) initiates the lectin pathway of complement activation.[87] Next to the classical and the alternative complement activation pathway, this pathway is now recognized as the third complement activation route.[88-90] The activated complement system has three main biological activities: 1) opsonization of pathogens, 2) chemotaxis and activation of leukocytes, and 3) direct killing of pathogens (figure 4).[91] The importance of MBL is underlined by several clinical studies that link low serum MBL levels with increased susceptibility to various infectious diseases.[92-98] Functional polymorphisms in the MBL2 gene encoding MBL have been described to determine the MBL serum level and could thus possibly also play a role in the development of recurrent OM.[99-103]

CD14 and Toll-like receptors

CD14 was initially characterized as the endotoxin/lipopolysaccharide (LPS) receptor.[104] Howev-

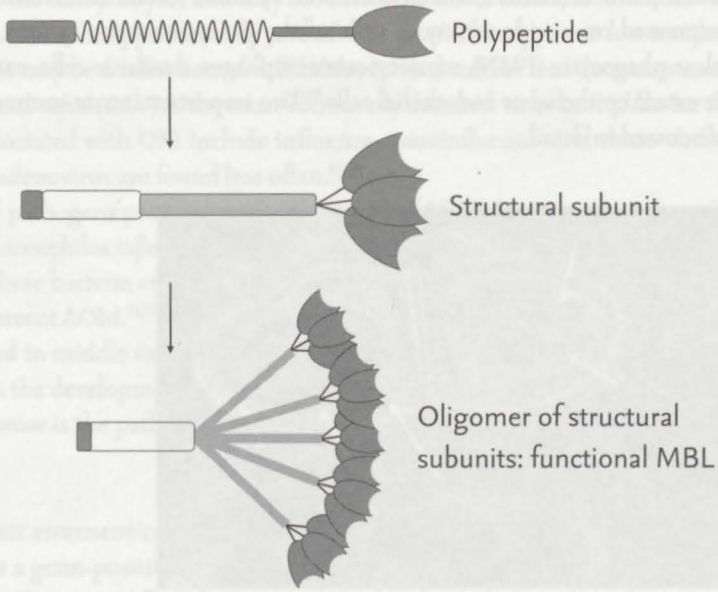


Figure 3 Structure of the Mannan-binding lectin molecule.
The MBL2 gene encodes for single MBL polypeptides. Three polypeptides fold together to form a structural subunit. Oligomers of 3 to 6 structural subunits together produce functional MBL.

er, evidence evolves suggesting CD14 is also involved in responses to lipoteichoic acid of gram-positive bacteria,[105] peptidoglycan of both gram-positive and gram-negative bacteria,[106] mycobacteria and possibly viruses.[107] Furthermore, involvement of CD14 in macrophage responses against purified capsular polysaccharide of *S. pneumoniae* has been described.[108] CD14 thus seems to play a central role in the first innate immune defense against many upper respiratory tract pathogens.[109]

Signal transduction of CD14 is mediated through a complex of Toll-like receptor 4 (TLR4) and MD-2.[110] TLRs are the principal membrane signaling molecules through which mammals sense infection and binding of a ligand to TLRs leads to activation of pro-inflammatory pathways.[111-113] A functional polymorphism in the CD14 promoter is known to exist, determining soluble CD14 levels.[114] This polymorphism has already been associated with several immunological disorders like allergy, atopy, tuberculosis and rheumatoid arthritis.[115-118] Since CD14 is important in the immune defense against many OM associated pathogens, functional CD 14 polymorphisms could be of importance in the development of OM.

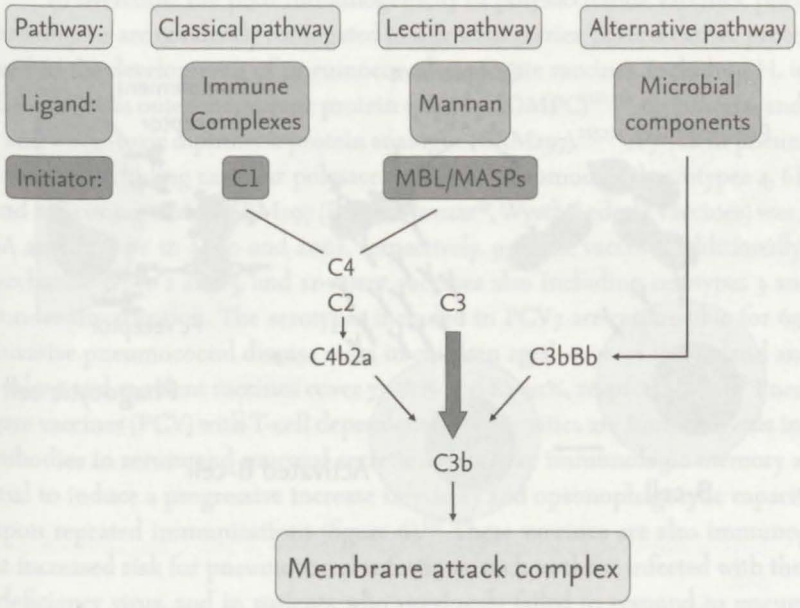


Figure 4 The three principal pathways and major components of the complement cascade.

Adaptive immune defense in OM

Next to the above-described innate immune defense systems, adaptive immunity plays a profound role in the protection against OM (figure 5). The role of antibodies directed against the pneumococcal capsular polysaccharide, induced after natural encounter with the pathogen and/or after vaccination, and Fc receptors expressed on phagocytic cells that interact with these antibodies will be discussed in detail below.

Pneumococcal serotype specific antibodies

Protective antibodies against encapsulated bacteria are directed against the bacterial polysaccharide capsule and are serotype specific. Polysaccharides are able to induce antibody responses in the absence of T-cells and are referred to as T-cell independent type 2 (TI-2) antigens. A number of features distinguish TI-2 antigens from proteins, which are classified as T-cell dependent (TD) antigens.[119] TI-2 antigens are high molecular weight molecules, carrying repeating epitopes, which are poorly metabolized.[120] TI-2 antigens do not induce classical immunologic memory and no avidity maturation is observed after repeated antigenic stimulus with TI-2 antigens.[121,122] Furthermore, the immune response to TI-2 antigens develops late in ontogeny, around 18-24 months of age, compared to TD antigens.[123]
Effective phagocytosis of pneumococci by PMN depends primarily of IgG_2-anti-pneumococcal antibodies in several in-vitro studies.[124-127] In children with recurrent OM both increased as well

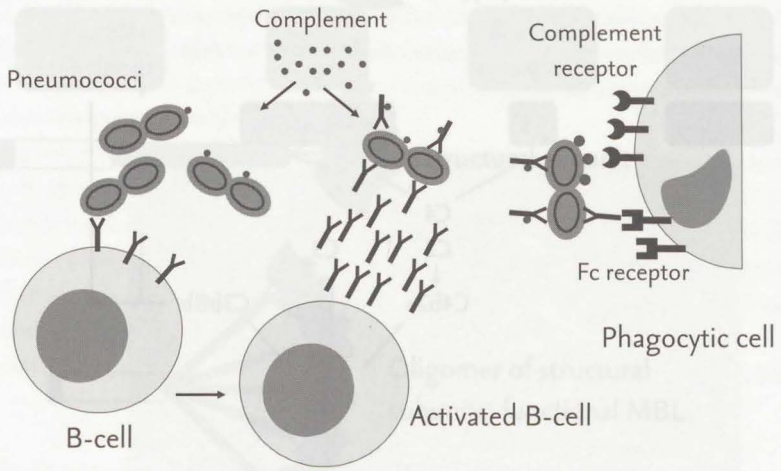


Figure 5 Main immune defense mechanisms against S. pneumoniae.
Immunity against S. pneumoniae mainly depends on complement and antibody mediated phagocytosis through complement- and Fc receptors expressed on neutrophils and monocytes/macrophages.

as normal serum IgA, IgM, IgG and IgG_1 levels, and for IgG_2 both normal as well as low values have been found.[128-132] Pneumococcal serotype-specific IgG and IgG_2 antibody titers seem to be lower in children with frequent recurrent AOM compared to healthy controls.[133-135]

Pneumococcal vaccines

Antibodies can be elicited upon both natural encounter with the pathogen or by vaccination. To protect from pneumococcal disease several vaccines based on the capsular polysaccharides have been developed. A first pneumococcal polysaccharide vaccine for the prevention of invasive pneumococcal infections was marketed as early as 1946. The currently available 23-valent pneumococcal polysaccharide vaccine (PPV23) contains 25 μg of capsular polysaccharides of each of the pneumococcal serotypes 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19F, 19A, 20, 22F, 23F and 33F. These serotypes were chosen on the basis of the relative distribution of the individual serotypes that cause around 90% of invasive pneumococcal infections.[136-141] Immuno-competent healthy adults produce protective anti-pneumococcal antibody titers two to three weeks after vaccination with PPV23. All immunoglobulin classes are included in these responses, although among IgG subclasses, IgG_2 predominates. In children below 2 years of age the immunogenicity of polysaccharide vaccines is poor because of the T-cell independent character of polysaccharide antigens (figure 6).[123] Clinical effectiveness of 8- and 14-valent pneumococcal polysaccharide vaccines in children has been evaluated in trials in the early 1980's, and was at best moderate in children older than 2 years of age and only marginal in younger children.[142-149] To overcome the poor immunogenicity of polysaccharide vaccines, pneumococcal capsular antigens are covalently conjugated to a protein carrier. Several carrier proteins have been utilized in the development of pneumococcal conjugate vaccines, including H. *influenzae* protein, N. *meningitidis* outer-membrane protein complex (OMPC)[150,151], diphtheria- and tetanus toxoids,[152] and a non-toxic diphtheria protein analogue (CRM197).[153,154] A 7-valent pneumococcal conjugate vaccine including capsular polysaccharides of pneumococcal serotypes 4, 6B, 9V, 14, 18C, 19F and 23F conjugated to CRM197 (PCV7, Prevnar®, Wyeth Lederle Vaccines) was licensed in the USA and Europe in 2000 and 2001, respectively. 9-valent vaccines additionally including polysaccharide types 1 and 5, and 11-valent vaccines also including serotypes 3 and 7F are currently under investigation. The serotypes included in PCV7 are responsible for 69-79% of reported invasive pneumococcal disease cases in children aged <5 years in England and Wales, and the 9-valent and 11-valent vaccines cover 77-87% and 82-91%, respectively.[155,156] Pneumococcal conjugate vaccines (PCV) with T-cell dependent characteristics are immunogenic in infants, induce antibodies in serum and mucosal secretions, provoke immunologic memory and have the potential to induce a progressive increase in avidity and opsonophagocytic capacity of antibodies upon repeated immunizations (figure 6).[157] These vaccines are also immunogenic in children at increased risk for pneumococcal infections, such as those infected with the human immuno-deficiency virus, and in patients who previously failed to respond to pneumococcal polysaccharide vaccinations.[158-160] Furthermore, PCV7 induces good antibody responses in children with recurrent AOM.[161,162] Despite induction of IgG antibodies and >95% efficacy of these conjugate vaccines against vaccine serotype invasive pneumococcal disease,[163,164] efficacy in preventing mucosal colonization and infection with conjugate vaccine pneumococcal serotypes is

at best approximately 50%.[157,165-170] However, along side this decrease an increase of non-conjugate vaccine serotypes has been observed in nasopharyngeal carriage, middle ear disease and even in invasive pneumococcal disease.[157,167,168,171-173]

Determination of IgG antibody concentrations by ELISA is at present the standard technique to measure immune responses upon vaccination with PCV or PPV. However as of yet, no definite threshold antibody levels or minimal protective antibody levels, which should be defined for each individual serotype in the vaccine and possibly even for different pneumococcal diseases, have been set. It is agreed that for prevention of mucosal disease higher antibody levels are required as compared with invasive disease. Functional antibody characteristics, like antibody avidity and the opsonophagocytic capacity of antibodies should be investigated when evaluating vaccine efficacy as well, because some of the poor immunogenic capsular antigens like serotype 6B seem to induce excellent clinical protection from mucosal and systemic disease, despite low ELISA antibody titers after vaccinations.[174,175]

Fc receptors

Fc receptors (FcR) are expressed on phagocytic cells and recognize the constant part of an antibody. Therefore FcR are often referred to as a crucial links between the cellular and humoral parts of the immune system.[176-178] The interaction between antibodies and FcRs can result in

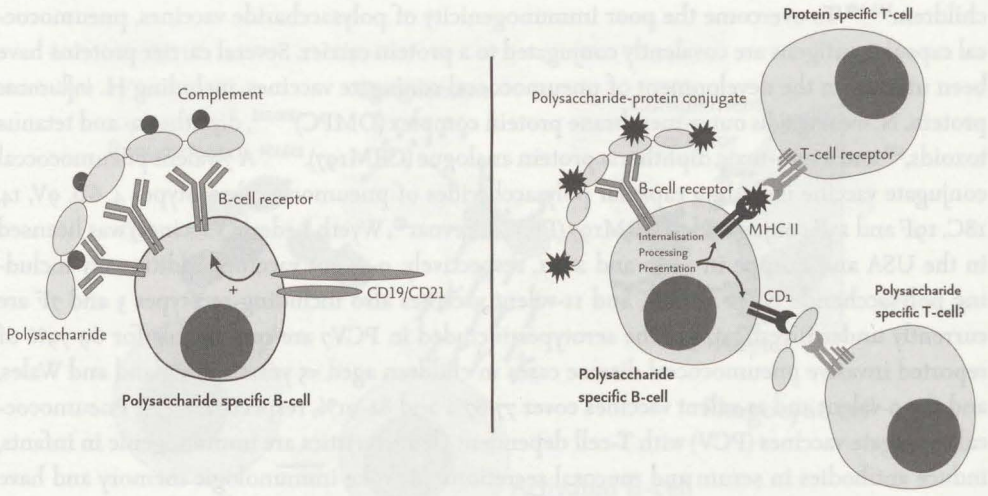


Figure 6 B-cell activation upon stimulation with polysaccharide or after a polysaccharide-protein conjugate.

Left: Polysaccharide activates B-cells independently of T-cells, by cross linking B-cell receptors.

Right: After binding of polysaccharide-protein conjugate to polysaccharide specific B-cell receptor, the conjugate is internalized, processed and presented. The protein part of a polysaccharide-protein conjugate attracts T-cell help, and therefore a T-cell dependent immune response is elicited, also influencing the immune response against the polysaccharide part. Possibly a polysaccharide specific T-cell is present as well (right).

activation of several effector functions, like phagocytosis, antibody-dependent cellular cytotoxicity (ADCC), antigen presentation, cytokine release, degranulation, and regulation of antibody synthesis. Polymorphisms that exist in FcRs for IgG (FcγRs), especially in FcγRIIa that interacts with IgG_2 antibodies,[179] are relevant for the susceptibility to respiratory tract infections and could possibly also play a role in the development of OM.[126,127,180,181]

Scope of this thesis

In this thesis we have studied the role of several factors of innate and adaptive immunity in two large and well-defined cohorts of children with either recurrent AOM or prolonged episodes of OME. Children participated in two randomized controlled trials on the prevention of OM by combined pneumococcal conjugate and polysaccharide vaccinations in the Netherlands.[41,165]
In **chapter 2** we studied genetically determined low levels of Mannan-binding lectin (MBL), which initiates the lectin pathway of complement activation, as a risk factor in the development of recurrent AOM. In **chapter 3**, the impact of a functional CD14 promoter polymorphism, described to determine soluble CD14 levels, on the number of AOM recurrences was studied. This clinical association might be related to the influence of this polymorphism on pneumococcal serotype specific IgG antibody levels, as described in this chapter. In **chapter 4** we set out to compare functional antibody synthesis in children with recurrent AOM, children with persistent OME and age-matched controls without OM. The antibody levels against a viral vaccine (measles), a bacterial vaccine (diphtheria, tetanus) and a conjugated polysaccharide-protein vaccine (*Haemophilus influenzae* type b) after regular childhood vaccinations were analysed. Previously, we described the adaptive IgG antibody response to combined pneumococcal conjugate and polysaccharide vaccinations in recurrent AOM and persistent OME.[165,182] In **chapter 5** we now studied antibody functionality of pneumococcal polysaccharide specific IgG antibodies after this vaccine schedule in children with recurrent AOM. In **chapter 6** the role of the Fcγ receptor IIa polymorphism, known to determine IgG_2 binding capacity, as a potential risk factor to develop recurrent AOM, is investigated. The influence of single nucleotide polymorphisms (SNPs) in IL-4, IL-4RA and IL-13 on pneumococcal polysaccharide specific IgG antibody responses, both in isolation and in haplotypes composed of these polymorphisms, are described in **chapter 7**. Finally, several immunologic risk factors (immunoglobulin levels, MBL and FcγR polymorphisms) in the development of OME are discussed in **chapter 8** and a summarizing discussion is presented in **chapter 9**.

References

1. Freid, V.M.; Makuc, D.M.; Rooks, R.N.: Ambulatory health care visits by children: principal diagnosis and place of visit. Vital Health Stat.13 **1998**; 1-23
2. Gonzales, R.; Malone, D.C.; Maselli, J.H.; Sande, M.A.: Excessive antibiotic use for acute respiratory infections in the United States. Clin.Infect.Dis. **2001**; 33:757-762
3. Gates, G.A.: Cost-effectiveness considerations in otitis media treatment. Otolaryngol.Head Neck Surg. **1996**; 114:525-530
4. Alsarraf, R.; Jung, C.J.; Perkins, J.; Crowley, C.; Alsarraf, N.W.; Gates, G.A.: Measuring the indirect and direct costs of acute otitis media. Arch.Otolaryngol.Head Neck Surg. **1999**; 125:12-18
5. Cripps, A.W.; Otczyk, D.C.; Kyd, J.M.: Bacterial otitis media: a vaccine preventable disease? Vaccine **2005**; 23:2304-2310
6. Gates, G.A.; Klein, J.O.; Lim, D.J.; Mogi, G.; Ogra, P.L.; Pararella, M.M.; Paradise, J.L.; Tos, M.: Recent advances in otitis media. 1. Definitions, terminology, and classification of otitis media. Ann.Otol.Rhinol.Laryngol.Suppl **2002**; 188:8-18
7. Ryding, M.; Konradsson, K.; Kalm, O.; Prellner, K.: Sequelae of recurrent acute otitis media. Ten-year follow-up of a prospectively studied cohort of children. Acta Paediatr. **1997**; 86:1208-1213
8. Alho, O.P.; Oja, H.; Koivu, M.; Sorri, M.: Chronic otitis media with effusion in infancy. How frequent is it? How does it develop? Arch.Otolaryngol.Head Neck Surg. **1995**; 121:432-436
9. Bluestone, C.D.: Pathogenesis of otitis media: role of eustachian tube. Pediatr.Infect.Dis.J **1996**; 15:281-291
10. Marchant, C.D.; Shurin, P.A.; Turczyk, V.A.; Wasikowski, D.E.; Tutihasi, M.A.; Kinney, S.E.: Course and outcome of otitis media in early infancy: a prospective study. J.Pediatr. **1984**; 104:826-831
11. Faden, H.; Duffy, L.; Boeve, M.: Otitis media: back to basics. Pediatr.Infect.Dis.J **1998**; 17:1105-1112
12. Wintermeyer, S.M. and Nahata, M.C.: Chronic suppurative otitis media. Ann.Pharmacother. **1994**; 28:1089-1099
13. Teele, D.W.; Klein, J.O.; Rosner, B.: Epidemiology of otitis media during the first seven years of life in children in greater Boston: a prospective, cohort study. J.Infect.Dis. **1989**; 160:83-94
14. Paradise, J.L.; Rockette, H.E.; Colborn, D.K.; Bernard, B.S.; Smith, C.G.; Kurs-Lasky, M.; Janosky, J.E.: Otitis media in 2253 Pittsburgh-area infants: prevalence and risk factors during the first two years of life. Pediatrics **1997**; 99:318-333
15. Stangerup, S.E. and Tos, M.: Epidemiology of acute suppurative otitis media. Am.J.Otolaryngol. **1986**; 7:47-54
16. Alho, O.P.; Koivu, M.; Sorri, M.; Rantakallio, P.: The occurrence of acute otitis media in infants. A life-table analysis. Int.J.Pediatr.Otorhinolaryngol. **1991**; 21:7-14
17. Alho, O.P.; Laara, E.; Oja, H.: What is the natural history of recurrent acute otitis media in infancy? J.Fam.Pract. **1996**; 43:258-264
18. Hendley, J.O.: Clinical practice. Otitis media. N.Engl.J.Med. **2002**; 347:1169-1174
19. Rosenfeld, R.M.; Culpepper, L.; Yawn, B.; Mahoney, M.C.: Otitis media with effusion clinical practice guideline. Am.Fam.Physician **2004**; 69:2776, 2778-2776, 2779
20. Zielhuis, G.A.; Rach, G.H.; van den, Broek P.: The natural course of otitis media with effusion in preschool children. Eur.Arch.Otorhinolaryngol. **1990**; 247:215-221
21. Glasziou, P.P.; Del Mar, C.B.; Sanders, S.L.; Hayem, M.: Antibiotics for acute otitis media in children. Cochrane. Database.Syst.Rev. **2004**; CD000219-
22. Williams, R.L.; Chalmers, T.C.; Stange, K.C.; Chalmers, F.T.; Bowlin, S.J.: Use of antibiotics in preventing recurrent acute otitis media and in treating otitis media with effusion. A meta-analytic attempt to resolve the brouhaha. JAMA **1993**; 270:1344-1351
23. Rovers, M.M.; Schilder, A.G.; Zielhuis, G.A.; Rosenfeld, R.M.: Otitis media. Lancet **2004**; 363:465-473
24. Rosenfeld, R.M.: Surgical prevention of otitis media. Vaccine **2000**; 19 Suppl 1:S134-S139
25. Gates, G.A.; Avery, C.A.; Prihoda, T.J.; Cooper, J.C., Jr.: Effectiveness of adenoidectomy and tympanostomy tubes in the treatment of chronic otitis media with effusion. N.Engl.J.Med. **1987**; 317:1444-1451
26. Mandel, E.M.; Rockette, H.E.; Bluestone, C.D.; Paradise, J.L.; Nozza, R.J.: Myringotomy with and without tympanostomy tubes for chronic otitis media with effusion. Arch.Otolaryngol.Head Neck Surg. **1989**; 115:1217-1224
27. Black, N.A.; Sanderson, C.F.; Freeland, A.P.; Vessey, M.P.: A randomised controlled trial of surgery for glue ear.

BMJ **1990**; 300:1551-1556

28. Maw, R. and Bawden, R.: Spontaneous resolution of severe chronic glue ear in children and the effect of adenoidectomy, tonsillectomy, and insertion of ventilation tubes (grommets). BMJ **1993**; 306:756-760
29. Paradise, J.L.; Feldman, H.M.; Campbell, T.F.; Dollaghan, C.A.; Colborn, D.K.; Bernard, B.S.; Rockette, H.E.; Janosky, J.E.; Pitcairn, D.L.; Sabo, D.L.; Kurs-Lasky, M.; Smith, C.G.: Effect of early or delayed insertion of tympanostomy tubes for persistent otitis media on developmental outcomes at the age of three years. N.Engl.J.Med. **2001**; 344:1179-1187
30. Rovers, M.M.; Straatman, H.; Ingels, K.; van der Wilt, G.J.; van den, Broek P.; Zielhuis, G.A.: The effect of ventilation tubes on language development in infants with otitis media with effusion: A randomized trial. Pediatrics **2000**; 106:E42-
31. Paradise, J.L.; Bluestone, C.D.; Rogers, K.D.; Taylor, F.H.; Colborn, D.K.; Bachman, R.Z.; Bernard, B.S.; Schwarzbach, R.H.: Efficacy of adenoidectomy for recurrent otitis media in children previously treated with tympanostomy-tube placement. Results of parallel randomized and nonrandomized trials. JAMA **1990**; 263:2066-2073
32. Paradise, J.L.; Bluestone, C.D.; Colborn, D.K.; Bernard, B.S.; Smith, C.G.; Rockette, H.E.; Kurs-Lasky, M.: Adenoidectomy and adenotonsillectomy for recurrent acute otitis media: parallel randomized clinical trials in children not previously treated with tympanostomy tubes. JAMA **1999**; 282:945-953
33. Coyte, P.C.; Croxford, R.; McIsaac, W.; Feldman, W.; Friedberg, J.: The role of adjuvant adenoidectomy and tonsillectomy in the outcome of the insertion of tympanostomy tubes. N.Engl.J.Med. **2001**; 344:1188-1195
34. Schilder, A.G.: Assessment of complications of the condition and of the treatment of otitis media with effusion. Int.J.Pediatr.Otorhinolaryngol. **1999**; 49 Suppl 1:S247-S251
35. Bluestone, C.D.: Clinical course, complications and sequelae of acute otitis media. Pediatr.Infect.Dis.J. **2000**; 19: S37-S46
36. van Kempen, M.J.; Rijkers, G.T.; Van Cauwenberge, P.B.: The immune response in adenoids and tonsils. Int.Arch. Allergy Immunol. **2000**; 122:8-19
37. Stenfors, L.E. and Raisanen, S.: Secretory IgA-, IgG- and C3b-coated bacteria in the nasopharynx of otitis-prone and non-otitis-prone children. Acta Otolaryngol. **1993**; 113:191-195
38. Barenkamp, S.J.; Kurono, Y.; Ogra, P.L.; Leiberman, A.; Bakaletz, L.O.; Murphy, T.F.; Chonmaitree, T.; Patel, J.A.; Heikkinen, T.; Sih, T.M.; Hurst, D.S.; St, G.J., III; Kawauchi, H.; Stenfors, L.E.: Recent advances in otitis media. 5. Microbiology and immunology. Ann.Otol.Rhinol.Laryngol.Suppl **2005**; 194:60-85
39. Wiertsema, S.P.; Veenhoven, R.H.; Sanders, E.A.; Rijkers, G.T.: Immunologic Screening of Children with Recurrent otitis media. Curr.Allergy Asthma Rep. **2005**; 5:302-307
40. Skotnicka, B. and Hassmann, E.: Cytokines in children with otitis media with effusion. Eur.Arch.Otorhinolaryngol. **2000**; 257:323-326
41. Straetemans, M.; van Heerbeek, N.; Tonnaer, E.; Ingels, K.J.; Rijkers, G.T.; Zielhuis, G.A.: A comprehensive model for the aetiology of otitis media with effusion. Med.Hypotheses **2001**; 57:784-791
42. Kubba, H.; Pearson, J.P.; Birchall, J.P.: The aetiology of otitis media with effusion: a review. Clin.Otolaryngol.Allied Sci. **2000**; 25:181-194
43. Casselbrant, M.L. and Mandel, E.M.: Genetic susceptibility to otitis media. Curr.Opin.Allergy Clin.Immunol. **2005**; 5:1-4
44. Rovers, M.; Haggard, M.; Gannon, M.; Koeppen-Schomerus, G.; Plomin, R.: Heritability of symptom domains in otitis media: a longitudinal study of 1,373 twin pairs. Am.J.Epidemiol. **2002**; 155:958-964
45. Casselbrant, M.L.; Mandel, E.M.; Fall, P.A.; Rockette, H.E.; Kurs-Lasky, M.; Bluestone, C.D.; Ferrell, R.E.: The heritability of otitis media: a twin and triplet study. JAMA **1999**; 282:2125-2130
46. Kvaerner, K.J.; Tambs, K.; Harris, J.R.; Magnus, P.: Distribution and heritability of recurrent ear infections. Ann. Otol.Rhinol.Laryngol. **1997**; 106:624-632
47. Stenstrom, C. and Ingvarsson, L.: Otitis-prone children and controls: a study of possible predisposing factors. 1. Heredity, family background and perinatal period. Acta Otolaryngol. **1997**; 117:87-93
48. Kalm, O.; Johnson, U.; Prellner, K.; Ninn, K.: HLA frequency in patients with recurrent acute otitis media. Arch. Otolaryngol.Head Neck Surg. **1991**; 117:1296-1299
49. Wiet, R.J.: Patterns of ear disease in the southwestern American Indian. Arch.Otolaryngol. **1979**; 105:381-385
50. Leach, A.J. and Morris, P.S.: Perspectives on infective ear disease in indigenous Australian children. J.Paediatr.

Child Health **2001**; 37:529-530
51. Leach, A.J.: Otitis media in Australian Aboriginal children: an overview. Int.J.Pediatr.Otorhinolaryngol. **1999**; 49 Suppl 1:S173-S178
52. Pedersen, C.B. and Zachau-Christiansen, B.: Otitis media in Greenland children: acute, chronic and secretory otitis media in three- to eight-year-olds. J.Otolaryngol. **1986**; 15:332-335
53. Curns, A.T.; Holman, R.C.; Shay, D.K.; Cheek, J.E.; Kaufman, S.F.; Singleton, R.J.; Anderson, L.J.: Outpatient and hospital visits associated with otitis media among American Indian and Alaska native children younger than 5 years. Pediatrics **2002**; 109:E41-E41
54. Daly, K.A.; Rovers, M.M.; Hoffman, H.J.; Uhari, M.; Casselbrant, M.L.; Zielhuis, G.; Kvaerner, K.J.: Recent advances in otitis media. 1. Epidemiology, natural history, and risk factors. Ann.Otol.Rhinol.Laryngol.Suppl **2005**; 194:8-15
55. Uhari, M.; Mantysaari, K.; Niemela, M.: A meta-analytic review of the risk factors for acute otitis media. Clin. Infect.Dis. **1996**; 22:1079-1083
56. DiFranza, J.R. and Lew, R.A.: Morbidity and mortality in children associated with the use of tobacco products by other people. Pediatrics **1996**; 97:560-568
57. Dewey, C.; Midgeley, E.; Maw, R.: The relationship between otitis media with effusion and contact with other children in a british cohort studied from 8 months to 3 1/2 years. The ALSPAC Study Team. Avon Longitudinal Study of Pregnancy and Childhood. Int.J.Pediatr.Otorhinolaryngol. **2000**; 55:33-45
58. Rovers, M.M.; Hofstad, E.A.; Franken-van den Brand KI; Straatman, H.; Ingels, K.; van der Wilt, G.J.; Zielhuis, G.A.: Prognostic factors for otitis media with effusion in infants. Clin.Otolaryngol.Allied Sci. **1998**; 23:543-546
59. Engel, J.; Anteunis, L.; Volovics, A.; Hendriks, J.; Marres, E.: Risk factors of otitis media with effusion during infancy. Int.J.Pediatr.Otorhinolaryngol. **1999**; 48:239-249
60. Ford, L.C.; Sulprizio, S.L.; Rasgon, B.M.: Otolaryngological manifestations of velocardiofacial syndrome: a retrospective review of 35 patients. Laryngoscope **2000**; 110:362-367
61. Reyes, M.R.; LeBlanc, E.M.; Bassila, M.K.: Hearing loss and otitis media in velo-cardio-facial syndrome. Int. J.Pediatr.Otorhinolaryngol. **1999**; 47:227-233
62. Dyce, O.; McDonald-McGinn, D.; Kirschner, R.E.; Zackai, E.; Young, K.; Jacobs, I.N.: Otolaryngologic manifestations of the 22q11.2 deletion syndrome. Arch.Otolaryngol.Head Neck Surg. **2002**; 128:1408-1412
63. Stenstrom, C.; Bylander-Groth, A.; Ingvarsson, L.: Eustachian tube function in otitis-prone and healthy children. Int.J.Pediatr.Otorhinolaryngol. **1991**; 21:127-138
64. Takahashi, H.; Hayashi, M.; Sato, H.; Honjo, I.: Primary deficits in eustachian tube function in patients with otitis media with effusion. Arch.Otolaryngol.Head Neck Surg. **1989**; 115:581-584
65. Heikkinen, T.: The role of respiratory viruses in otitis media. Vaccine **2000**; 19 Suppl 1:S51-5.:S51-S55
66. Chonmaitree, T. and Heikkinen, T.: Viruses and acute otitis media. Pediatr.Infect.Dis.J **2000**; 19:1005-1007
67. Heikkinen, T.; Thint, M.; Chonmaitree, T.: Prevalence of various respiratory viruses in the middle ear during acute otitis media. N.Engl.J Med. **1999**; 340:260-264
68. Heikkinen, T. and Chonmaitree, T.: Importance of respiratory viruses in acute otitis media. Clin.Microbiol.Rev. **2003**; 16:230-241
69. Klein, J.O.: Otitis media. Clin.Infect.Dis. **1994**; 19:823-833
70. Luotonen, J.; Herva, E.; Karma, P.; Timonen, M.; Leinonen, M.; Makela, P.H.: The bacteriology of acute otitis media in children with special reference to Streptococcus pneumoniae as studied by bacteriological and antigen detection methods. Scand.J.Infect.Dis. **1981**; 13:177-183
71. Bluestone, C.D.; Stephenson, J.S.; Martin, L.M.: Ten-year review of otitis media pathogens. Pediatr.Infect.Dis.J. **1992**; 11:S7-11
72. Block, S.L.: Causative pathogens, antibiotic resistance and therapeutic considerations in acute otitis media. Pediatr.Infect.Dis.J. **1997**; 16:449-456
73. Faden, H.; Waz, M.J.; Bernstein, J.M.; Brodsky, L.; Stanievich, J.; Ogra, P.L.: Nasopharyngeal flora in the first three years of life in normal and otitis-prone children. Ann.Otol.Rhinol.Laryngol. **1991**; 100:612-615
74. Faden, H.; Duffy, L.; Wasielewski, R.; Wolf, J.; Krystofik, D.; Tung, Y.: Relationship between nasopharyngeal colonization and the development of otitis media in children. Tonawanda/Williamsville Pediatrics. J.Infect.Dis. **1997**; 175:1440-1445
75. Magee, A.D. and Yother, J.: Requirement for capsule in colonization by Streptococcus pneumoniae. Infect.Immun.

2001; 69:3755-3761
76. Watson, D.A. and Musher, D.M.: Interruption of capsule production in Streptococcus pneumonia serotype 3 by insertion of transposon Tn916. Infect.Immun. 1990; 58:3135-3138
77. Watson, D.A.; Musher, D.M.; Verhoef, J.: Pneumococcal virulence factors and host immune responses to them. Eur.J.Clin.Microbiol.Infect.Dis. 1995; 14:479-490
78. Henrichsen, J.: Six newly recognized types of Streptococcus pneumoniae. J.Clin.Microbiol. 1995; 33:2759-2762
79. Ghaffar, F.; Friedland, I.R.; McCracken, G.H., Jr.: Dynamics of nasopharyngeal colonization by Streptococcus pneumoniae. Pediatr.Infect.Dis.J. 1999; 18:638-646
80. Ekdahl, K.; Ahlinder, I.; Hansson, H.B.; Melander, E.; Molstad, S.; Soderstrom, M.; Persson, K.: Duration of nasopharyngeal carriage of penicillin-resistant Streptococcus pneumoniae: experiences from the South Swedish Pneumococcal Intervention Project. Clin.Infect.Dis. 1997; 25:1113-1117
81. Hoffmann, J.A.; Kafatos, F.C.; Janeway, C.A.; Ezekowitz, R.A.: Phylogenetic perspectives in innate immunity. Science 1999; 284:1313-1318
82. Janeway, C.A., Jr. and Medzhitov, R.: Innate immune recognition. Annu.Rev.Immunol. 2002; 20:197-216
83. Mukhopadhyay, S.; Herre, J.; Brown, G.D.; Gordon, S.: The potential for Toll-like receptors to collaborate with other innate immune receptors. Immunology 2004; 112:521-530
84. Holmskov, U.; Thiel, S.; Jensenius, J.C.: Collections and ficolins: humoral lectins of the innate immune defense. Annu.Rev.Immunol. 2003; 21:547-578
85. Holmskov, U.; Malhotra, R.; Sim, R.B.; Jensenius, J.C.: Collectins: collagenous C-type lectins of the innate immune defense system. Immunol.Today 1994; 15:67-74
86. Nuytinck, L. and Shapiro, F.: Mannose-binding lectin: laying the stepping stones from clinical research to personalized medicine. Personalized Medicine 2004; 1:35-52
87. Dahl, M.R.; Thiel, S.; Matsushita, M.; Fujita, T.; Willis, A.C.; Christensen, T.; Vorup-Jensen, T.; Jensenius, J.C.: MASP-3 and its association with distinct complexes of the mannan-binding lectin complement activation pathway. Immunity. 2001; 15:127-135
88. Gal, P. and Ambrus, G.: Structure and function of complement activating enzyme complexes: C1 and MBL-MASPs. Curr.Protein Pept.Sci. 2001; 2:43-59
89. Fujita, T.: Evolution of the lectin-complement pathway and its role in innate immunity. Nat.Rev.Immunol. 2002; 2:346-353
90. Gadjeva, M.; Thiel, S.; Jensenius, J.C.: The mannan-binding-lectin pathway of the innate immune response. Curr. Opin.Immunol. 2001; 13:74-78
91. Whaley, K. and Schwaeble, W.: Complement and complement deficiencies. Semin.Liver Dis. 1997; 17:297-310
92. Kilpatrick, D.C.: Mannan-binding lectin: clinical significance and applications. Biochim.Biophys.Acta 2002; 1572:401-413
93. Turner, M.W.: The role of mannose-binding lectin in health and disease. Mol.Immunol. 2003; 40:423-429
94. Super, M.; Thiel, S.; Lu, J.; Levinsky, R.J.; Turner, M.W.: Association of low levels of mannan-binding protein with a common defect of opsonisation. Lancet 1989; 2:1236-1239
95. Koch, A.; Melbye, M.; Sorensen, P.; Homoe, P.; Madsen, H.O.; Molbak, K.; Hansen, C.H.; Andersen, L.H.; Hahn, G.W.; Garred, P.: Acute respiratory tract infections and mannose-binding lectin insufficiency during early childhood. JAMA 2001; 285:1316-1321
96. Roy, S.; Knox, K.; Segal, S.; Griffiths, D.; Moore, C.E.; Welsh, K.I.; Smarason, A.; Day, N.P.; McPheat, W.L.; Crook, D.W.; Hill, A.V.: MBL genotype and risk of invasive pneumococcal disease: a case-control study. Lancet 2002; 359:1569-1573
97. Turner, M.W.: Deficiency of mannan binding protein--a new complement deficiency syndrome. Clin.Exp.Immunol. 1991; 86 Suppl 1:53-56
98. Summerfield, J.A.; Ryder, S.; Sumiya, M.; Thursz, M.; Gorchein, A.; Monteil, M.A.; Turner, M.W.: Mannose binding protein gene mutations associated with unusual and severe infections in adults. Lancet 1995; 345:886-889
99. Madsen, H.O.; Garred, P.; Kurtzhals, J.A.; Lamm, L.U.; Ryder, L.P.; Thiel, S.; Svejgaard, A.: A new frequent allele is the missing link in the structural polymorphism of the human mannan-binding protein. Immunogenetics 1994; 40:37-44
100. Madsen, H.O.; Garred, P.; Thiel, S.; Kurtzhals, J.A.; Lamm, L.U.; Ryder, L.P.; Svejgaard, A.: Interplay between

promoter and structural gene variants control basal serum level of mannan-binding protein. J.Immunol. **1995**; 155:3013-3020

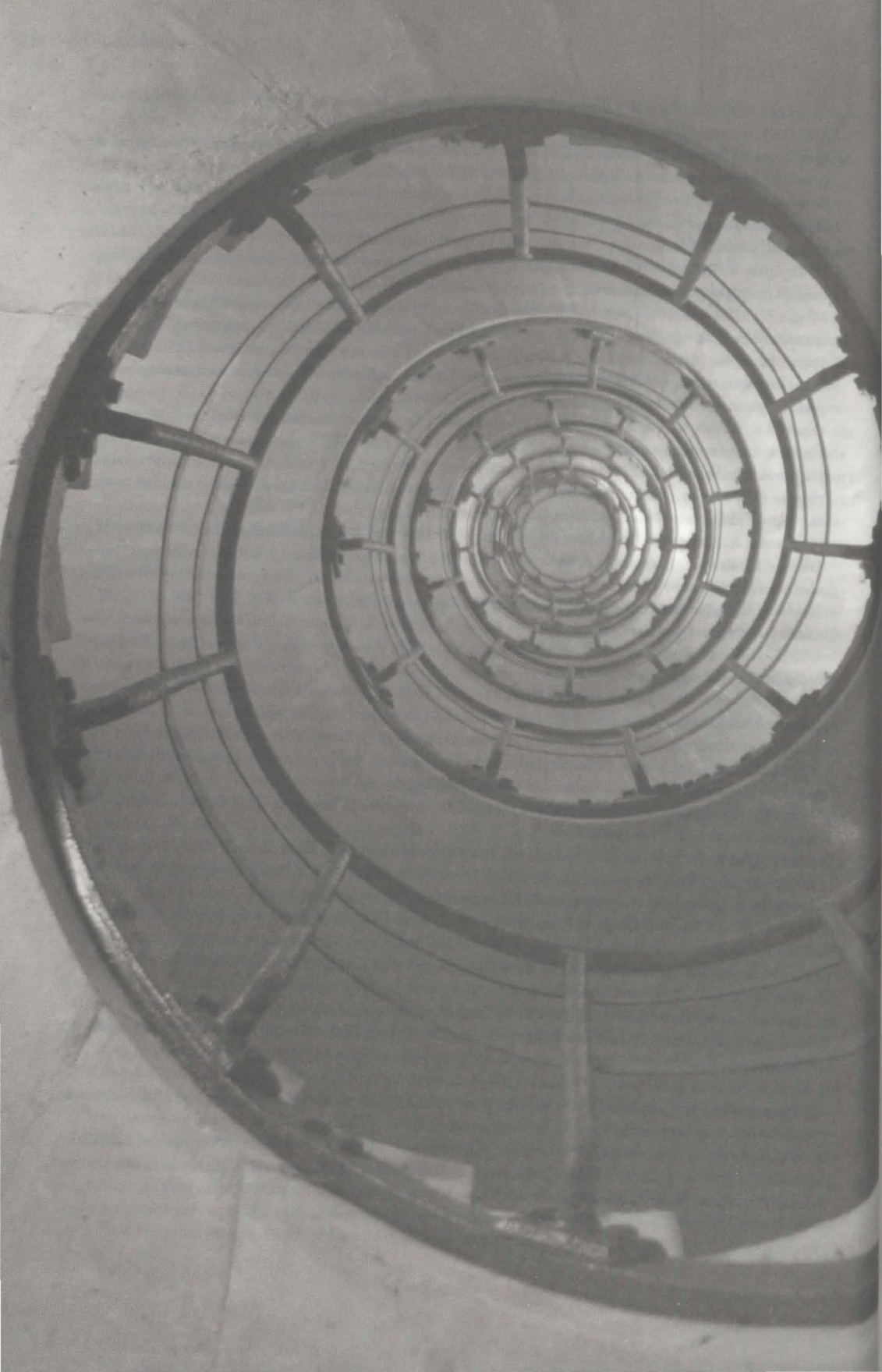
101. Crosdale, D.J.; Ollier, W.E.; Thomson, W.; Dyer, P.A.; Jensenious, J.; Johnson, R.W.; Poulton, K.V.: Mannose binding lectin (MBL) genotype distributions with relation to serum levels in UK Caucasoids. Eur.J.Immunogenet. **2000**; 27:111-117
102. Sumiya, M.; Super, M.; Tabona, P.; Levinsky, R.J.; Arai, T.; Turner, M.W.; Summerfield, J.A.: Molecular basis of opsonic defect in immunodeficient children. Lancet **1991**; 337:1569-1570
103. Lipscombe, R.J.; Sumiya, M.; Summerfield, J.A.; Turner, M.W.: Distinct physicochemical characteristics of human mannose binding protein expressed by individuals of differing genotype. Immunology **1995**; 85:660-667
104. Wright, S.D.; Ramos, R.A.; Tobias, P.S.; Ulevitch, R.J.; Mathison, J.C.: CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. Science **1990**; 249:1431-1433
105. Cleveland, M.G.; Gorham, J.D.; Murphy, T.L.; Tuomanen, E.; Murphy, K.M.: Lipoteichoic acid preparations of gram-positive bacteria induce interleukin-12 through a CD14-dependent pathway. Infect.Immun. **1996**; 64:1906-1912
106. Gupta, D.; Kirkland, T.N.; Viriyakosol, S.; Dziarski, R.: CD14 is a cell-activating receptor for bacterial peptidoglycan. J.Biol.Chem. **1996**; 271:23310-23316
107. Dobrovolskaia, M.A. and Vogel, S.N.: Toll receptors, CD14, and macrophage activation and deactivation by LPS. Microbes.Infect. **2002**; 4:903-914
108. Um, S.H.; Son, E.W.; Kim, B.O.; Moon, E.Y.; Rhee, D.K.; Pyo, S.: Activation of murine peritoneal macrophages by Streptococcus pneumoniae type II capsular polysaccharide: involvement of CD14-dependent pathway. Scand. J.Immunol. **2000**; 52:39-45
109. Pugin, J.; Heumann, I.D.; Tomasz, A.; Kravchenko, V.V.; Akamatsu, Y.; Nishijima, M.; Glauser, M.P.; Tobias, P.S.; Ulevitch, R.J.: CD14 is a pattern recognition receptor. Immunity. **1994**; 1:509-516
110. da Silva, C.J.; Soldau, K.; Christen, U.; Tobias, P.S.; Ulevitch, R.J.: Lipopolysaccharide is in close proximity to each of the proteins in its membrane receptor complex. transfer from CD14 to TLR4 and MD-2. J.Biol.Chem. **2001**; 276:21129-21135
111. Akira, S.: Toll-like receptor signaling. J.Biol.Chem. **2003**; 278:38105-38108
112. Aderem, A. and Ulevitch, R.J.: Toll-like receptors in the induction of the innate immune response. Nature **2000**; 406:782-787
113. Beutler, B.; Hoebe, K.; Du, X.; Ulevitch, R.J.: How we detect microbes and respond to them: the Toll-like receptors and their transducers. J.Leukoc.Biol. **2003**; 74:479-485
114. Baldini, M.; Lohman, I.C.; Halonen, M.; Erickson, R.P.; Holt, P.G.; Martinez, F.D.: A Polymorphism* in the 5' flanking region of the CD14 gene is associated with circulating soluble CD14 levels and with total serum immunoglobulin E. Am.J.Respir.Cell Mol.Biol. **1999**; 20:976-983
115. Koppelman, G.H.; Reijmerink, N.E.; Colin, S.O.; Howard, T.D.; Whittaker, P.A.; Meyers, D.A.; Postma, D.S.; Bleecker, E.R.: Association of a promoter polymorphism of the CD14 gene and atopy. Am.J.Respir.Crit Care Med. **2001**; 163:965-969
116. Koppelman, G.H. and Postma, D.S.: The genetics of CD14 in allergic disease. Curr.Opin.Allergy Clin.Immunol. **2003**; 3:347-352
117. Pacheco, E.; Fonseca, C.; Montes, C.; Zabaleta, J.; Garcia, L.F.; Arias, M.A.: CD14 gene promoter polymorphism in different clinical forms of tuberculosis. FEMS Immunol.Med.Microbiol. **2004**; 40:207-213
118. Repo, H.; Anttonen, K.; Kilpinen, S.K.; Palotie, A.; Salven, P.; Orpana, A.; Leirisalo-Repo, M.: CD14 and TNfa promoter polymorphisms in patients with acute arthritis. Special reference to development of chronic spondyloarthropathy. Scand.J.Rheumatol. **2002**; 31:355-361
119. Vos, Q.; Lees, A.; Wu, Z.Q.; Snapper, C.M.; Mond, J.J.: B-cell activation by T-cell-independent type 2 antigens as an integral part of the humoral immune response to pathogenic microorganisms. Immunol.Rev. **2000**; 176:154-170
120. Feldmann, M. and Easten, A.: The relationship between antigenic structure and the requirement for thymus-derived cells in the immune response. J.Exp.Med. **1971**; 134:103-119
121. Insel, R.A.; Kittelberger, A.; Anderson, P.: Isoelectric focusing of human antibody to the Haemophilus influenzae b capsular polysaccharide: restricted and identical spectrotypes in adults. J.Immunol. **1985**; 135:2810-2816
122. Kayhty, H.; Karanko, V.; Peltola, H.; Makela, P.H.: Serum antibodies after vaccination with Haemophilus influen-

zae type b capsular polysaccharide and responses to reimmunization: no evidence of immunologic tolerance or memory. Pediatrics **1984**; 74:857-865

123. Weintraub, A.: Immunology of bacterial polysaccharide antigens. Carbohydr.Res. **2003**; 338:2539-2547
124. Kaniuk, A.S.; Lortan, J.E.; Monteil, M.A.: Specific IgG subclass antibody levels and phagocytosis of serotype 14 pneumococcus following immunization. Scand.J.Immunol.Suppl **1992**; 11:96-98
125. Lortan, J.E.; Kaniuk, A.S.; Monteil, M.A.: Relationship of in vitro phagocytosis of serotype 14 Streptococcus pneumoniae to specific class and IgG subclass antibody levels in healthy adults. Clin.Exp.Immunol. **1993**; 91:54-57
126. Rodriguez, M.E.; van der Pol, W.L.; Sanders, L.A.; van de Winkel, J.G.: Crucial role of FcgammaRIIa (CD32) in assessment of functional anti-Streptococcus pneumoniae antibody activity in human sera. J Infect.Dis. **1999**; 179:423-433
127. Jansen, W.T.; Breukels, M.A.; Snippe, H.; Sanders, L.A.; Verheul, A.F.; Rijkers, G.T.: Fcgamma receptor polymorphisms determine the magnitude of in vitro phagocytosis of Streptococcus pneumoniae mediated by pneumococcal conjugate sera. J Infect.Dis. **1999**; 180:888-891
128. Berman, S.; Lee, B.; Nuss, R.; Roark, R.; Giclas, P.C.: Immunoglobulin G, total and subclass, in children with or without recurrent otitis media. J.Pediatr. **1992**; 121:249-251
129. Sorensen, C.H. and Nielsen, L.K.: Plasma IgG, IgG subclasses and acute-phase proteins in children with recurrent acute otitis media. APMIS **1988**; 96:676-680
130. Freijd, A.; Oxelius, V.A.; Rynnel-Dagoo, B.: A prospective study demonstrating an association between plasma IgG2 concentrations and susceptibility to otitis media in children. Scand.J.Infect.Dis. **1985**; 17:115-120
131. Jorgensen, F.; Andersson, B.; Hanson, L.A.; Nylen, O.; Eden, C.S.: Gamma-globulin treatment of recurrent acute otitis media in children. Pediatr.Infect.Dis.J. **1990**; 9:389-394
132. Veenhoven, R.; Rijkers, G.; Schilder, A.; Adelmeijer, J.; Uiterwaal, C.; Kuis, W.; Sanders, E.: Immunoglobulins in Otitis-Prone Children. Pediatr.Res. **2003**;
133. Freijd, A.; Hammarstrom, L.; Persson, M.A.; Smith, C.I.: Plasma anti-pneumococcal antibody activity of the IgG class and subclasses in otitis prone children. Clin.Exp.Immunol. **1984**; 56:233-238
134. Prellner, K.; Kalm, O.; Harsten, G.; Heldrup, J.; Oxelius, V.A.: Pneumococcal serum antibody concentrations during the first three years of life: a study of otitis-prone and non-otitis-prone children. Int.J.Pediatr.Otorhinolaryngol. **1989**; 17:267-279
135. Hotomi, M.; Yamanaka, N.; Saito, T.; Shimada, J.; Suzumoto, M.; Suetake, M.; Faden, H.: Antibody responses to the outer membrane protein P6 of non-typeable Haemophilus influenzae and pneumococcal capsular polysaccharides in otitis-prone children. Acta Otolaryngol. **1999**; 119:703-707
136. Plouffe, J.F.; Breiman, R.F.; Facklam, R.R.: Bacteremia with Streptococcus pneumoniae. Implications for therapy and prevention. Franklin County Pneumonia Study Group. JAMA **1996**; 275:194-198
137. Rudolph, K.M.; Parkinson, A.J.; Reasonover, A.L.; Bulkow, L.R.; Parks, D.J.; Butler, J.C.: Serotype distribution and antimicrobial resistance patterns of invasive isolates of Streptococcus pneumoniae: Alaska, 1991-1998. J.Infect. Dis. **2000**; 182:490-496
138. Laurichesse, H.; Grimaud, O.; Waight, P.; Johnson, A.P.; George, R.C.; Miller, E.: Pneumococcal bacteraemia and meningitis in England and Wales, 1993 to 1995. Commun.Dis.Public Health **1998**; 1:22-27
139. McKenzie, H.; Reid, N.; Dijkhuizen, R.S.: Clinical and microbiological epidemiology of Streptococcus pneumoniae bacteraemia. J.Med.Microbiol. **2000**; 49:361-366
140. Sankilampi, U.; Herva, E.; Haikala, R.; Liimatainen, O.; Renkonen, O.V.; Leinonen, M.: Epidemiology of invasive Streptococcus pneumoniae infections in adults in Finland. Epidemiol.Infect. **1997**; 118:7-15
141. Jorgenson, J.H.; Howell, A.W.; Maher, L.A.; Facklam, R.R.: Serotypes of respiratory isolates of Streptococcus pneumoniae compared with the capsular types included in the current pneumococcal vaccine. J.Infect.Dis. **1991**; 163:644-646
142. Makela, P.H.; Sibakov, M.; Herva, E.; Henrichsen, J.; Luotonen, J.; Timonen, M.; Leinonen, M.; Koskela, M.; Pukander, J.; Pontynen, S.; Gronroos, P.; Karma, P.: Pneumococcal vaccine and otitis media. Lancet **1980**; 2:547-551
143. Sloyer, J.L., Jr.; Ploussard, J.H.; Howie, V.M.: Efficacy of pneumococcal polysaccharide vaccine in preventing acute otitis media in infants in Huntsville, Alabama. Rev.Infect.Dis. **1981**; 3 Suppl:S119-S123
144. Teele, D.W.; Klein, J.O.; Bratton, L.; Fisch, G.R.; Mathieu, O.R.; Porter, P.J.; Starobin, S.G.; Tarlin, L.D.; Younes, R.P.: Use of pneumococcal vaccine for prevention of recurrent acute otitis media in infants in Boston. The

Greater Boston Collaborative otitis media Study Group. Rev.Infect.Dis. **1981**; 3 Suppl:S113-S118
145. Schuller, D.E.: Prophylaxis of otitis media in asthmatic children. Pediatr.Infect.Dis. **1983**; 2:280-283
146. Douglas, R.M. and Miles, H.B.: Vaccination against Streptococcus pneumoniae in childhood: lack of demonstrable benefit in young Australian children. J.Infect.Dis. **1984**; 149:861-869
147. Rosen, C.; Christensen, P.; Henrichsen, J.; Hovelius, B.; Prellner, K.: Beneficial effect of pneumococcal vaccination on otitis media in children over two years old. Int.J.Pediatr.Otorhinolaryngol. **1984**; 7:239-246
148. Karma, P.; Pukander, J.; Sipila, M.; Timonen, M.; Pontynen, S.; Herva, E.; Gronroos, P.; Makela, H.: Prevention of otitis media in children by pneumococcal vaccination. Am.J.Otolaryngol. **1985**; 6:173-184
149. Douglas, R.M.; Hansman, D.; McDonald, B.; Paton, J.; Kirke, K.: Pneumococcal vaccine in aboriginal children--a randomized controlled trial involving 60 children. Community Health Stud. **1986**; 10:189-196
150. Blum, M.D.; Dagan, R.; Mendelman, P.M.; Pinsk, V.; Giordani, M.; Li, S.; Bohidar, N.; McNeely, T.B.: A comparison of multiple regimens of pneumococcal polysaccharide-meningococcal outer membrane protein complex conjugate vaccine and pneumococcal polysaccharide vaccine in toddlers. Vaccine **2000**; 18:2359-2367
151. Anderson, E.L.; Kennedy, D.J.; Geldmacher, K.M.; Donnelly, J.; Mendelman, P.M.: Immunogenicity of heptavalent pneumococcal conjugate vaccine in infants. J.Pediatr. **1996**; 128:649-653
152. Nurkka, A.; Ahman, H.; Yaich, M.; Eskola, J.; Kayhty, H.: Serum and salivary anti-capsular antibodies in infants and children vaccinated with octavalent pneumococcal conjugate vaccines, PncD and PncT. Vaccine **2001**; 20:194-201
153. Rennels, M.B.; Edwards, K.M.; Keyserling, H.L.; Reisinger, K.S.; Hogerman, D.A.; Madore, D.V.; Chang, I.; Paradiso, P.R.; Malinoski, F.J.; Kimura, A.: Safety and immunogenicity of heptavalent pneumococcal vaccine conjugated to CRM197 in United States infants. Pediatrics **1998**; 101:604-611
154. Shinefield, H.R.; Black, S.; Ray, P.; Chang, I.; Lewis, N.; Fireman, B.; Hackell, J.; Paradiso, P.R.; Siber, G.; Kohberger, R.; Madore, D.V.; Malinowski, F.J.; Kimura, A.; Le, C.; Landaw, I.; Aguilar, J.; Hansen, J.: Safety and immunogenicity of heptavalent pneumococcal CRM197 conjugate vaccine in infants and toddlers. Pediatr.Infect.Dis.J. **1999**; 18:757-763
155. Miller, E.; Waight, P.; Efstratiou, A.; Brisson, M.; Johnson, A.; George, R.: Epidemiology of invasive and other pneumococcal disease in children in England and Wales 1996-1998. Acta Paediatr.Suppl **2000**; 89:11-16
156. Sleeman, K.; Knox, K.; George, R.; Miller, E.; Waight, P.; Griffiths, D.; Efstratiou, A.; Broughton, K.; Mayon-White, R.T.; Moxon, E.R.; Crook, D.W.: Invasive pneumococcal disease in England and Wales: vaccination implications. J.Infect.Dis. **2001**; 183:239-246
157. Eskola, J.; Kilpi, T.; Palmu, A.; Jokinen, J.; Haapakoski, J.; Herva, E.; Takala, A.; Kayhty, H.; Karma, P.; Kohberger, R.; Siber, G.; Makela, P.H.: Efficacy of a pneumococcal conjugate vaccine against acute otitis media. N.Engl.J Med. **2001**; 344:403-409
158. Klugman, K.P.; Madhi, S.A.; Huebner, R.E.; Kohberger, R.; Mbelle, N.; Pierce, N.: A trial of a 9-valent pneumococcal conjugate vaccine in children with and those without HIV infection. N.Engl.J.Med. **2003**; 349:1341-1348
159. Zielen, S.; Buhring, I.; Strnad, N.; Reichenbach, J.; Hofmann, D.: Immunogenicity and tolerance of a 7-valent pneumococcal conjugate vaccine in nonresponders to the 23-valent pneumococcal vaccine. Infect.Immun. **2000**; 68:1435-1440
160. Sorensen, R.U.; Leiva, L.E.; Giangrosso, P.A.; Butler, B.; Javier, F.C., III; Sacerdote, D.M.; Bradford, N.; Moore, C.: Response to a heptavalent conjugate Streptococcus pneumoniae vaccine in children with recurrent infections who are unresponsive to the polysaccharide vaccine. Pediatr.Infect.Dis.J **1998**; 17:685-691
161. Barnett, E.D.; Pelton, S.I.; Cabral, H.J.; Eavey, R.D.; Allen, C.; Cunningham, M.J.; McNamara, E.R.; Klein, J.O.: Immune response to pneumococcal conjugate and polysaccharide vaccines in otitis-prone and otitis-free children. Clin.Infect.Dis. **1999**; 29:191-192
162. Breukels, M.A.; Rijkers, G.T.; Voorhorst-Ogink, M.M.; Zegers, B.J.; Sanders, L.A.: Pneumococcal conjugate vaccine primes for polysaccharide-inducible IgG2 antibody response in children with recurrent otitis media acuta. J Infect.Dis. **1999**; 179:1152-1156
163. Black, S.; Shinefield, H.; Fireman, B.; Lewis, E.; Ray, P.; Hansen, J.R.; Elvin, L.; Ensor, K.M.; Hackell, J.; Siber, G.; Malinoski, F.; Madore, D.; Chang, I.; Kohberger, R.; Watson, W.; Austrian, R.; Edwards, K.: Efficacy, safety and immunogenicity of heptavalent pneumococcal conjugate vaccine in children. Northern California Kaiser Permanente Vaccine Study Center Group. Pediatr.Infect.Dis.J **2000**; 19:187-195

164. Shinefield, H.R. and Black, S.: Efficacy of pneumococcal conjugate vaccines in large scale field trials. *Pediatr. Infect.Dis.J.* **2000**; 19:394-397
165. Veenhoven, R.; Bogaert, D.; Uiterwaal, C.; Brouwer, C.; Kiezebrink, H.; Bruin, J.; IJzerman, E.; Hermans, P.; de Groot, R.; Zegers, B.; Kuis, W.; Rijkers, G.; Schilder, A.; Sanders, E.: Effect of conjugate pneumococcal vaccine followed by polysaccharide pneumococcal vaccine on recurrent acute otitis media: a randomised study. *Lancet* **2003**; 361:2189-2195
166. Dagan, R.; Melamed, R.; Muallem, M.; Piglansky, L.; Greenberg, D.; Abramson, O.; Mendelman, P.M.; Bohidar, N.; Yagupsky, P.: Reduction of nasopharyngeal carriage of pneumococci during the second year of life by a heptavalent conjugate pneumococcal vaccine. *J.Infect.Dis.* **1996**; 174:1271-1278
167. Mbelle, N.; Huebner, R.E.; Wasas, A.D.; Kimura, A.; Chang, I.; Klugman, K.P.: Immunogenicity and impact on nasopharyngeal carriage of a nonavalent pneumococcal conjugate vaccine. *J.Infect.Dis.* **1999**; 180:1171-1176
168. Dagan, R.; Givon-Lavi, N.; Zamir, O.; Sikuler-Cohen, M.; Guy, L.; Janco, J.; Yagupsky, P.; Fraser, D.: Reduction of nasopharyngeal carriage of Streptococcus pneumoniae after administration of a 9-valent pneumococcal conjugate vaccine to toddlers attending day care centers. *J.Infect.Dis.* **2002**; 185:927-936
169. Obaro, S.K.; Adegbola, R.A.; Banya, W.A.; Greenwood, B.M.: Carriage of pneumococci after pneumococcal vaccination. *Lancet* **1996**; 348:271-272
170. Dagan, R.; Muallem, M.; Melamed, R.; Leroy, O.; Yagupsky, P.: Reduction of pneumococcal nasopharyngeal carriage in early infancy after immunization with tetravalent pneumococcal vaccines conjugated to either tetanus toxoid or diphtheria toxoid. *Pediatr.Infect.Dis.J.* **1997**; 16:1060-1064
171. Veenhoven, R.H.; Bogaert, D.; Schilder, A.G.; Rijkers, G.T.; Uiterwaal, C.S.; Kiezebrink, H.H.; van Kempen, M.J.; Dhooge, I.J.; Bruin, J.; IJzerman, E.P.; de Groot, R.; Kuis, W.; Hermans, P.W.; Sanders, E.A.: Nasopharyngeal pneumococcal carriage after combined pneumococcal conjugate and polysaccharide vaccination in children with a history of recurrent acute otitis media. *Clin.Infect.Dis.* **2004**; 39:911-919
172. Block, S.L.; Hedrick, J.; Harrison, C.J.; Tyler, R.; Smith, A.; Findlay, R.; Keegan, E.: Community-wide vaccination with the heptavalent pneumococcal conjugate significantly alters the microbiology of acute otitis media. *Pediatr. Infect.Dis.J.* **2004**; 23:829-833
173. Hsu, K.; Pelton, S.; Karumuri, S.; Heisey-Grove, D.; Klein, J.: Population-based surveillance for childhood invasive pneumococcal disease in the era of conjugate vaccine. *Pediatr.Infect.Dis.J.* **2005**; 24:17-23
174. Jodar, L.; Butler, J.; Carlone, G.; Dagan, R.; Goldblatt, D.; Kayhty, H.; Klugman, K.; Plikaytis, B.; Siber, G.; Kohberger, R.; Chang, I.; Cherian, T.: Serological criteria for evaluation and licensure of new pneumococcal conjugate vaccine formulations for use in infants. *Vaccine* **2003**; 21:3265-3272
175. Lee, L.H.; Frasch, C.E.; Falk, L.A.; Klein, D.L.; Deal, C.D.: Correlates of immunity for pneumococcal conjugate vaccines. *Vaccine* **2003**; 21:2190-2196
176. Van de Winkel, J.G. and Capel, P.J.: Human IgG Fc receptor heterogeneity: molecular aspects and clinical implications. *Immunol.Today* **1993**; 14:215-221
177. Ravetch, J.V.: Fc receptors. *Curr.Opin.Immunol.* **1997**; 9:121-125
178. Ravetch, J.V. and Bolland, S.: IgG Fc receptors. *Annu.Rev.Immunol.* **2001**; 19:275-290
179. Warmerdam, P.A.; van de Winkel, J.G.; Vlug, A.; Westerdaal, N.A.; Capel, P.J.: A single amino acid in the second Ig-like domain of the human Fc gamma receptor II is critical for human IgG2 binding. *J.Immunol.* **1991**; 147:1338-1343
180. Sanders, L.A.; van de Winkel, J.G.; Rijkers, G.T.; Voorhorst-Ogink, M.M.; De Haas, M.; Capel, P.J.; Zegers, B.J.: Fc gamma receptor IIa (CD32) heterogeneity in patients with recurrent bacterial respiratory tract infections. *J.Infect. Dis.* **1994**; 170:854-861
181. Sanders, E.A.; van de Winkel, J.G.; Feldman, R.G.; Voorhorst-Ogink, M.M.; Rijkers, G.T.; Capel, P.J.; Zegers, B.J.: Fc gamma IIA receptor phenotype and opsonophagocytosis in two patients with recurrent bacterial infections. *Immunodeficiency* **1993**; 4:163-165
182. van Heerbeek, N.; Straetemans, M.; Wiertsema, S.P.; Ingels, K.; Rijkers, G.T.; Schilder, A.G.; Sanders, E.A.; Zielhuis, G.A.: The effect of combined pneumococcal conjugate and polysaccharide vaccination on recurrent otitis media with effusion. *Pediatrics* **2005**



2

Functional polymorphisms in the MBL2 gene: Impact on MBL levels and otitis media

Selma P. Wiertsema[1], Bjorn Herpers[2], Reinier H. Veenhoven[3], Marcel M.M. Salimans[4], Henk J.T. Ruven[5], Elisabeth A.M. Sanders[1], Ger T. Rijkers[1]

1 Department of Pediatric Immunology, University Medical Center Utrecht, the Netherlands
2 Department of Medical Microbiology, St. Antonius Hospital, Nieuwegein, the Netherlands
3 Department of Pediatrics, Spaarne Hospital, Hoofddorp, the Netherlands
4 Department of Medical Microbiology, Meander MC, Amersfoort, the Netherlands
5 Department of Clinical Chemistry, St. Antonius Hospital, Nieuwegein, the Netherlands

Submitted

Summary

Mannan-binding lectin (MBL) can bind highly conserved structures on a wide range of microorganisms, leading to initiation of the lectin pathway of complement activation. Aberrant functional MBL serum levels, caused by MBL2 gene polymorphisms, are a possible risk factor for recurrent infections. Within the seven common previously described MBL haplotypes, still considerable variation in MBL serum levels and activity is known to exist.

12 genetic variants in the MBL2 gene and functional MBL serum levels were determined in a large and well-defined cohort of children with a history of recurrent acute otitis media. Haplotypes were constructed and associated with functional MBL serum levels and the number of otitis episodes in the previous year.

The 7 common MBL2 haplotypes mainly determine the level of functional MBL in serum. Additionally, the 3130G>C SNP, located in exon 4, further significantly influenced functional MBL levels within the LXPA haplotype. LXPA carriers with 3130G showed a significantly lower geometric mean functional MBL serum level of 0.19 μg/ml (95% CI 0.07-0.5) compared to 0.70 μg/ml (95% CI 0.4-1.2) in 3130C carriers (p=0.026). Clinically, non-wild type MBL2 carriers between 12-24 months of age had a significantly increased number of acute otitis media episodes (5.1/year) compared to wild type MBL2 carriers (4.1/year) (p=0.027). In older children this association was not found anymore.

We conclude that additional SNPs within the seven common haplotypes can further explain the observed variation in functional MBL serum levels. MBL seems to be of particular clinical importance during early childhood, when maternally derived antibodies have waned, and protective adaptive immunity is not well developed yet.

Introduction

Mannan-binding lectin (MBL) is a C-type lectin with a collagen-like domain[1] that plays an important role in innate immune defense against infections.[2] MBL functions as a pattern-recognition receptor (PRR) that can bind highly conserved structures on a wide range of microorganisms, known as pathogen-associated molecular patterns (PAMPs).[3,4] MBL subunits fold into oligomers, which upon binding of mannan or alternative carbohydrate ligands, recruit MBL-associated serine proteases (MASPs). This complex initiates the lectin pathway of complement activation.[5-8] As compared with other components of the complement system, there is an enormous range in serum MBL concentrations in humans, from 0.1-10,000 ng/ml. MBL deficiency, defined as an MBL serum concentration below 100 ng/ml, has been estimated to occur in 5-10% of the general Caucasian population.[9] This condition seems to be associated with an increased risk for infections, especially when a coexisting primary or secondary immune deficiency is present and during the immunologic frail period of early childhood, when the adaptive immune system has not yet matured and protective maternally derived antibodies have waned.[10-12] Furthermore, MBL insufficiency is thought to influence autoimmune conditions, like SLE and rheumatoid arthritis, and the severity and course of several other diseases, like AIDS, hepatitis, and cystic fibrosis.[10,13-15]

The differences in circulating serum MBL levels can to a large extent be explained by genetic variation in the MBL2 gene, which encodes MBL.[16] Three single nucleotide polymorphisms (SNPs) in exon 1, known as the D, B and C alleles, all interfere with the formation of higher MBL oligomers, leading to alterations in circulating MBL levels (Arg52Cys, D-variant; Gly54Asp, B-variant; Gly57Glu, C-variant).[17-19] An MBL coding region carrying any of the three polymorphisms is collectively termed "o", whereas the wild-type allele is referred to as "A".[20] In addition, three pairs of allelic dimorphisms in the downstream promoter and the 5'-untranslated region of the MBL gene have been described to further affect MBL serum levels: -619G>C (H/L), -290G>C (Y/X) and -66C>T (P/Q).[20-22] Many other polymorphisms within the MBL2 gene exist; whether these SNPs also influence functional MBL serum levels is unknown.[23] Since strong linkage disequilibrium exists between the known genetic MBL variants, at present only seven common haplotypes have been described.[20,24]

The most frequently diagnosed infection in early childhood is otitis media (OM), in which viral, especially respiratory syncytial virus (RSV), as well as bacterial pathogens like *Streptococcus pneumoniae* and *Haemophilus influenzae* are involved.[25,26] MBL activity could contribute to protection against pathogens involved in the development of OM and likewise, MBL deficiency could be one of the factors predisposing to recurrent OM. We studied haplotypes of MBL2 exon 1 and Y/X promoter polymorphisms in a well-defined group of 383 children with a history of recurrent acute otitis media (AOM). Haplotypes were associated with functional MBL serum levels and with the number of AOM episodes the child had experienced in the previous year. Additionally, haplotypes composed of 12 polymorphisms across the MBL2 gene were investigated for their influence on functional serum MBL levels in these children with recurrent AOM.

Material and Methods

Study population

Serum and DNA was available from children with a history of AOM, participating in a randomized controlled pneumococcal vaccine efficacy trial as described in detail elsewhere.[27] Inclusion criteria were age between 1 and 7 years and a history of 2 or more physician diagnosed AOM episodes in the previous year. The ethical committees of the participating hospitals previously approved the study. Written informed parental consent was obtained from all subjects. Serum and whole blood for DNA isolation were collected at study entry, i.e. before any vaccination was given. Serum and blood was stored at -20 °C until analysis.

MBL2 exon 1 and Y/X promoter haplotyping

Genomic DNA was extracted from whole blood using a QIAamp DNA Blood Kit (Qiagen, Hilden, Germany) and stored at -20°C until analysis. Haplotypes of MBL2 exon 1 and Y/X promoter polymorphisms were determined using a previously described denaturing gradient gel electrophoresis (DGGE) assay with slight modifications in a nested PCR protocol.[28] Two PCR assays specific for the promoter X haplotype (forward primer ATT TGT TCT CAC TGC CAC C; reverse primer GAG CTG AAT CTC TGT TTT GAG TT; 25 cycles, annealing temperature 63°C) or Y haplotype (forward primer TTT GTT CTC ACT GCC ACG) were run. The PCR products were diluted 1:100 in distilled water. MBL2 exon 1 was amplified from these dilutions with an extra GC-clamp attached (forward primer with clamp ccg ccc gcc gcg ccc cgc gcc cgg ccc gcc gcc ccc gcc ccG TGT TCA TTA ACT GAG ATT AAC CTT C; reverse primer CAG AAC AGC CCA ACA CG). The amplified DNA was run overnight on a 6% polyacrylamide gel containing a denaturing gradient linearly increasing from 35% to 55% formamide and urea. Upon electrophoresis, all exon 1 haplotypes have a unique pattern of migration. The corresponding Y/X promoter haplotypes could be inferred from the presence or absence of a product in the nested PCR.

Single Nucleotide Polymorphisms in the MBL2 gene

11 Single Nucleotide Polymorphisms in the MBL2 gene were determined using polymerase chain reaction with sequence-specific primers (SSP-PCR). SNPs and primer sequences are described in Table 1.

Because of the reported difficulties in SSP-PCR for the -619G>C (H/L) SNP (rs11003125)[20] this SNP was determined using a TaqMan SNP Genotyping assay. Two locus-specific PCR primers (forward primer GGA GTT TGC TTC CCC TTG GT; reverse primer GGG CCA ACG TAG TAA GAA ATT TCC A) and two allele-specific oligonucleotide TaqMan probes (CAA GCC TGT CTA AAA C-Vic; AAG CCT GTG TAA AA C-Fam) were used.

Functional MBL levels

Functional mannan-binding lectin levels in serum were determined using the assay described by Kuipers et al.[29] This assay is based on the principle of yeast-induced bystander lysis of chicken erythrocytes. In short, serum samples were diluted and incubated with a standardized

Table 1 Single Nucleotide Polymorphisms determined using SSP-PCR

dbSNP identifier	SNP*	Amino-acid change	Name	Primer
rs930506	−5156A>G	promoter		R-AAAGGCAGCTCAGTAGTTAGGA**T** R- AAGGCAGCTCAGTAGTTAGGA**C** F-CAATGATGGTCACCCACTAGG
rs920725	−1640C>T	promoter		R-CCTTACGGGCATCGCCA**A** R- CTTACGGGCATCGCCA**G** F-GTGATGATAGCACAACCGTGT
rs7084554	−418A>G	promoter		R-CTCCATGTCCTCTTCGGG**T** R- TCCATGTCCTCTTCGGG**C** F-GCTAGGCTGCTGAGGTTTCTT
rs7096206	−290G>C	promoter	Y/X	F-CATTTGTTCTCACTGCCAC**G/C** R-GATGCCAGAGAATGAGAGCTG
rs7095891	−66C>T	promoter	P/Q	F-GTAGGACAGAGGGCATGCT**T** F- TAGGACAGAGGGCATGCT**C** R-GATGCCAGAGAATGAGAGCTG
rs5030737	154C>T	D52C	D-variant	R- T (C) CCTTGGTG (C) CATCAC**G**† R- CT (T) CCTTGGTG (C) CATCAC**G**† R- CT (C) CCTTGGTG (T) CATCAC**G**† R-TTCT (T) CCTTGGTG (T) CATCAC**G**† R- CT(C/T)CCTTGGTG(C/T)CATCAC**A**† F-ACGGTCCCATTTGTTCTCACT
rs1800450	161G>A	G54D	B-variant	R-CCCCTTTTCT(C/T)CCTTGGTG**T**† R- CCCTTTTCT(C/T)CCTTGGTG**C**† F-ACGGTCCCATTTGTTCTCACT
rs1800451	170G>A	G57E	C-variant	R-ACCTGGTTCCCCCTTTTCT**T** R- CCTGGTTCCCCCTTTTCT**C** F-ACGGTCCCATTTGTTCTCACT
rs1982266	760C>T	intron		F-TCCTGGGTCAAGGTCTCC**T** F- CCTGGGTCAAGGTCTCC**C** R-TTTTTCCAGGGTCTCCTTTTTG
rs930507	3130G>C	L126L		F-CCACTTTTTCACATTTTAGGGCT**G/C** R-ATAGGGAACTCACAGACGGC
rs2506	5887T>G			R-TGTCTTTCAGGAGTCTATACCTG**A** R- GTCTTTCAGGAGTCTATACCTG**C** F-ATGAGGTTTCTACTGGGACCAC

Notes. * Basepair substitutions are defined based on the coding strand

† Different primers were used in one run for overlapping sequences in exon1

amount of freshly cultured baker's yeast (S. cerevisiae) to activate the MBL pathway. To standardize for complement components of the classical, amplification and terminal route, MBL deficient serum was added. Chicken erythrocytes were used as the target for hemolysis due to MBL pathway activation. Hemoglobin release was measured in an ELISA reader at 405 nm. Percentages of hemolysis were calculated using controls for 100% (water lysed) and 0% (buffer control) hemolysis. MBL activity is expressed as µg equivalent MBL per ml, relative to a reference human serum pool with an established antigenic MBL level of 1.67 µg/ml.

Statistical analyses

Differences in functional MBL concentrations according to MBL2 Exon1 and Y/X haplotypes were tested for significance using a general linear model correcting for age and gender. The difference in the mean number of documented AOM episodes in the previous year according to haplotype was tested for significance using Mann-Whitney U-test. Haplotypes for the 12 SNP positions were estimated from unphased genotype data using the Baysian statistical method in PHASE 2.1[30,31] (http://www.stat.washington.edu/stephens/software.html). Two not previously described haplotypes (HYPB, LYQB) were found. These two additional haplotypes might be imperfectly constructed using PHASE or these individuals might have been incorrectly genotyped. Therefore these subjects were not included in statistical analyses.
Differences in functional MBL levels according to the phased haplotypes were tested for significance using a general linear model correcting for age and gender.
SPSS 12.0.1 for windows (SPSS Inc., Chicago, IL) was used for all statistical analyses. $P<0.05$ was considered significant.

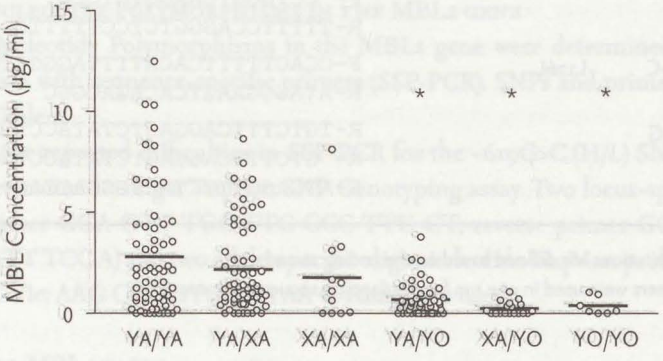


Figure 1 Functional MBL concentrations (µg/ml) according to MBL haplotype.
* Indicates $p<0.01$ compared to wild-type haplotype YA/YA

Results

Functional and genetic MBL analyses were performed in material from a pneumococcal vaccine efficacy trial, in which 383 children were initially included. Of 244 of these children DNA was available to determine haplotypes of MBL2 exon 1 and Y/X promoter polymorphisms using DGGE. More than 98% of the patients were of Caucasian ethnicity. Functional MBL concentrations were determined in available serum from 204 of these 244 subjects. We correlated haplotypes of these individuals with their serum MBL level. Highest mean functional MBL levels were observed in serum of wild-type YA/YA haplotype carriers (figure 1). Mean serum MBL levels were lower when MBL2 exon 1 and/or Y/X promoter polymorphisms were present. Differences in MBL serum levels between YA/YO, XA/YO or YO/YO and wild-type YA/YA haplotype carriers were statistically significant ($p<0.01$).

Additionally, functional serum MBL concentrations were investigated according to haplotypes constructed of 12 polymorphisms in the MBL2 gene in 195 individuals with full genotype data on these 12 SNP positions (figure 2). PHASE 2.1 software constructed 33 different haplotypes from the genotype data. Based on the MBL2 exon 1 and the H/L, Y/X, and P/Q promoter SNPs, the seven common (HYPA, LYPA, LYQA, LXPA, HYPD, LYPB, and LYQC) and one of the rare (HXPA) haplotypes were obtained [20,32]. Of 181 children with haplotype data functional MBL levels were available. We now investigated geometric mean functional MBL concentrations according to the 8 recognized haplotypes. MBL levels were lower when an MBL2 exon 1 polymorphism was present (Table 2).

Of the additional 6 SNPs that were studied, the exon 4 SNP 3130G>C, common within the LXPA haplotype, was found to affect functional MBL levels. Haplotypes with 3130G showed a significantly lower geometric mean functional MBL serum level of 0.19 μg/ml (95% CI 0.07-0.5) compared to 0.70 μg/ml (95% CI 0.4-1.2) in haplotypes including 3130C carriers ($p=0.026$) (figure 2).

The 12 SNP haplotype analysis revealed linkage between a number of SNPs. The P/Q SNP at

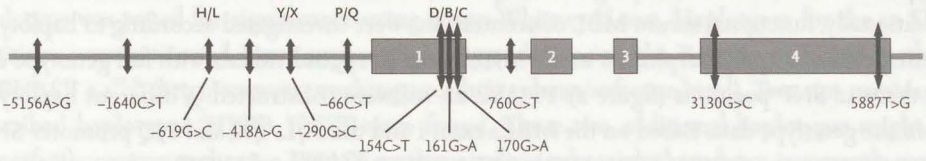
Table 2 Geometric mean MBL levels (μg/ml) and 95% confidence intervals according to recognized haplotypes

	N	Mean functional MBL level (μg/ml)	95% CI	p-values					
				LYPA	LYQA	LXPA	HYPD	LYPB	LYQC
HYPA	119	0.62	0.42-0.91	0.2	0.4	0.4	0.05	<0.001	0.009
LYPA	22	1.18	0.48-2.89		0.5	0.07	0.01	<0.001	0.003
LYQA	60	0.84	0.49-1.44			0.1	0.02	<0.001	0.004
LXPA	94	0.47	0.30-0.73				0.1	<0.001	0.02
HYPD	17	0.20	0.07-0.57					0.3	0.2
LYPB	44	0.10	0.05-0.20						0.5
LYQC	5	0.05	0.01-0.32						

Note. p-values comparing these levels are calculated using a general linear model correcting for age and gender

position –66 was linked with the –418A>G promoter SNP and with the –5156A>G promoter SNP (figure 2).

Clinical relevance of the MBL2 haplotypes was investigated by comparing the mean number of AOM episodes in the previous year between carriers of the wild-type YA/YA haplotype with non-wild-type carriers (Table 3). For the group as a whole there was no difference in the number of previous AOM episodes according to haplotype. Since MBL is thought to be of particular importance in young children, we split the cohort based on age. Indeed, in the group of youngest children between 12-24 months of age, wild-type carriers had experienced significantly less AOM episodes as compared to non-wild-type carriers (p=0.027). For the older children the MBL haplotype had no effect on the number of AOM episodes (p=0.89).



	–5156	–1640	–619	–418	–290	–66	Exon 1	760	3130	5887	N	MBL level (95% CI)
HYPA	A	C	H	A	Y	P	A	C	G	T	115	0.57 (0.4 - 0.8)
	A	C	H	A	Y	P	A	T	G	T	3	2.75 (0.2 - 30.6)
	A	T	H	A	Y	P	A	C	G	T	1	nd
LYPA	A	T	L	A	Y	P	A	C	G	G	13	0.96 (0.3 - 3.1)
	A	C	L	A	Y	P	A	C	G	G	5	1.36 (0.2 - 8.9)
	A	T	L	A	Y	P	A	T	G	G	1	nd
	A	C	L	A	Y	P	A	T	C	G	1	nd
	A	C	L	A	Y	P	A	C	G	T	1	nd
	A	T	L	A	Y	P	A	C	G	T	1	nd
LYQA	G	C	L	G	Y	Q	A	T	G	T	45	0.80 (0.4 - 1.5)
	G	T	L	G	Y	Q	A	T	G	T	6	0.62 (0.1 - 3.5)
	G	C	L	G	Y	Q	A	T	G	G	5	0.69 (0.1 - 4.5)
	G	T	L	G	Y	Q	A	T	G	G	2	1.47 (0.1 - 28.2)
	G	T	L	A	Y	Q	A	T	G	T	1	nd
	A	C	L	G	Y	Q	A	T	G	G	1	nd
LXPA	A	C	L	A	X	P	A	T	C	T	71	0.64 (0.4 - 1.1)
	A	C	L	A	X	P	A	T	G	G	15	0.18 (0.1 - 0.5)
	A	C	L	A	X	P	A	T	G	T	4	0.24 (0.0 - 2.0)
	A	C	L	A	X	P	A	T	C	G	2	0.87 (0.0 - 16.8)
	A	C	L	A	X	P	A	C	C	T	1	nd
	A	T	L	A	X	P	A	T	C	T	1	nd
HXPA	A	C	H	A	X	P	A	C	G	T	1	nd
HYPD	A	C	H	A	Y	P	D	C	G	T	15	0.15 (0.1 - 0.5)
	A	T	H	A	Y	P	D	C	G	T	2	1.53 (0.1 - 29.6)
LYPB	A	C	L	A	Y	P	B	C	G	G	38	0.12 (0.1 - 0.2)
	A	C	L	A	Y	P	B	C	G	T	3	0.01 (0.0 - 0.1)
	A	C	L	A	Y	P	B	C	C	G	2	0.08 (0.0 - 1.6)
	A	C	L	A	Y	P	B	C	G	G	1	nd
LYQC	G	C	L	G	Y	Q	C	T	G	G	5	0.05 (0.0 - 0.3)

Figure 2 Schematic representation of single nucleotide polymorphisms studied across the MBL2 gene and overview of haplotypes with functional MBL levels (µg/ml) constructed from genotype data on the 12 SNP positions.

N indicates frequency of haplotypes.

nd: Mean MBL level is not determined when haplotype frequency (N) is 1.

Wild-type positions are indicated in light grey, polymorphic positions in dark grey.

Discussion

MBL levels and function vary greatly within the general population. Part of this variation can be explained by polymorphisms within the structural gene as well as by promoter polymorphisms. Yet, since considerable variation in MBL activity exists even within the seven common MBL haplotypes, it has been speculated that additional variants may contribute to functional MBL levels. To address this issue, we have correlated both the 7 recognized MBL2 gene haplotypes as well as several new polymorphisms within existing haplotypes with functional serum MBL levels. Indeed we found that a common SNP in exon 4 (3130G>C) regulates functional MBL serum levels within the LXPA haplotype. Furthermore, we showed that functional MBL2 gene polymorphisms are of clinical importance with respect to susceptibility to AOM in children below two years of age when adaptive immune responses are still immature and the child is without maternally derived protective antibodies.

Recently Bernig *et al.* published 87 polymorphic sites across the MBL2 gene.[23] The high frequency of MBL2 polymorphic haplotypes in certain ethnic populations suggests a possible selective advantage for heterozygosity.[20,21] Carriers of MBL2 genetic variants may be protected against intracellular replicating organisms like *Mycobacteria* spp and *Leishmania* spp or the lower MBL levels serve to reduce the harmful effects of extreme complement activation via the lectin pathway.[33-36]

In the study of Bernig *et al.* a high degree of linkage between 51 SNPs with a minor-allele frequency greater than 0.05, occurring in a two-block structure, was observed. We also found a high degree of linkage in our current study, especially within the 5' end of the MBL2 gene. Whereas

Table 3 Association between MBL haplotype and number of AOM episodes from retrospectively obtained data.

		Total cohort	12-24 months	>24 months
YA/YA				
	Number of children	76	32	44
	Median AOM episodes	3	3	3
	Mean AOM episodes (95% CI)	4.3 (3.7 - 4.9)	4.1 (3.2 - 5.0)	4.4 (3.6 - 5.2)
Non-YA/YA				
	Number of children	168	81	87
	Median AOM episodes	4	4	3
	Mean AOM episodes (95% CI)	4.7 (4.3 - 5.1)	5.1 (4.5 - 5.6)	4.4 (3.8 - 5.0)
p-value		0.12	0.027	0.89

Note. p-values are determined comparing the number of AOM episodes between wild-type and non-wild-type carriers using Mann-Whitney U-test.

Bernig *et al.* defined the 5' block boundary at position –2703, we now observed linkage of the more 5' SNP at position –5156 with the SNPs at positions –418 and –66 of the MBL2 promoter. This could implicate that the boundaries of the 5' block lie further downstream in the promoter region, and future analysis beyond the –5156 boundary, possibly including neighboring genes, may be required for optimal correlation of MBL2 haplotypes with MBL serum levels.

The density of common SNPs is comparable for the two-block structures identified by Bernig *et al.*, but the region that separates the two blocks shows an increased recombination rate and thus probably includes a recombination hot spot. Within this recombination hot spot we now identified an SNP (3130G>C) that apparently influences functional MBL levels within the LXPA haplotype. As compared to homozygous carriers of the other common haplotypes (HYPA, LYPA or LYQA), LXPA homozygotes display a much larger variation in MBL levels.[24] Our finding that the 3130G>C SNP further splits-up the LXPA haplotype in two haplotypes with different MBL activities, could be the basis for that large variation. Subtyping of LXPA homozygotes for the 3130G>C SNP would further establish the relation between this particular genotype and MBL function.

The 3130G>C SNP has previously been shown to be mainly observed within the LXPA haplotype in Caucasians.[23] In other ethnicity groups 3130C can also be found in the HYPD, LYPA, and LYQC haplotypes.[23] Our study population consists for approximately 95% of Caucasian children and we thus found the 3130C primarily within the LXPA haplotype.

The 3130G>C SNP in exon 4, which encodes the carbohydrate recognition domain, does not lead to an amino acid substitution. However, it is know that non-coding SNPs might influence exon splicing or stability of the mRNA.[23,37] Alternatively, Bernig *et al.* showed the 3130G>C SNP to be in linkage disequilibrium with the intronic IVS3+709G>C SNP. Possibly this SNP or another SNP in linkage with 3130G>C causes the difference in functional MBL serum levels we observed within the LXPA haplotype.

Previously variant MBL2 genotypes as well as low circulating levels of MBL have been associated with susceptibility to invasive pneumococcal and meningococcal disease, immunodeficiency and severe infections.[38-43] Additionally, several studies showed an association of upper respiratory tract infections and/or acute otitis media with aberrant MBL levels and/or genotypes,[11,44-46] but this was not confirmed by others.[47,48] These conflicting results might be caused by variation in age of subjects under study, since in one prospective study in children from Greenland the maximal effect of MBL deficiency was observed in children between 6 and 17 months of age, whereas the effect could not be demonstrated in younger or older children.[11] The fact that we find MBL relevance only below the age of 24 months makes sense, since adaptive immunity against polysaccharide encapsulated bacteria, a main group of causative pathogens for AOM, matures around this age.[49] Similar to Koch *et al.* we included the Y/X promoter polymorphism in our clinical association study, whereas other studies only investigated the structural MBL2 exon 1 variants. Since others and we showed that the Y/X promoter polymorphism further down-regulates functional MBL levels, this also could explain the conflicting data in literature.[20] Finally, the studies that did not find an association between otitis media and MBL2 genotype were rather small (n=73, n=89), especially when looking at otitis media sub-groups.[47,48] Probably large and well-characterized cohorts of defined ethnicity are necessary to establish the effect of

a single gene in a multi-factorial disease like otitis media.
Overall, our data show that both recognized and new MBL2 polymorphisms influence functional MBL serum levels and are of clinical relevance in young children suffering from recurrent acute otitis media. Possibly additional SNPs within common MBL2 haplotypes are of clinical importance. Therefore, future association studies will probably require a more locus-wide approach, as already suggested by Bernig *et al.* Since data on MBL2 haplotype distribution in a childhood population without recurrent OM is not available, and healthy adult populations will include persons with a history of recurrent OM, a large prospective birth cohort is probably required to determine the relative contribution of MBL2 haplotypes to the development of recurrent OM. Whether MBL replacement therapy[50-52] will be a treatment option for these children will depend also on the relative impact of MBL on AOM susceptibility.

Acknowledgements

We want to thank Piet Aerts (Eijkman-Winkler Center for Microbiology, Inflammation and Infectious Diseases, University Medical Center Utrecht, the Netherlands) for functional MBL determinations. Wim Gerritsen, Jan Broess, Helga Dissel and Marieke Quaak (all from the Department of Clinical Chemistry, St. Antonius Hospital, Nieuwegein, the Netherlands) and Frouke Stegeman (Department of Microbiology, Meander MC, Amersfoort, the Netherlands) assisted with SNP analysis. Ben de Jong (Department of Medical Microbiology, St. Antonius Hospital, Nieuwegein, the Netherlands) assisted with MBL haplotype determinations using DGGE. Furthermore, we would like to thank Drs. Kors van der Ent and Martijn Slieker for stimulating discussions.
The work described in this paper was financially supported by the Netherlands Organization for Health Research and Development ZonMW (grant numbers 002828480 and 90461092), and the Health Insurance Company Zilveren Kruis-Achmea.

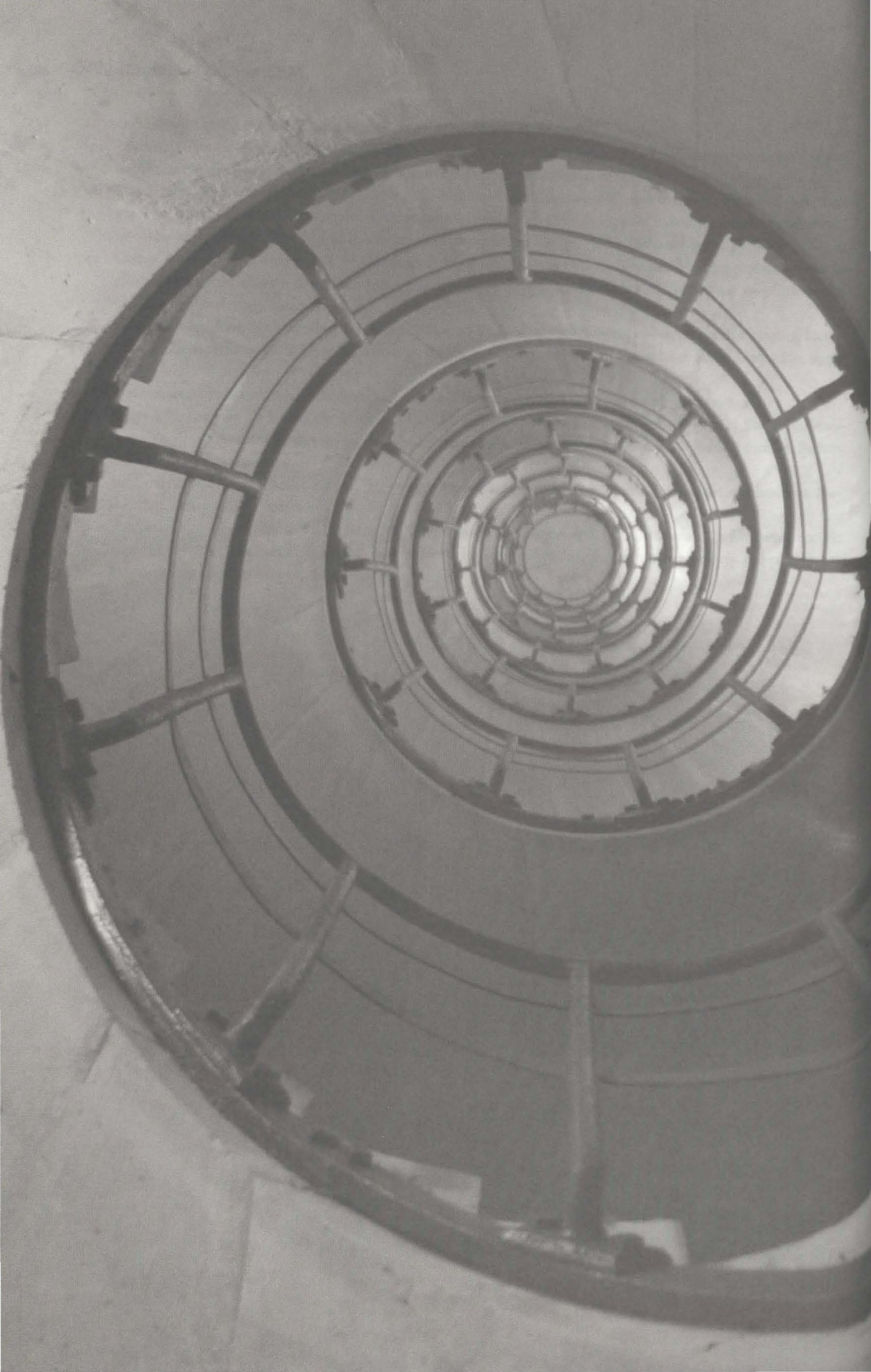
References

1. Holmskov, U.; Thiel, S.; Jensenius, J.C.: Collections and ficolins: humoral lectins of the innate immune defense. Annu.Rev.Immunol. **2003**; 21:547-578
2. Kilpatrick, D.C.: Mannan-binding lectin and its role in innate immunity. Transfus.Med. **2002**; 12:335-352
3. Janeway, C.A., Jr. and Medzhitov, R.: Innate immune recognition. Annu.Rev.Immunol. **2002**; 20:197-216
4. Medzhitov, R. and Janeway, C., Jr.: Innate immunity. N.Engl.J.Med. **2000**; 343:338-344
5. Jack, D.L. and Turner, M.W.: Anti-microbial activities of mannose-binding lectin. Biochem.Soc.Trans. **2003**; 31:753-757
6. Townsend, R.; Read, R.C.; Turner, M.W.; Klein, N.J.; Jack, D.L.: Differential recognition of obligate anaerobic bacteria by human mannose-binding lectin. Clin.Exp.Immunol. **2001**; 124:223-228
7. van Emmerik, L.C.; Kuijper, E.J.; Fijen, C.A.; Dankert, J.; Thiel, S.: Binding of mannan-binding protein to various bacterial pathogens of meningitis. Clin.Exp.Immunol. **1994**; 97:411-416
8. Ikeda, K.; Sannoh, T.; Kawasaki, N.; Kawasaki, T.; Yamashina, I.: Serum lectin with known structure activates complement through the classical pathway. J.Biol.Chem. **1987**; 262:7451-7454
9. Turner, M.W.: Deficiency of mannan binding protein--a new complement deficiency syndrome. Clin.Exp.Immunol. **1991**; 86 Suppl 1:53-56
10. Super, M.; Thiel, S.; Lu, J.; Levinsky, R.J.; Turner, M.W.: Association of low levels of mannan-binding protein with a common defect of opsonisation. Lancet **1989**; 2:1236-1239
11. Koch, A.; Melbye, M.; Sorensen, P.; Homoe, P.; Madsen, H.O.; Molbak, K.; Hansen, C.H.; Andersen, L.H.; Hahn, G.W.; Garred, P.: Acute respiratory tract infections and mannose-binding lectin insufficiency during early childhood. JAMA **2001**; 285:1316-1321
12. Aittoniemi, J.; Baer, M.; Soppi, E.; Vesikari, T.; Miettinen, A.: Mannan binding lectin deficiency and concomitant immunodefects. Arch.Dis.Child **1998**; 78:245-248
13. Kilpatrick, D.C.: Mannan-binding lectin: clinical significance and applications. Biochim.Biophys.Acta **2002**; 1572:401-413
14. Turner, M.W.: The role of mannose-binding lectin in health and disease. Mol.Immunol. **2003**; 40:423-429
15. Carlsson, M.; Sjoholm, A.G.; Eriksson, L.; Thiel, S.; Jensenius, J.C.; Segelmark, M.; Truedsson, L.: Deficiency of the mannan-binding lectin pathway of complement and poor outcome in cystic fibrosis: bacterial colonization may be decisive for a relationship. Clin.Exp.Immunol. **2005**; 139:306-313
16. Crosdale, D.J.; Ollier, W.E.; Thomson, W.; Dyer, P.A.; Jensenious, J.; Johnson, R.W.; Poulton, K.V.: Mannose binding lectin (MBL) genotype distributions with relation to serum levels in UK Caucasoids. Eur.J.Immunogenet. **2000**; 27:111-117
17. Madsen, H.O.; Garred, P.; Kurtzhals, J.A.; Lamm, L.U.; Ryder, L.P.; Thiel, S.; Svejgaard, A.: A new frequent allele is the missing link in the structural polymorphism of the human mannan-binding protein. Immunogenetics **1994**; 40:37-44
18. Sumiya, M.; Super, M.; Tabona, P.; Levinsky, R.J.; Arai, T.; Turner, M.W.; Summerfield, J.A.: Molecular basis of opsonic defect in immunodeficient children. Lancet **1991**; 337:1569-1570
19. Lipscombe, R.J.; Sumiya, M.; Summerfield, J.A.; Turner, M.W.: Distinct physicochemical characteristics of human mannose binding protein expressed by individuals of differing genotype. Immunology **1995**; 85:660-667
20. Madsen, H.O.; Garred, P.; Thiel, S.; Kurtzhals, J.A.; Lamm, L.U.; Ryder, L.P.; Svejgaard, A.: Interplay between promoter and structural gene variants control basal serum level of mannan-binding protein. J.Immunol. **1995**; 155:3013-3020
21. Madsen, H.O.; Satz, M.L.; Hogh, B.; Svejgaard, A.; Garred, P.: Different molecular events result in low protein levels of mannan-binding lectin in populations from southeast Africa and South America. J Immunol. **1998**; 161:3169-3175
22. den Dunnen, J.T. and Antonarakis, S.E.: Mutation nomenclature extensions and suggestions to describe complex mutations: a discussion. Hum.Mutat. **2000**; 15:7-12
23. Bernig, T.; Taylor, J.G.; Foster, C.B.; Staats, B.; Yeager, M.; Chanock, S.J.: Sequence analysis of the mannose-binding lectin (MBL2) gene reveals a high degree of heterozygosity with evidence of selection. Genes Immun. **2004**; 5:461-476

24. Steffensen, R.; Thiel, S.; Varming, K.; Jersild, C.; Jensenius, J.C.: Detection of structural gene mutations and promoter polymorphisms in the mannan-binding lectin (MBL) gene by polymerase chain reaction with sequence-specific primers. J.Immunol.Methods **2000**; 241:33-42
25. Block, S.L.: Causative pathogens, antibiotic resistance and therapeutic considerations in acute otitis media. Pediatr.Infect.Dis.J. **1997**; 16:449-456
26. Teele, D.W.; Klein, J.O.; Rosner, B.: Epidemiology of otitis media during the first seven years of life in children in greater Boston: a prospective, cohort study. J.Infect.Dis. **1989**; 160:83-94
27. Veenhoven, R.; Bogaert, D.; Uiterwaal, C.; Brouwer, C.; Kiezebrink, H.; Bruin, J.; IJzerman, E.; Hermans, P.; de Groot, R.; Zegers, B.; Kuis, W.; Rijkers, G.; Schilder, A.; Sanders, E.: Effect of conjugate pneumococcal vaccine followed by polysaccharide pneumococcal vaccine on recurrent acute otitis media: a randomised study. Lancet **2003**; 361:2189-2195
28. Gabolde, M.; Muralitharan, S.; Besmond, C.: Genotyping of the three major allelic variants of the human mannose-binding lectin gene by denaturing gradient gel electrophoresis. Hum.Mutat. **1999**; 14:80-83
29. Kuipers, S.; Aerts, P.C.; Sjoholm, A.G.; Harmsen, T.; van Dijk, H.: A hemolytic assay for the estimation of functional mannose-binding lectin levels in human serum. J.Immunol.Methods **2002**; 268:149-157
30. Stephens, M. and Donnelly, P.: A comparison of bayesian methods for haplotype reconstruction from population genotype data. Am.J.Hum.Genet. **2003**; 73:1162-1169
31. Stephens, M.; Smith, N.J.; Donnelly, P.: A new statistical method for haplotype reconstruction from population data. Am.J.Hum.Genet. **2001**; 68:978-989
32. Sullivan, K.E.; Wooten, C.; Goldman, D.; Petri, M.: Mannose-binding protein genetic polymorphisms in black patients with systemic lupus erythematosus. Arthritis Rheum. **1996**; 39:2046-2051
33. Garred, P.; Richter, C.; Andersen, A.B.; Madsen, H.O.; Mtoni, I.; Svejgaard, A.; Shao, J.: Mannan-binding lectin in the sub-Saharan HIV and tuberculosis epidemics. Scand.J.Immunol. **1997**; 46:204-208
34. Hoal-Van Helden, E.G.; Epstein, J.; Victor, T.C.; Hon, D.; Lewis, L.A.; Beyers, N.; Zurakowski, D.; Ezekowitz, A.B.; Van Helden, P.D.: Mannose-binding protein B allele confers protection against tuberculous meningitis. Pediatr. Res. **1999**; 45:459-464
35. Santos, I.K.; Costa, C.H.; Krieger, H.; Feitosa, M.F.; Zurakowski, D.; Fardin, B.; Gomes, R.B.; Weiner, D.L.; Harn, D.A.; Ezekowitz, R.A.; Epstein, J.E.: Mannan-binding lectin enhances susceptibility to visceral leishmaniasis. Infect.Immun. **2001**; 69:5212-5215
36. Takahashi, K.; Gordon, J.; Liu, H.; Sastry, K.N.; Epstein, J.E.; Motwani, M.; Laursen, I.; Thiel, S.; Jensenius, J.C.; Carroll, M.; Ezekowitz, R.A.: Lack of mannose-binding lectin-A enhances survival in a mouse model of acute septic peritonitis. Microbes.Infect. **2002**; 4:773-784
37. Cartegni, L.; Chew, S.L.; Krainer, A.R.: Listening to silence and understanding nonsense: exonic mutations that affect splicing. Nat.Rev.Genet. **2002**; 3:285-298
38. Summerfield, J.A.; Ryder, S.; Sumiya, M.; Thursz, M.; Gorchein, A.; Monteil, M.A.; Turner, M.W.: Mannose binding protein gene mutations associated with unusual and severe infections in adults. Lancet **1995**; 345:886-889
39. Bax, W.A.; Cluysenaer, O.J.; Bartelink, A.K.; Aerts, P.C.; Ezekowitz, R.A.; van Dijk, H.: Association of familial deficiency of mannose-binding lectin and meningococcal disease. Lancet **1999**; 354:1094-1095
40. Salimans, M.M.; Bax, W.A.; Stegeman, F.; van Deuren, M.; Bartelink, A.K.; van Dijk, H.: Association between familial deficiency of mannose-binding lectin and mutations in the corresponding gene and promoter region. Clin.Diagn.Lab Immunol. **2004**; 11:806-807
41. Roy, S.; Knox, K.; Segal, S.; Griffiths, D.; Moore, C.E.; Welsh, K.I.; Smarason, A.; Day, N.P.; McPheat, W.L.; Crook, D.W.; Hill, A.V.: MBL genotype and risk of invasive pneumococcal disease: a case-control study. Lancet **2002**; 359:1569-1573
42. Hibberd, M.L.; Sumiya, M.; Summerfield, J.A.; Booy, R.; Levin, M.: Association of variants of the gene for mannose-binding lectin with susceptibility to meningococcal disease. Meningococcal Research Group. Lancet **1999**; 353:1049-1053
43. Garred, P.; Madsen, H.O.; Hofmann, B.; Svejgaard, A.: Increased frequency of homozygosity of abnormal mannan-binding-protein alleles in patients with suspected immunodeficiency. Lancet **1995**; 346:941-943
44. Thorarinsdottir, H.K.; Ludviksson, B.R.; Vikingsdottir, T.; Leopoldsdottir, M.O.; Ardal, B.; Jonsson, T.; Valdimarsson, H.; Arason, G.J.: Childhood levels of immunoglobulins and mannan-binding lectin in relation to infections

and allergy. Scand.J.Immunol. **2005**; 61:466-474

45. Tezcan, I.; Yilmaz, Y.; Oner, F.; Yel, L.; Sanal, O.; Ersoy, F.; Onerci, M.; Berkel, A.I.: Defective serum opsonization activity in children aged 6-48 months having acute purulent otitis media. Turk.J.Pediatr. **1997**; 39:453-457
46. Straetemans, M.; Wiertsema, S.P.; Sanders, E.A.; Rijkers, G.T.; Graamans, K.; van der, B.B.; Zielhuis, G.A.: Immunological status in the aetiology of recurrent otitis media with effusion: serum immunoglobulin levels, functional mannose-binding lectin and Fc receptor polymorphisms for IgG. J.Clin.Immunol. **2005**; 25:78-86
47. Garred, P.; Brygge, K.; Sorensen, C.H.; Madsen, H.O.; Thiel, S.; Svejgaard, A.: Mannan-binding protein--levels in plasma and upper-airways secretions and frequency of genotypes in children with recurrence of otitis media. Clin.Exp.Immunol. **1993**; 94:99-104
48. Homoe, P.; Madsen, H.O.; Sandvej, K.; Koch, A.; Garred, P.: Lack of association between mannose-binding lectin, acute otitis media and early Epstein-Barr virus infection among children in Greenland. Scand.J.Infect.Dis. **1999**; 31:363-366
49. Weintraub, A.: Immunology of bacterial polysaccharide antigens. Carbohydr.Res. **2003**; 338:2539-2547
50. Garred, P.; Pressler, T.; Lanng, S.; Madsen, H.O.; Moser, C.; Laursen, I.; Balstrup, F.; Koch, C.; Koch, C.: Mannose-binding lectin (MBL) therapy in an MBL-deficient patient with severe cystic fibrosis lung disease. Pediatr. Pulmonol. **2002**; 33:201-207
51. Valdimarsson, H.: Infusion of plasma-derived mannan-binding lectin (MBL) into MBL-deficient humans. Biochem.Soc.Trans. **2003**; 31:768-769
52. Valdimarsson, H.; Vikingsdottir, T.; Bang, P.; Saevarsdottir, S.; Gudjonsson, J.E.; Oskarsson, O.; Christiansen, M.; Blou, L.; Laursen, I.; Koch, C.: Human plasma-derived mannose-binding lectin: a phase I safety and pharmacokinetic study. Scand.J.Immunol. **2004**; 59:97-102



3

ASSOCIATION OF THE CD14 PROMOTER POLYMORPHISM WITH OTITIS MEDIA AND PNEUMOCOCCAL VACCINE RESPONSES

Selma P. Wiertsema[1], Siew-Kim Khoo[2], Gareth Baynam[2], Reinier H. Veenhoven[3], Ingrid A. Laing[2], Gerhard A. Zielhuis[4], Ger T. Rijkers[1], Jack Goldblatt[2,5], Peter N. LeSouëf[2], Elisabeth A.M. Sanders[1]

1 Department of Pediatric Immunology, University Medical Center Utrecht, the Netherlands
2 School of Paediatrics and Child Health, University of Western Australia, Perth, Australia
3 Department of Pediatrics, Spaarne Hospital, Hoofddorp, the Netherlands
4 Department of Epidemiology and Biostatistics, Radboud University Nijmegen Medical Centre, the Netherlands
5 Genetic Services of Western Australia, Perth, Australia

Submitted

Summary

Innate immunity may be of particular importance for protection against infections in infants and toddlers, before the full maturation of the adaptive immune system. CD14 plays a role in the innate immune defense against pathogens associated with otitis media, which is a major health care issue in these young children. The CD14 C-159T promoter polymorphism, regulating soluble CD14 levels, may have functional consequences for immune defense against otitis pathogens.

The objective of this study was to investigate the potential role of the functional CD14 C-159T polymorphism in the susceptibility to recurrent acute otitis media of toddlers.

The association between CD14 promoter genotype and the number of acute otitis media episodes was evaluated in a large and well-defined cohort of 300 children participating in a randomized controlled vaccination trial. Since S. pneumoniae is a major bacterial pathogen in otitis media, serotype specific IgG antibody responses after pneumococcal vaccinations were examined according to CD14 genotype in a separate group of children with otitis media.

Children aged between 12-24 months with the CD14 -159TT genotype had significantly fewer episodes of acute otitis media compared to age-matched CC homozygotes (mean number of AOM episodes 4.3 and 5.7, respectively; p=0.003), while this was no longer significant in older children. TT homozygotes showed higher serotype specific anti-pneumococcal IgG antibody levels.

CD14 -159TT homozygous children seem to be less prone to recurrent middle ear infections at early age when adaptive immunity is not yet fully developed. Possibly, higher sCD14 levels in carriers of the T allele, lead to an enhanced immune response in reaction to pathogens as observed after pneumococcal vaccinations.

Introduction

Otitis media (OM) is the most common reason for a child under 3 years of age to visit a general practitioner and represents a major pediatric health care issue.[1,2] A wide variety of bacterial pathogens, such as *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Moraxella catarrhalis*, and viruses, such as Respiratory Syncytial Virus, play a role in the onset of inflammation of the middle ear cavity. Innate defense systems capable of quickly distinguishing potential pathogens from self-structures are important for protection from infection, particularly at early age when the adaptive immune response to many microorganisms, like encapsulated bacteria, is still immature.[3]

CD14 plays a central role in innate immune defense against many microorganisms causing otitis media. After first being characterized as the endotoxin/lipopolysaccharide (LPS) receptor,[4] CD14 seems also to be involved in responses to lipoteichoic acid from gram-positive bacteria,[5] peptidoglycan of both gram-positive and gram-negative bacteria,[6] mycobacteria and viruses.[7] The CD14 crystal structure provides a basis for this ligand diversity.[8] Involvement of CD14 in macrophage responses against purified capsular polysaccharide of *S. pneumoniae*, the most important bacterial pathogen associated with OM, makes CD14 a potential candidate in the immune defense against middle ear infection.[9] CD14 is expressed in a membrane bound form (mCD14) on the cell surface of monocytes and neutrophils, but is also present in serum as a soluble protein. Soluble CD14 (sCD14) has the ability to confer pathogen responsiveness to cells that do not constitutively express CD14 on their membrane, such as epithelial-, endothelial- and dendritic-cells,[10,11] and to enhance mCD14 mediated responses to both LPS and peptidoglycan.[12] CD14 also is an important supplementary molecule needed for optimal functioning of Toll-like receptor 4,[13] which is a principal membrane signaling molecule through which mammals sense infection.[14]

A functional polymorphism in the promoter of the CD14 gene (C–159T) has been described.[15] The T allele is associated with enhanced transcriptional activity in a monocytic reporter assay and increased sCD14 levels in epidemiological studies.[15-17]

Since CD14 might be important in the immune defense against OM related pathogens, carriers of the CD14–159T allele may have a lower susceptibility to middle ear infections.

In a cohort of 300 children with a history of recurrent acute otitis media (AOM) the association between the CD14 C–159T genotype and the number of AOM episodes children experienced in the last year was investigated. It is known that innate immune systems can also influence the adaptive immune response by up-regulation of co-stimulatory molecules and increased cytokine production. Therefore the humoral immune response to *S. pneumoniae*, which is the crucial part of adaptive immunity against this microorganism, was examined in relation to the CD14 promoter polymorphism. IgG antibody responses to 7-valent pneumococcal conjugate vaccination (PCV7) followed by boosting with the 23-valent pneumococcal polysaccharide vaccine (PPV23) were evaluated in children with either a history of recurrent AOM (n=48) or prolonged periods of otitis media with effusion (OME) (n=23).

Methods

Study population to investigate occurrence of AOM

DNA for genotyping of the CD14 C-159T polymorphism was available from 300 children who participated in a randomized controlled trial on the clinical efficacy of pneumococcal vaccines in prevention of AOM.[18] All children were between 1 and 7 years of age and had a history of 2 or more physician documented AOM episodes in the year before study entry.

Study population to investigate pneumococcal vaccine responses

The potential association between the CD14 promoter polymorphism and the response to pneumococcal conjugate followed by polysaccharide vaccinations was investigated in seventy-one children affected with recurrent or prolonged OM. Pneumococcal serotype specific IgG vaccine responses were available from children participating in two large vaccination trials investigating prevention of recurrence of otitis media by pneumococcal vaccinations in the Netherlands.[18,19]

The first group consisted of 48 children aged 2 to 7 years, with a history of 2 or more physician diagnosed episodes of acute otitis media in the previous 12 months, randomly selected from the study population participating in the randomized controlled vaccination study described above (AOM group). All children received the 7-valent pneumococcal conjugate vaccine (Prevnar®, Wyeth Pharmaceuticals, Philadelphia, PA) followed by the 23-valent pneumococcal polysaccharide vaccine 6 months later (Pneumune®, Wyeth Pharmaceuticals, Philadelphia, PA).

The second group consisted of 23 children aged 2 to 7 years, who had suffered from at least two prolonged periods of bilateral otitis media with effusion, each lasting 3 months or longer and documented by an ENT specialist, and who were therefore referred for ventilation tube placement (OME group). These children were also vaccinated with the 7-valent pneumococcal conjugate vaccine followed by the 23-valent pneumococcal polysaccharide vaccine but with a 4 months interval.

The 7-valent pneumococcal conjugate vaccine (PCV7) consisted of 2 μg each of capsular polysaccharides from pneumococcal serotypes 4, 9V, 14, 19F, and 23F, 4 μg of serotype 6B polysaccharide, and 2 μg of serotype 18C oligosaccharide, each conjugated individually to mutant nontoxic diphtheria toxin (CRM197). The 23-valent pneumococcal polysaccharide vaccine (PPV23) consisted of 25 μg of capsular polysaccharides from each of the pneumococcal serotypes 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19F, 19A, 20, 22F, 23F, and 33F.

These studies were approved by the participating hospitals and institutions ethics committees. Parental written informed consent was obtained for all subjects.

DNA extraction and genotyping

Genomic DNA was extracted from whole blood collected at study entry using a QIAamp DNA Blood Kit (Qiagen, Hilden, Germany). Patients were genotyped for the CD14 C-159T polymorphism using polymerase chain reaction (PCR) and restriction enzyme digestion with AvaII (Promega Corporation, Madison, WI), as described by Baldini and coworkers.[15]

Measurement of pneumococcal antibodies and immunoglobulins

Blood samples for determination of pneumococcal antibodies were obtained 4 weeks after the pneumococcal polysaccharide booster vaccination. Serum was separated and stored at -20°C until analysis. Post vaccination IgG antibody levels to all PCV7 serotypes 4, 6B, 9V, 14, 18C, 19F and 23F were measured by ELISA as described previously.[20] Minimal detection levels for these pneumococcal serotypes in our assay were 0.03, 0.09, 0.12, 0.61, 0.05, 0.11, and 0.08 μg/ml, respectively. All sera were pre-incubated overnight at 4°C with pneumococcal cell wall polysaccharide (CPS) in diluting buffer to block non-specific anti-CPS antibodies (50 μg/ml; Statens Serum Institute, Copenhagen, Denmark).[21] The pneumococcal antibody reference serum (lot 89-SF) was used for assay standardization.[22]

Serum immunoglobulin levels (IgM, IgG, IgA) and IgG subclasses were measured by rate nephelometry in sera obtained at enrollment of the study and stored at -20°C until analysis. Total serum IgE levels were determined by ImmunoCAP® (Pharmacia, Uppsala, Sweden).

Statistical methods

ANOVA, Chi-square and Kruskal-Wallis tests were used to determine the significance of differences in general and immunological characteristics.

Poisson regression was employed for the analysis of the mean number of AOM episodes according to CD14 genotype in the group of children between 12-24 months and above 24 months of age, correcting for age, gender and log total IgE.

To test differences between antibody levels after pneumococcal vaccination for the three genotype groups and using a model recessive for the C allele, general linear models were used, adjusting for age, gender, type of ear disease and log total IgE levels.

P-values <0.05 were considered statistically significant. SPSS 12.0.1 for windows (SPSS Inc., Chicago, IL) was used for all statistical analyses.

Results

CD14 promoter polymorphism and number of acute otitis media infections

General characteristics and immunologic data on the cohort of children with recurrent AOM are described in Table 1. Overall, there were no statistically significant differences in the distribution of genotypes for age or gender. Serum IgM, IgA, IgG and IgG subclass levels did not differ significantly according to genotype. Also environmental risk factors that are known to predispose to recurrence of AOM did not differ across CD14 genotype. Notably, total serum IgE levels were significantly higher in CC and CT carriers compared to TT homozygotes (Table 1).

When analyzing the number of AOM episodes in the year before study entry in the group of children between 12-24 months of age according to CD14 genotype, those homozygous for -159TT had a history of significantly fewer AOM episodes compared to children homozygous for -159CC (mean number of AOM episodes 4.3 and 5.7, respectively; p=0.003). Heterozygotes had an intermediate number of episodes (Table 2). A significant linear trend was found across all three genotype groups with an increasing number of AOM episodes with each additional

Table 1 General and immunologic characteristics of the children under study for occurrence of AOM

		CD14 CC	CD14 CT	CD14 TT	p-value*
Number of children		83	149	68	
Mean Age (95% CI)		3.2 (2.8-3.5)	3.1 (2.8-3.4)	2.8 (2.4-3.1)	0.3
Boys (%)		50 (60.2)	87 (58.4)	48 (70.6)	0.2
≥3 months breastfeeding (%)		36 (43.4)	63 (42.3)	29 (42.6)	0.9
Mean number of siblings (95% CI)		1.1 (0.9-1.3)	1.2 (1.0-1.3)	1.0 (0.8-1.2)	0.6
Daycare attendance (%)	12-24 months	10 (35.7)	24 (46.2)	13 (44.8)	0.7
	≥24 months	22 (81.5)	49 (85.9)	21 (84.0)	0.9
Smoking in home (%)		23 (27.7)	47 (31.5)	29 (42.6)	0.1
IgM (g/l)		1.4	1.4	1.3	0.5
IgA (g/l)		0.7	0.8	0.7	0.4
IgG (g/l)		9.3	9.2	8.8	0.6
IgG_1 (g/l)		7.2	7.3	7.2	0.9
IgG_2 (g/l)		0.9	0.9	0.9	0.6
IgG_3 (g/l)		0.4	0.4	0.4	0.9
IgG_4 (g/l)		0.3	0.2	0.1	0.06
GM IgE (kU/l)		16.9	17.7	8.8	0.01

Note. * p-values are calculated comparing all three genotype groups, using Anova (age, immunoglobulin and IgE levels), Chi-square (gender, breastfeeding, daycare attendance, smoking) and Kruskal-Wallis (siblings) analyses.

Table 2 Association between CD14 C–159T genotype and number of AOM episodes: Retrospectively obtained data from the year before study entry

	12-24 months				≥24 months			
CD14 C–159T	N	Mean AOM* (95% CI)	IRR# (95% CI)	p	N	Mean AOM (95% CI)	IRR (95% CI)	p
CC	28	5.7 (4.5-6.9)	1		55	4.9 (4.1-5.6)	1	
CT	52	5.1 (4.5-5.8)	0.8 (0.6-1.0)	0.07	97	4.8 (4.3-5.4)	1.01 (0.8-1.2)	0.9
TT	29	4.3 (3.3-5.3)	0.7 (0.5-0.9)	0.003	39	5.0 (4.2-5.8)	1.01 (0.8-1.2)	0.9

Notes. p-value calculated compared to baseline CC genotype

* p value for linear trend in mean number of AOM episodes in children between 12-24 months: 0.003

\# IRR=Incidence Rate Ratio

C allele (p=0.003). This association was not identified in children more than 24 months of age (mean number of AOM episodes, 5.0 and 4.9 in those with -159TT and CC, respectively; p=0.9).

CD14 PROMOTER POLYMORPHISM AND PNEUMOCOCCAL VACCINE RESPONSES

Since it has been described that the otitis-prone condition might be related to poor responses tot pneumococcal polysaccharide vaccine,[20,23-25] and since innate immune systems might significantly influence adaptive immune responses,[26] we investigated the IgG specific antibody response after combined pneumococcal polysaccharide and conjugate vaccinations according to CD14 genotype in a separate group of children with either AOM or OME. General characteristics and serum immunoglobulin levels of this study cohort according to genotype are shown in Table 3. There were no significant differences in distribution of genotypes by age or gender. For the study of antibody responses, more children with recurrent episodes of AOM participated (68%) compared with children with prolonged episodes of OME (32%). However, since we have previously shown that both groups of children display similar antibody responses after vaccination,[27] these groups were pooled for data analysis. Total IgM, IgA, IgG and IgG subclass levels did not differ significantly according to genotype.

Individuals with the CC genotype, associated with a higher number of previous otitis episodes

Table 3 General characteristics and serum immunoglobulin levels according to CD14 genotype of the children under study for antibody responses

		CD14 CC	CD14 CT	CD14 TT	p-value*
N		19	39	13	
Gender	Boys (%)	13 (68.4)	28 (71.8)	5 (38.5)	0.1
Ear disease	AOM (%)	14 (73.7)	26 (66.7)	8 (61.5)	0.8
Mean Age (95% CI)		4.2 (3.5-4.9)	4.5 (3.9-5.1)	4.1 (2.9-5.2)	0.7
IgM (g/l)		1.4	1.3	1.4	0.7
IgA (g/l)		1.0	1.1	0.8	0.6
IgG (g/l)		10.2	10.0	9.4	0.8
IgG_1 (g/l)		8.3	8.1	8.0	0.9
IgG_2 (g/l)		1.2	1.3	1.2	0.9
IgG_3 (g/l)		0.5	0.5	0.5	0.9
IgG_4 (g/l)		0.4	0.3	0.2	0.4

Note. * p-values are calculated comparing all three genotype groups, using Anova (age, immunoglobulin levels), and Chi-square (gender, ear disease).

in the above described AOM cohort, had lower IgG antibody responses against all serotypes of the 7-valent pneumococcal conjugate vaccine compared to CT or TT genotypes (figure 1). Antibody levels were significantly lower in CC homozygotes compared to CT/TT individuals for pneumococcal serotypes 9V (p=0.02), 18C (p=0.04) and 23F (p=0.02) and a trend for serotype 4 (p=0.09).

Discussion

An association was found between the CD14 C-159T polymorphism and the number of AOM episodes in a cohort of 300 children with a history of documented episodes of AOM. TT homozygotes suffered from significantly fewer AOM episodes compared to CC carriers. This was found only in the youngest patients from our study cohort and not in children more than 2 years of age. In a subset of children with AOM and OME, the specific IgG response to pneumococcal conjugate vaccine serotypes after pneumococcal conjugate vaccination followed by pneumococcal polysaccharide vaccination was higher in TT homozygotes.

The higher antibody levels in TT carriers suggest a more vigorous adaptive immune defense against S. *pneumoniae*, one of the main causative pathogens in AOM. This enhanced immune response may contribute to the reduced frequency of middle ear infections in the youngest children, especially since IgG anti-capsular antibodies after pneumococcal vaccinations were previously shown to protect against recurrent episodes of acute otitis media in infants.[28,29] We

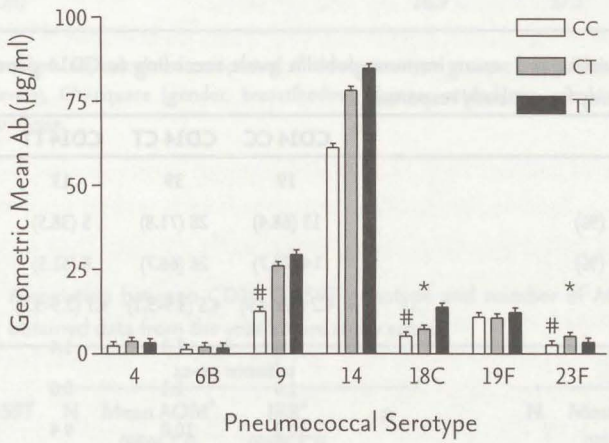


Figure 1 Adjusted geometric mean IgG antibody titers against 7 pneumococcal serotypes after pneumococcal conjugate vaccination followed by pneumococcal polysaccharide booster vaccination according to CD14 C-159T genotype
open bars: CD14 CC; gray bars: CD14 CT; closed bars CD14 TT
Error-bars indicate standard errors
* indicates p<0.05 comparing CC vs CT vs TT (overall) using a general linear model
\# indicates p<0.05 comparing CC vs CT/TT (grouped) using a general linear model

also previously reported otitis-prone patients with 4 or more AOM episodes per year to have lower total IgG levels[30] and lower responses upon pneumococcal vaccinations (unpublished data). This is consistent with the currently described association between CD14 genotype, infection frequency and the pneumococcal specific antibody response. In the present study we investigated number of ear infections and antibody responses in two separate cohorts. Future research in one large cohort is required to confirm our findings.

The fact that CD14, as a component of innate immunity, plays a more prominent role in immune defense early in life is in accordance with age-related data on other innate immune pathways, such as the mannan-binding lectin pathway.[31] MBL was found to be only important in the development of upper respiratory tract infections in children between 6-17 months of age.[32] The generally accepted explanation for this is that innate immune defense pathways are of prime importance between 6 and 24 months of age when maternally derived antibodies have waned and the adaptive immune system of the young child is not yet fully developed. Furthermore it is known that IgG antibody responsiveness after pneumococcal polysaccharide vaccinations and infections gradually matures only after 18 to 24 months of age.[3] Children aged below 24 months with the TT variant may therefore benefit from their higher sCD14 levels directly, because of an enhanced innate immune defense or indirectly, through earlier adaptive IgG anti-pneumococcal immune responsiveness. This advantage is lost after 2 years when the adaptive immune system of all children has matured, and becomes of prime importance.

We can only speculate on a possible mechanism in which CD14 might influence the magnitude of pneumococcal specific antibody responses, and the development of otitis media. In preliminary experiments, we observed higher IL-6, TNFα and IL-10 cytokine levels in supernatants of un-stimulated PBMC cultures from CD14 –159TT homozygote donors, known to have increased sCD14 levels. Cytokine levels were also high in supernatants of PBMC cultures from –159CC donors after recombinant sCD14 was added. Additionally, increasing levels of IL1α and IL1β were found in the latter situation. This would be compatible with previous findings of Cauwels *et al.* showing a pro-inflammatory role for sCD14 during the progression of bacterial meningitis in mice and showing that co-injection of *S. pneumoniae* with recombinant sCD14 resulted in enhanced release of IL-6 and TNFα.[33] The protective role of these pro-inflammatory cytokines in the innate immune defense against bacteria is well established, and it is known that the events that mediate these innate responses to pathogens have a profound influence on the quality and intensity of the subsequent adaptive immune response.[26,34,35] Furthermore, an important role for these pro-inflammatory cytokines, i.e IL-1, IL-6, TNFα and IL-10, in enhancing humoral immune responses to polysaccharide antigens, has been described.[36-39] An enhanced immune response upon natural exposure to pathogens able to cause AOM, might explain the observed lower otitis media frequency in carriers of the TT genotype.

Since the CD14 promoter polymorphism may be associated with both the clinical course of recurrent AOM in young children, as well as with antibody responses upon pneumococcal vaccination, further studies into additional components of this innate immune pathway are warranted. The interaction between CD14 and TLR4, which forms an essential link between innate and adaptive immune responses,[26] leads to initiation of downstream signal transduction cascades, ultimately resulting in activation of transcription factors.[40,41] Thus, investigations

of polymorphisms within these downstream components of the CD14/TLR4 pathway, such as Myd88,[42] IRAK4,[43] NEMO,[44] and IκBα[45] may also be worthwhile.
Environmental factors such as breast-feeding, number of siblings, day care attendance, and tobacco smoke exposure are known to contribute to the development of recurrent otitis media.[46] In the study cohort of 300 children described here, these environmental factors were equally distributed across CD14 genotype, suggesting an effect of the CD14 promoter polymorphism in our population separate from these known environmental factors. It remains possible that our findings are the result of another polymorphism in linkage disequilibrium with the studied polymorphism.
As previously demonstrated by others,[15,47,48] we found significantly lower geometric mean total IgE levels in TT homozygotes, who showed higher pneumococcal specific IgG levels. Our data may suggest an inverse relationship between IgG and IgE levels according to CD14 genotype. Possibly, the atopic condition as such may predispose to increased susceptibility to pneumococcal- or respiratory tract infections.[49-51]
Together our data support a role for CD14 in early immune defense, possibly by linking innate and adaptive humoral immune responses. A large prospective birth cohort however is required to determine the true impact of innate immunity pathways associated with CD14, in relation to infection susceptibility at young age.

Acknowledgements

The work described in this paper was financially supported by the Netherlands Organisation for Health Research and Development ZonMW (grant numbers 002828480 and 90461092), the Netherlands Health Insurance Company Zilveren Kruis-Achmea, an Australia-Europe Scholarship 2004 funded by the Australian Government through the Department of Education, Science and Training and promoted by AEI, the Australian Government International Education Network and the Ter Meulen Fund, Royal Netherlands Academy of Arts and Sciences.
We thank all children and parents who participated in our studies.
We would like to thank Dr. Zhang (Telethon Institute for Child Health Research, Perth, Australia) for help with statistical analyses and Dr. Hayden (Telethon Institute for Child Health Research, Perth, Australia) for fruitful discussions on the work described in this paper.

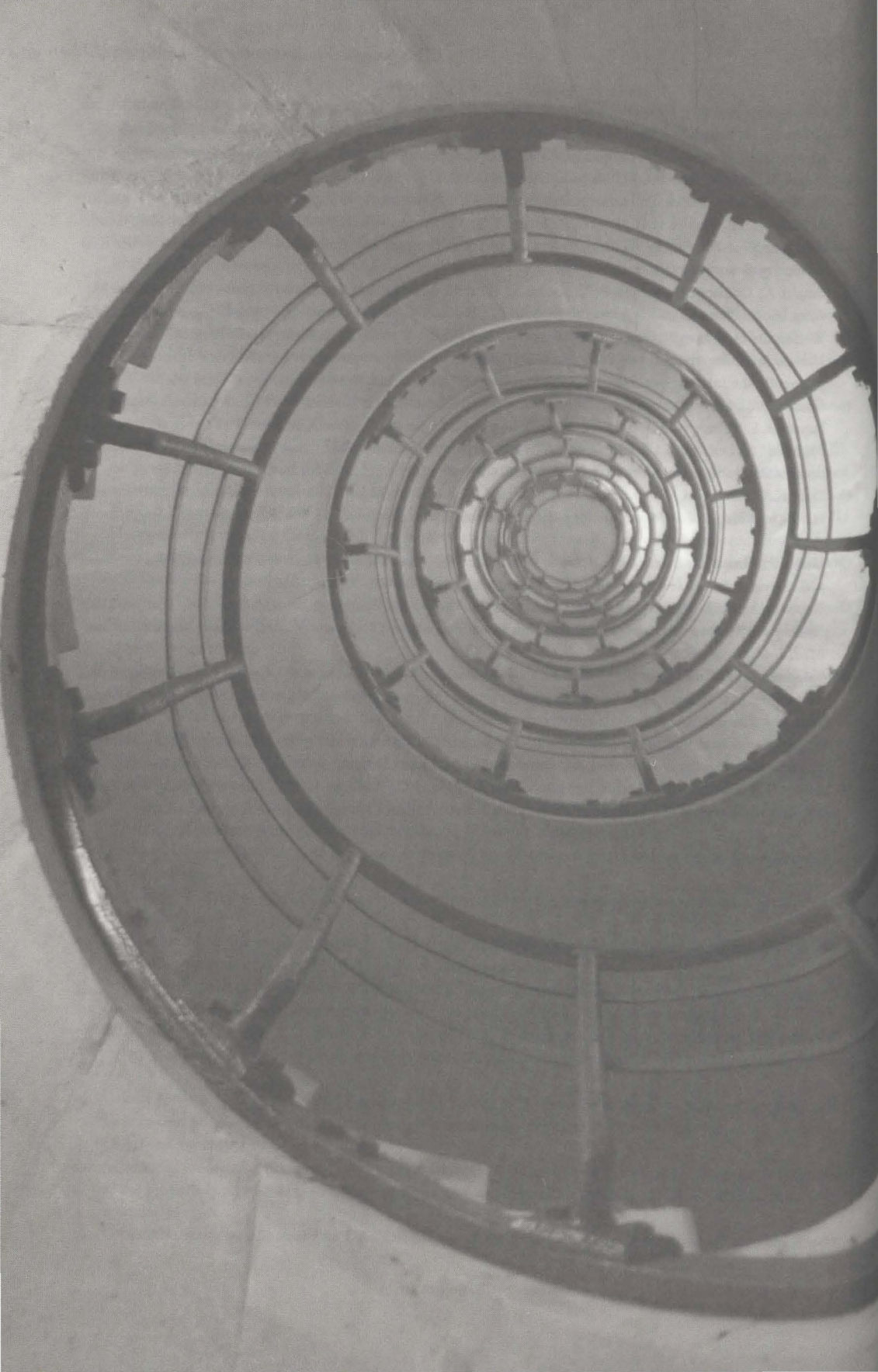
References

1. Freid, V.M.; Makuc, D.M.; Rooks, R.N.: Ambulatory health care visits by children: principal diagnosis and place of visit. Vital Health Stat.13 1998; 1-23
2. Teele, D.W.; Klein, J.O.; Rosner, B.: Epidemiology of otitis media during the first seven years of life in children in greater Boston: a prospective, cohort study. J.Infect.Dis. 1989; 160:83-94
3. Weintraub, A.: Immunology of bacterial polysaccharide antigens. Carbohydr.Res. 2003; 338:2539-2547
4. Wright, S.D.; Ramos, R.A.; Tobias, P.S.; Ulevitch, R.J.; Mathison, J.C.: CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. Science 1990; 249:1431-1433
5. Cleveland, M.G.; Gorham, J.D.; Murphy, T.L.; Tuomanen, E.; Murphy, K.M.: Lipoteichoic acid preparations of gram-positive bacteria induce interleukin-12 through a CD14-dependent pathway. Infect.Immun. 1996; 64:1906-1912
6. Gupta, D.; Kirkland, T.N.; Viriyakosol, S.; Dziarski, R.: CD14 is a cell-activating receptor for bacterial peptidoglycan. J.Biol.Chem. 1996; 271:23310-23316
7. Dobrovolskaia, M.A. and Vogel, S.N.: Toll receptors, CD14, and macrophage activation and deactivation by LPS. Microbes.Infect. 2002; 4:903-914
8. Kim, J.I.; Lee, C.J.; Jin, M.S.; Lee, C.H.; Paik, S.G.; Lee, H.; Lee, J.O.: Crystal structure of CD14 and its implications for lipopolysaccharide signaling. J.Biol.Chem. 2005;
9. Um, S.H.; Son, E.W.; Kim, B.O.; Moon, E.Y.; Rhee, D.K.; Pyo, S.: Activation of murine peritoneal macrophages by Streptococcus pneumoniae type II capsular polysaccharide: involvement of CD14-dependent pathway. Scand. J.Immunol. 2000; 52:39-45
10. Pugin, J.; Heumann, I.D.; Tomasz, A.; Kravchenko, V.V.; Akamatsu, Y.; Nishijima, M.; Glauser, M.P.; Tobias, P.S.; Ulevitch, R.J.: CD14 is a pattern recognition receptor. Immunity. 1994; 1:509-516
11. Vercelli, D.: Learning from discrepancies: CD14 polymorphisms, atopy and the endotoxin switch. Clin.Exp.Allergy 2003; 33:153-155
12. Dziarski, R.; Viriyakosol, S.; Kirkland, T.N.; Gupta, D.: Soluble CD14 enhances membrane CD14-mediated responses to peptidoglycan: structural requirements differ from those for responses to lipopolysaccharide. Infect. Immun. 2000; 68:5254-5260
13. Lien, E.; Means, T.K.; Heine, H.; Yoshimura, A.; Kusumoto, S.; Fukase, K.; Fenton, M.J.; Oikawa, M.; Qureshi, N.; Monks, B.; Finberg, R.W.; Ingalls, R.R.; Golenbock, D.T.: Toll-like receptor 4 imparts ligand-specific recognition of bacterial lipopolysaccharide. J.Clin.Invest 2000; 105:497-504
14. Beutler, B.; Hoebe, K.; Du, X.; Ulevitch, R.J.: How we detect microbes and respond to them: the Toll-like receptors and their transducers. J.Leukoc.Biol. 2003; 74:479-485
15. Baldini, M.; Lohman, I.C.; Halonen, M.; Erickson, R.P.; Holt, P.G.; Martinez, F.D.: A Polymorphism* in the 5' flanking region of the CD14 gene is associated with circulating soluble CD14 levels and with total serum immunoglobulin E. Am.J.Respir.Cell Mol.Biol. 1999; 20:976-983
16. LeVan, T.D.; Bloom, J.W.; Bailey, T.J.; Karp, C.L.; Halonen, M.; Martinez, F.D.; Vercelli, D.: A common single nucleotide polymorphism in the CD14 promoter decreases the affinity of Sp protein binding and enhances transcriptional activity. J.Immunol. 2001; 167:5838-5844
17. Kabesch, M.; Hasemann, K.; Schickinger, V.; Tzotcheva, I.; Bohnert, A.; Carr, D.; Baldini, M.; Hackstein, H.; Leupold, W.; Weiland, S.K.; Martinez, F.D.; Mutius, E.; Bein, G.: A promoter polymorphism in the CD14 gene is associated with elevated levels of soluble CD14 but not with IgE or atopic diseases. Allergy 2004; 59:520-525
18. Veenhoven, R.; Bogaert, D.; Uiterwaal, C.; Brouwer, C.; Kiezebrink, H.; Bruin, J.; IJzerman, E.; Hermans, P.; de Groot, R.; Zegers, B.; Kuis, W.; Rijkers, G.; Schilder, A.; Sanders, E.: Effect of conjugate pneumococcal vaccine followed by polysaccharide pneumococcal vaccine on recurrent acute otitis media: a randomised study. Lancet 2003; 361:2189-2195
19. Straetemans, M.; van Heerbeek, N.; Tonnaer, E.; Ingels, K.J.; Rijkers, G.T.; Zielhuis, G.A.: A comprehensive model for the aetiology of otitis media with effusion. Med.Hypotheses 2001; 57:784-791
20. Sanders, L.A.; Rijkers, G.T.; Tenbergen-Meekes, A.M.; Voorhorst-Ogink, M.M.; Zegers, B.J.: Immunoglobulin isotype-specific antibody responses to pneumococcal polysaccharide vaccine in patients with recurrent bacterial respiratory tract infections. Pediatr.Res. 1995; 37:812-819

21. Musher, D.M.; Luchi, M.J.; Watson, D.A.; Hamilton, R.; Baughn, R.E.: Pneumococcal polysaccharide vaccine in young adults and older bronchitics: determination of IgG responses by ELISA and the effect of adsorption of serum with non-type-specific cell wall polysaccharide. J.Infect.Dis. 1990; 161:728-735
22. Quataert, S.A.; Kirch, C.S.; Wiedl, L.J.; Phipps, D.C.; Strohmeyer, S.; Cimino, C.O.; Skuse, J.; Madore, D.V.: Assignment of weight-based antibody units to a human antipneumococcal standard reference serum, lot 89-S. Clin. Diagn.Lab Immunol. 1995; 2:590-597
23. Sanders, L.A.; Rijkers, G.T.; Kuis, W.; Tenbergen-Meekes, A.J.; Graeff-Meeder, B.R.; Hiemstra, I.; Zegers, B.J.: Defective antipneumococcal polysaccharide antibody response in children with recurrent respiratory tract infections. J.Allergy Clin.Immunol. 1993; 91:110-119
24. Dhooge, I.J.; van Kempen, M.J.; Sanders, L.A.; Rijkers, G.T.: Deficient IgA and IgG2 anti-pneumococcal antibody levels and response to vaccination in otitis prone children. Int.J.Pediatr.Otorhinolaryngol. 2002; 64:133-141
25. Epstein, M.M. and Gruskay, F.: Selective deficiency in pneumococcal antibody response in children with recurrent infections. Ann.Allergy Asthma Immunol. 1995; 75:125-131
26. Hoebe, K.; Janssen, E.; Beutler, B.: The interface between innate and adaptive immunity. Nat.Immunol. 2004; 5:971-974
27. Wiertsema, S.P.; Sanders, E.A.; Veenhoven, R.H.; van Heerbeek, N.; van Den, H.S.; Berbers, G.A.; Rijkers, G.T.: Antibody levels after regular childhood vaccinations in the immunological screening of children with recurrent otitis media. J.Clin.Immunol. 2004; 24:354-360
28. Black, S.; Shinefield, H.; Fireman, B.; Lewis, E.; Ray, P.; Hansen, J.R.; Elvin, L.; Ensor, K.M.; Hackell, J.; Siber, G.; Malinoski, F.; Madore, D.; Chang, I.; Kohberger, R.; Watson, W.; Austrian, R.; Edwards, K.: Efficacy, safety and immunogenicity of heptavalent pneumococcal conjugate vaccine in children. Northern California Kaiser Permanente Vaccine Study Center Group. Pediatr.Infect.Dis.J 2000; 19:187-195
29. Eskola, J.; Kilpi, T.; Palmu, A.; Jokinen, J.; Haapakoski, J.; Herva, E.; Takala, A.; Kayhty, H.; Karma, P.; Kohberger, R.; Siber, G.; Makela, P.H.: Efficacy of a pneumococcal conjugate vaccine against acute otitis media. N.Engl.J Med. 2001; 344:403-409
30. Veenhoven, R.; Rijkers, G.; Schilder, A.; Adelmeijer, J.; Uiterwaal, C.; Kuis, W.; Sanders, E.: Immunoglobulins in Otitis-Prone Children. Pediatr.Res. 2003;
31. Turner, M.W.: The role of mannose-binding lectin in health and disease. Mol.Immunol. 2003; 40:423-429
32. Koch, A.; Melbye, M.; Sorensen, P.; Homoe, P.; Madsen, H.O.; Molbak, K.; Hansen, C.H.; Andersen, L.H.; Hahn, G.W.; Garred, P.: Acute respiratory tract infections and mannose-binding lectin insufficiency during early childhood. JAMA 2001; 285:1316-1321
33. Cauwels, A.; Frei, K.; Sansano, S.; Fearns, C.; Ulevitch, R.; Zimmerli, W.; Landmann, R.: The origin and function of soluble CD14 in experimental bacterial meningitis. J.Immunol. 1999; 162:4762-4772
34. Medzhitov, R. and Janeway, C.A., Jr.: Innate immunity: the virtues of a nonclonal system of recognition. Cell 1997; 91:295-298
35. Medzhitov, R. and Janeway, C.A., Jr.: Innate immunity: impact on the adaptive immune response. Curr.Opin.Immunol. 1997; 9:4-9
36. Chelvarajan, R.L.; Gilbert, N.L.; Bondada, S.: Neonatal murine B lymphocytes respond to polysaccharide antigens in the presence of IL-1 and IL-6. J.Immunol. 1998; 161:3315-3324
37. Luo, W.; Fine, J.; Garg, M.; Kaplan, A.M.; Bondada, S.: Interleukin-10 enhances immune responses to pneumococcal polysaccharides and sheep erythrocytes in young and aged mice. Cell Immunol. 1999; 195:1-9
38. Khan, A.Q.; Shen, Y.; Wu, Z.Q.; Wynn, T.A.; Snapper, C.M.: Endogenous pro- and anti-inflammatory cytokines differentially regulate an in vivo humoral response to Streptococcus pneumoniae. Infect.Immun. 2002; 70:749-761
39. Colino, J.; Shen, Y.; Snapper, C.M.: Dendritic cells pulsed with intact Streptococcus pneumoniae elicit both protein- and polysaccharide-specific immunoglobulin isotype responses in vivo through distinct mechanisms. J.Exp.Med. 2002; 195:1-13
40. da Silva, C.J.; Soldau, K.; Christen, U.; Tobias, P.S.; Ulevitch, R.J.: Lipopolysaccharide is in close proximity to each of the proteins in its membrane receptor complex. transfer from CD14 to TLR4 and MD-2. J.Biol.Chem. 2001; 276:21129-21135
41. Cook, D.N.; Pisetsky, D.S.; Schwartz, D.A.: Toll-like receptors in the pathogenesis of human disease. Nat.Immu-

nol. 2004; 5:975-979

42. Takeuchi, O.; Takeda, K.; Hoshino, K.; Adachi, O.; Ogawa, T.; Akira, S.: Cellular responses to bacterial cell wall components are mediated through MyD88-dependent signaling cascades. Int.Immunol. 2000; 12:113-117
43. Picard, C.; Puel, A.; Bonnet, M.; Ku, C.L.; Bustamante, J.; Yang, K.; Soudais, C.; Dupuis, S.; Feinberg, J.; Fieschi, C.; Elbim, C.; Hitchcock, R.; Lammas, D.; Davies, G.; Al Ghonaium, A.; Al Rayes, H.; Al Jumaah, S.; Al Hajjar, S.; Al Mohsen, I.Z.; Frayha, H.H.; Rucker, R.; Hawn, T.R.; Aderem, A.; Tufenkeji, H.; Haraguchi, S.; Day, N.K.; Good, R.A.; Gougerot-Pocidalo, M.A.; Ozinsky, A.; Casanova, J.L.: Pyogenic bacterial infections in humans with IRAK-4 deficiency. Science 2003; 299:2076-2079
44. Smahi, A.; Courtois, G.; Vabres, P.; Yamaoka, S.; Heuertz, S.; Munnich, A.; Israel, A.; Heiss, N.S.; Klauck, S.M.; Kioschis, P.; Wiemann, S.; Poustka, A.; Esposito, T.; Bardaro, T.; Gianfrancesco, F.; Ciccodicola, A.; D'Urso, M.; Woffendin, H.; Jakins, T.; Donnai, D.; Stewart, H.; Kenwrick, S.J.; Aradhya, S.; Yamagata, T.; Levy, M.; Lewis, R.A.; Nelson, D.L.: Genomic rearrangement in NEMO impairs NF-kappaB activation and is a cause of incontinentia pigmenti. The International Incontinentia Pigmenti (IP) Consortium. Nature 2000; 405:466-472
45. Courtois, G.; Smahi, A.; Reichenbach, J.; Doffinger, R.; Cancrini, C.; Bonnet, M.; Puel, A.; Chable-Bessia, C.; Yamaoka, S.; Feinberg, J.; Dupuis-Girod, S.; Bodemer, C.; Livadiotti, S.; Novelli, F.; Rossi, P.; Fischer, A.; Israel, A.; Munnich, A.; Le Deist, F.; Casanova, J.L.: A hypermorphic IkappaBalpha mutation is associated with autosomal dominant anhidrotic ectodermal dysplasia and T cell immunodeficiency. J.Clin.Invest 2003; 112:1108-1115
46. Daly, K.A.; Rovers, M.M.; Hoffman, H.J.; Uhari, M.; Casselbrant, M.L.; Zielhuis, G.; Kvaerner, K.J.: Recent advances in otitis media. 1. Epidemiology, natural history, and risk factors. Ann.Otol.Rhinol.Laryngol.Suppl 2005; 194:8-15
47. Gao, P.S.; Mao, X.Q.; Baldini, M.; Roberts, M.H.; Adra, C.N.; Shirakawa, T.; Holt, P.G.; Martinez, F.D.; Hopkin, J.M.: Serum total IgE levels and CD14 on chromosome 5q31. Clin.Genet. 1999; 56:164-165
48. Koppelman, G.H.; Reijmerink, N.E.; Colin, S.O.; Howard, T.D.; Whittaker, P.A.; Meyers, D.A.; Postma, D.S.; Bleecker, E.R.: Association of a promoter polymorphism of the CD14 gene and atopy. Am.J.Respir.Crit Care Med. 2001; 163:965-969
49. Koopman, L.P.; Smit, H.A.; Heijnen, M.L.; Wijga, A.; van Strien, R.T.; Kerkhof, M.; Gerritsen, J.; Brunekreef, B.; de Jongste, J.C.; Neijens, H.J.: Respiratory infections in infants: interaction of parental allergy, child care, and siblings--The PIAMA study. Pediatrics 2001; 108:943-948
50. Illi, S.; von Mutius, E.; Lau, S.; Bergmann, R.; Niggemann, B.; Sommerfeld, C.; Wahn, U.: Early childhood infectious diseases and the development of asthma up to school age: a birth cohort study. BMJ 2001; 322:390-395
51. Talbot, T.R.; Hartert, T.V.; Mitchel, E.; Halasa, N.B.; Arbogast, P.G.; Poehling, K.A.; Schaffner, W.; Craig, A.S.; Griffin, M.R.: Asthma as a risk factor for invasive pneumococcal disease. N.Engl.J.Med. 2005; 352:2082-2090



4

Antibody levels after regular childhood vaccinations in the immunological screening of children with recurrent otitis media

Selma P. Wiertsema[1], Elisabeth A.M. Sanders[1], Reinier H. Veenhoven[2], Niels van Heerbeek[3], Susan van den Hof[4], Guy A.M. Berbers[5], Ger T. Rijkers[1]

1 Department of Pediatric Immunology, University Medical Center Utrecht, The Netherlands
2 Department of Pediatrics, Spaarne Hospital, Hoofddorp, the Netherlands
3 Department of Otorhinolaryngology, University Medical Center Nijmegen, the Netherlands
4 Department of Infectious Diseases Epidemiology, National Institute for Public Health and the Environment, Bilthoven, the Netherlands
5 Laboratory for Vaccine preventable Diseases, National Institute for Public Health and the Environment, Bilthoven, the Netherlands

J Clin Immunol. 2004 Jul;24(4): 354-60.

Summary

Recurrent otitis media may be related to defects in specific antibody production, as suggested previously. This might be reflected in lower antibody responses to vaccinations administered in the context of the national childhood vaccination program in children suffering from recurrent otitis media.

In a cross sectional study we determined the levels of anti-diphtheria, anti-tetanus, anti-Haemophilus influenzae type b (anti-Hib) and anti-measles antibodies in sera of 163 children with two or more episodes of acute otitis media per year and in 143 children with repeated periods of persistent otitis media with effusion each lasting at least 3 months. The control group consisted of 521 age-matched healthy children, who were free of recurrent respiratory tract infections. Children with recurrent acute otitis media, including highly otitis-prone children, showed higher anti-diphtheria and anti-tetanus antibody titers compared to controls. No differences were observed in anti-Hib and anti-measles antibody levels between children with recurrent acute otitis media and controls, nor did any of the antibody levels in children with persistent otitis media with effusion differ from those in controls. Therefore, the results of our study do not point toward a generalized immunological hypo-responsiveness in children with recurrent acute otitis media and persistent otitis media with effusion. Determination of antibody responses to regular vaccines is not indicative for otitis proneness.

Introduction

Otitis media (OM) is one of the most common childhood infections and is the cause of 12% of children's office visits to physicians in the United States.[1] To rule out immunodeficiency, children whose frequency of infections exceeds the norm, as well as children who experience serious complications of common infections, are often evaluated for antibody deficiency. Exclusion of agammaglobulinemia via measurement of serum immunoglobulin concentrations of the major classes of immunoglobulins IgA, IgM, IgG, with or without measurement of IgG subclasses, is the starting point for humoral immunity. Since the early 1980's, several studies have reported on groups of patients with recurrent respiratory tract infections who have normal or near normal serum immunoglobulin concentrations, but do show specific antibody deficiencies.[2,3] Therefore, apart from overall immunoglobulin levels, it is nowadays advized to measure specific antibody synthesis as response to vaccination. In previous smaller studies, specific antibody deficiency is suggested to occur in otitis-prone children. This is based on low antibody titers in otitis-prone children after diphtheria, tetanus, and rubella vaccination[4] or in response to repeated contact with pathogens like *Haemophilus influenzae*.[5,6] To further investigate potential immunological hypo-responsiveness in children with recurrent ear infections, we now studied specific antibody levels after regular childhood vaccinations in large and well-defined groups of children with recurrent acute otitis media (AOM) or persistent otitis media with effusion (OME). These values were compared with data of 521 healthy age matched controls.

Methods

Study populations

Three groups of children were immunologically evaluated as described in detail below: 1) children with a history of at least two documented episodes of AOM in the year prior to study; 2) children with at least two documented prolonged episodes of OME; and 3) healthy control children.

1. The AOM patient population included 163 children aged between 24 and 84 months (median age 4 years) with a history of at least two physician-diagnosed episodes of AOM in the previous 12 months.[7] Approximately 50% of the children had a reported history of atopy, defined as having eczema, hay fever, or recurrent wheezing or asthma.[7] A subdivision in the AOM group was made on the basis of the number of AOM episodes the child experienced the previous year: a) 2–3 AOM episodes; b) 4–5 AOM episodes; and c) >6 AOM episodes.
2. The OME group consisted of 143 children aged between 26 and 97 months (median age 5 years) who experienced at least two periods of bilateral OME lasting 3 months or longer, documented by an ENT specialist, and who were therefore referred for ventilation-tube placement. In both the AOM and the OME patient groups, patients with previously recognized congenital or acquired immunodeficiencies, cystic fibrosis, immotile cilia syndrome, craniofacial abnormalities (i.e. cleft palate), or chromosomal abnormalities (i.e. Down's syndrome) were excluded.

3. The control group consisted of 521 age-matched healthy children selected from a cross-sectional, population-based surveillance study performed in the Netherlands from October 1995 through December 1996 by the National Institute of Public Health and the Environment to evaluate the national immunization program (NIP). At the time of blood sampling, parents of participants filled out a questionnaire to obtain information regarding the determinants of the immune and general health status of the child.[8] For the present study we selected children aged between 24 and 84 months old (median age 4 years), of whom parents stated in the questionnaire that 1) the child participated in the Dutch NIP; 2) the child did not experience any ear infection, infection of the nasal cavity, or (maxillary) sinusitis during the last 12 months for which a physician was consulted or medication required; and 3) the child's general health condition was "good" to "very good"(on a scale from "poor" to "very good").

The ethical committees of the participating hospitals and institutions previously approved all three studies. Written informed parental consent was obtained from all subjects.

Dutch national immunization program

DTP-IPV and hib: All children under study received combined diphtheria, tetanus, whole cell pertussis, and inactivated poliomyelitis vaccination (DTP-IPV produced by the Netherlands Vaccine Institute (NVI), Bilthoven, The Netherlands) at the age of 3, 4, 5, and 11 months. DTIPV booster vaccination is administered to children 4 and 9 years of age. Since 1993, PRP-T conjugated *Haemophilus influenzae* type b vaccination (Pasteur Mérieux SV, Lyon, France) is given simultaneously but in a separate syringe, with the first series of DTP-IPV vaccinations.
MMR: Live attenuated measles, mumps, and rubella (MMR) viral vaccine (Merck & Co., Inc. Rahway, NJ, produced by the NVI) is administered to children at the age of 14 months and repeated at 9 years of age.

Measurement of specific antibody concentrations

Blood samples were obtained at random time points after vaccination by venepuncture, and serum was isolated. Patient sera were stored at –20°C and control sera at –80°C until analysis. The levels of anti-diphtheria and anti-tetanus antibodies were measured at the Laboratory for Vaccine preventable Diseases at the National Institute for Public Health and the Environment with a toxin-binding inhibition assay described elsewhere in detail.[9] In brief, twofold serum dilution series were incubated with a fixed amount of diphtheria- or tetanus-toxin overnight. The toxin that was not neutralized by serum antibodies was measured in an enzyme linked immunosorbent assay (ELISA). Horse anti-toxin (diphtheria and tetanus, respectively) purified from hyperimmune serum was used for coating and peroxidase labeled horse anti-diphtheria or anti-tetanus IgG as a conjugate. Antibody titers are expressed in international units, which were calculated according to the WHO reference standard serum (10 IU/ml) by the four-parameter fit method in Kineticalc (KC4, Biolyse, USA) with aBio-Tek plate reader (EL312d). IgG anti-Hib antibody titers in the patient groups were determined at the Laboratory of Pediatric Immunology at the Wilhelmina Children's Hospital by ELISA as described previously.[10] Wells

of a 96-well polystyrene microtiter plate (Greiner Labortechnik, Langerthal, Germany) were coated with Hib polysaccharide conjugated to human serum albumin (HbO-HALot 17, produced by Wyeth Lederle Vaccines; obtained through NISCB, UK, 1 μg/ml in PBS). Subsequently, plates were incubated with serial dilutions of serum samples, thereafter with alkaline phosphatase-conjugated goat-anti-human IgG (Sanbio Biosource, Camarillo, CA; 1:1000 in diluting buffer), and p-nitrophenylphosphate (Sigma–Aldrich Chemie Gmbh, Steinheim, Germany). The IgG anti-Hib antibody titers in the control group were determined at the Laboratory for Vaccine preventable Diseases at the National Institute for Public Health and the Environment by ELISA, according to methods previously described elsewhere.[11] A number of samples were exchanged between the two institutes to test for consistency of the quantitative measurements, which proved to be good (R^2 >0.98). Specific anti-measles IgG was determined at the Laboratory for Vaccine preventable Diseases at the National Institute for Public Health and the Environment using ELISA.[12] Briefly, IgG antibody concentrations were measured against purified measles virus (strain Edmonston). Microtiter plates (Greiner Labortechnik) were coated with 2 μg/ml measles antigen. International unitage was calculated relative to the second international standard for human anti-measles serum by four-parameter fit.

Statistics

Geometric mean titers (GMTs) and 95% confidence intervals (CIs) were calculated for diphtheria, tetanus, Hib, and measles antibody levels in the groups of children with AOM, OME, and in controls. GMTs were tested for significant differences between the three groups, using analysis of covariance (ANCOVA) in the statistical analysis program SPSS 11.5 for windows (SPSS Inc., Chicago, IL). P-values below 0.05 were considered significant. On the basis of literature, age, sex, attendance at a day-care center or school, and older siblings at home were tested as potential covariates.[13–17] The percentages of children attending a day-care center or school or with older siblings at home were very high (Table 1), therefore, these covariates did not influence results and were not taken into account in further analyses. Age and sex appeared to be relevant, therefore these two factors were introduced as covariates in the ANCOVA model.

Table 1 General characteristics of the three studied groups

	AOM		OME		Control	
Number	163		143		521	
Number of males (%)	98	(61.6)	85	(59.4)	276	(53.0)
Median age in years	4		5		4	
Daycare or school attendance (%)	150	(94.3)	138	(96.5)	452	(86.8)
Sibling(s) at home (%)	134	(84.3)	130	(90.9)	456	(87.7)

Results

To evaluate the hypothesis of a broader specific antibody deficiency in children suffering from repeated episodes of AOM or OME, we evaluated immune responses in two large cohorts of children with recurrent otitis media (AOM, n=163; OME, n=143) and compared them with 521 healthy age-matched controls. General characteristics of the children in the three groups were compared (Table 1). The three groups were matched for age and sex, however the OME group consisted of slightly older children (median age 5 years) compared to the AOM and the control group (median age 4 years in both groups). The frequency of boys in the AOM and OME groups is higher (62 and 59%, respectively) compared to the control group (53%), which is in accordance with the observation that OM seems to occur more often in boys.[13-15]

Diphtheria and tetanus. Unexpectedly, statistically significant higher anti-diphtheria (GMT, 0.39 IU/ml) and anti-tetanus (GMT, 1.63 IU/ml) antibody levels were found in children with recurrent AOM compared to healthy controls (GMT, 0.26 and 0.90 IU/ml, respectively; $p<0.005$; Table 2). The differences between children with recurrent AOM and healthy controls were most outspoken in 4-year-olds, the age at which they received the DT-IPV booster vaccination (Fig. 1). At the age of 5, antibody levels in both groups declined again and differences were no longer distinguishable. Subdivision of the AOM group based on the number of AOM episodes the child experienced in the previous 12 months revealed a trend that anti-diphtheria and anti-tetanus antibody levels decreased with an increasing number of AOM episodes. However, for those children with four or five AOM episodes per year, and for the group of children with six or more AOM episodes per year, anti-diphtheria antibody titers were still similar and anti-tetanus antibody levels were even higher compared to controls (Table 3). No differences were observed in geometric mean anti-diphtheria and anti-tetanus antibody titers between children with persistent OME and controls.

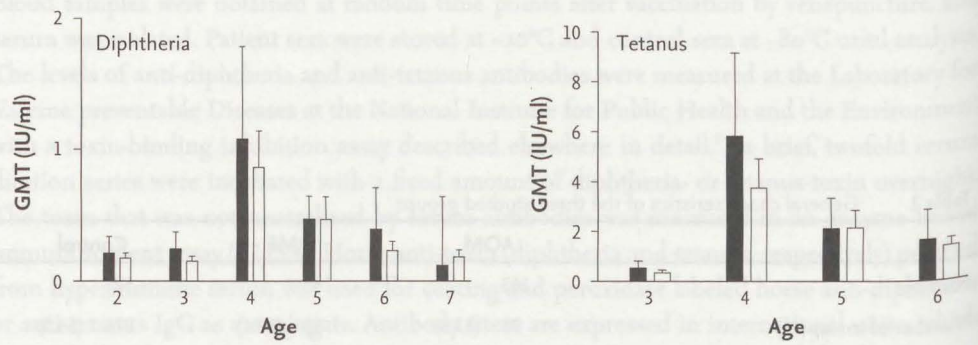


Figure 1 Geometric mean anti-diphtheria and anti-tetanus antibody levels in children with AOM and healthy controls per year of age.
Error bars indicate upper 95% confidence intervals of the geometric means.
Closed bars: AOM patients; Open bars: Controls.

Table 2 *Geometric mean antibody titers in children with AOM, OME and healthy controls*

	AOM		OME		Control		P-values		
	N	GMT (95%CI)	N	GMT (95%CI)	N	GMT (95%CI)	AOM-OME	AOM-Control	OME-Control
Diphtheria (IU/ml)	162	0.39 (0.32-0.49)	94	0.27 (0.20-0.35)	519	0.26 (0.23-0.29)	0.092	0.002	1.000
Tetanus (IU/ml)	132	1.63 (1.26-2.11)	110	1.15 (0.87-1.52)	519	0.90 (0.79-1.02)	0.214	<0.001	0.340
Hib (µg/ml)	163	1.38 (1.16-1.64)	143	1.06 (0.86-1.31)	122	1.48 (1.16-1.88)	0.165	1.000	0.202
Measles (IU/ml)	163	1.16 (0.98-1.37)	141	1.18 (0.99-1.41)	521	1.36 (1.24-1.49)	1.000	0.333	0.525

Note. Geometric mean antibody titers for diphtheria, tetanus, Hib, and measles in children with AOM, OME and healthy controls. Between brackets the 95% confidence intervals for the geometric means are given.

Table 3 Geometric mean antibody titers in children with AOM according to the number of AOM episodes in the year prior to study

	Controls		2-3 episodes		4-5 episodes		>6 episodes		P-values		
	N	GMT (95% CI)	N	GMT (95% CI)	N	GMT (95% CI)	N	GMT (95% CI)	2/3 vs. 4/5	2/3 vs. >6	4/5 vs. >6
Diphtheria (IU/mL)	519	0.26 (0.23-0.29)	60	0.55 (0.39-0.77)	43	0.35 (0.23-0.53)	55	0.28 (0.19-0.40)	0.308	0.024	1.000
Tetanus (IU/mL)	519	0.90 (0.79-1.02)	48	1.81 (1.16-2.81)	32	1.89 (1.10-3.24)	50	1.36 (0.88-2.09)	1.000	1.000	1.000
Hib (µg/mL)	122	1.48 (1.16-1.88)	60	1.46 (1.13-1.89)	43	1.47 (1.08-1.99)	56	1.22 (0.94-1.59)	1.000	1.000	1.000
Measles (IU/mL)	521	1.36 (1.24-1.49)	60	1.17 (0.94-1.45)	43	1.22 (0.94-1.57)	56	1.29 (1.03-1.61)	1.000	1.000	1.000

Note. Geometric mean antibody titers for diphtheria, tetanus, Hib, and measles in children with different numbers of AOM episodes in the year prior to study. Between brackets the 95% confidence intervals for the geometric means.

Hib. Because Hib-conjugate vaccination was introduced in the Dutch NIP in 1993 and the control surveillance study was conducted in 1995/1996, a control group of only 122 two and three years old children was available for the anti-Hib antibody determination. No differences in geometric mean anti-Hib antibody levels were found between children with AOM, OME, and in controls (GMT of 1.38, 1.06, and 1.48 μg/ml, respectively; Table 2). Analysis of the AOM subgroup on the basis of number of AOM episodes the child experienced in the preceding year, showed no difference in mean anti-Hib antibody titer between children with 2–3, 4–5, or more than 6 AOM episodes in 12 months (Table 3).

Measles. No differences in mean anti-measles antibody titers were observed between children with AOM, OME, and in controls (GMT of 1.16, 1.18, and 1.36 IU/mL, respectively; Table 2), nor did comparison between the AOM subgroups elucidate any significant differences (Table 3).

Discussion

There have been previous suggestions of specific antibody deficiency in otitis-prone children,[4-6] but now our study addresses, for the first time, this issue in large cohorts of well-defined patient populations and a large group of healthy control children. As a tool for immunological evaluation we determined antibody titers related to vaccinations of the NIP. In contrast with previous studies that described decreased antibody responses, suggesting a more general immunologic divergence to occur in otitis-prone children, we observed significantly higher anti-diphtheria and anti-tetanus antibody levels in children with recurrent episodes of AOM compared to healthy controls. This was particularly true for the age group of 4-year olds who received a DT-IPV booster vaccination, which seems to provoke an even more pronounced response in children with recurrent AOM as compared to healthy age matched controls. We did observe a trend in decreasing anti-diphtheria and anti-tetanus antibody levels with an increasing number of AOM episodes. However, in those children considered otitis-prone, with four or five AOM episodes per year, as well as in the severe otitis-prone group with six or more AOM episodes per year, similar anti-diphtheria and even higher anti-tetanus antibody levels were observed compared to controls. Furthermore, no differences were observed in anti-measles and anti-Hib GMTs between children with AOM and healthy controls. The fact that no antibody differences were found suggests that the immune responses in children with AOM will be at least as good as in healthy controls. Moreover, when the AOM group was further divided in an otitis-prone (4–5 AOM episodes in 12 months) and a non-otitis-prone (2–3 AOM episodes in 12 months) group, high anti-measles and anti-Hib antibody levels were seen in both groups. Even in the group of severe otitis-prone children with six or more AOM episodes per year, high anti-measles and anti-Hib antibody levels were observed, which were not different from those in healthy controls. Nasal atopy is considered a predisposing factor for AOM. Indeed, 50% of the AOM patients were reported as having atopy. The antibody production in response to protein and polysaccharide antigens of routine vaccinations did not differ between atopic and non-atopic AOM patients (data not shown). These results all indicate a well-functioning humoral immune system in response to protein, polysaccharide-protein conjugate, and viral vaccines, that is in

fact stimulated in children with recurrent AOM rather than hypo-responsive, as previously suggested.[4–6] Prellner *et al.* described significantly lower anti-rubella antibody levels after vaccination in sera of 13 children with recurrent AOM, defined as having 6 or more AOM episodes in 12 months, compared to sera of 29 healthy children. This trend, although not significant, was also shown for anti-tetanus toxoid antibody levels.[4] Yamanaka *et al.* described antibody levels against the P6 outer membrane protein of *Haemophilus influenzae* in 13 healthy children and in 30 otitis-prone children, specified as having experienced four or more episodes of OM in the first year of life or six or more episodes by the second year of life. Significantly lower antibody levels were found in the otitis-prone group after the age of 18 months.[5] Hotomi *et al.* described 20 otitis-prone children, all with AOM due to non-typeable *Haemophilus influenzae*. The otitis-prone condition was defined as having more than 3 AOM episodes in 6 months, more than 4 AOM episodes in 1 year, or more than 4 AOM episodes by 2 years of age. Of these 20 otitis-prone children, 11 showed subnormal levels of anti-P6 IgG.[6] Differences between our study and the previous studies are that we did not study children directly after an immunological challenge, either by vaccination, like Prellner *et al.* described, or by natural exposure, like Yamanaka and Hotomi *et al.* investigated. However, anti-diphtheria and anti-tetanus antibody responses in 4-year-olds do reflect the immune response after DT-IPV booster vaccination, showing good immunological responsiveness in the subjects in our study. Because measles booster vaccination is given to 9-year-old children and Hib booster vaccination is administered at 11 months, we could not evaluate responses directly after stimulation with these two vaccines in our study populations. However, overall antibody levels do not point at a hypo-responsive humoral immune system. Furthermore, the studies in literature that are suggesting a hypo-responsive immune system in children with recurrent AOM were conducted in very small groups of children, while our study was carried out in a well defined and substantial larger cohort of children using a large group of control subjects. Within the group with recurrent AOM, children with 2 or 3 episodes of AOM, showed no differences with otitis-prone children with 4–5 or even 6 or more AOM episodes. Our results therefore indicate that the previous suggestions of a generalized specific antibody deficiency in otitis-prone children should be reconsidered. Gross *et al.* stated that the measurement of immunoglobulin levels in children with recurrent respiratory infections is likely to identify a high proportion of children with values outside the normal range.[18] However, such children are likely to represent the small percentage of the normal population with values more than 2 SD below the mean for age. Therefore, it is more informative in the immunological evaluation of children with recurrent infections to study the capacity of a child to form antibodies. In addition to the antibody levels reported here, we observed normal to high immunoglobulin levels in children with recurrent AOM and OME as well.[19] These data again are not indicative for a hypo-responsive immune system but rather an activated one. Furthermore, Gross *et al.* described normal antibody responses after vaccination with tetanus toxoid in a group of 203 children with recurrent respiratory infections, which is in agreement with our current results. In addition, Gross *et al.* evaluated antibody responses to polysaccharide antigens by immunizing 66 patients with a pneumococcal polysaccharide (PPS) vaccine. This revealed a proportion of 79% of patients that responded poorly to one or more specific PPS types. Thus, these data from Gross *et al.* as well as our previous data[20] indicate that if children with recurrent

respiratory infections, like OM, are immunologically evaluated, the response to specific PPS's is the best discriminating test to elucidate immunodeficiency. This is supported by data from Herrod *et al.* who reported on otitis-prone children with normal serum immunoglobulin levels and normal titers of anti-tetanus toxoid antibodies, who were unable to respond to specific Hib capsular polysaccharides.[21]

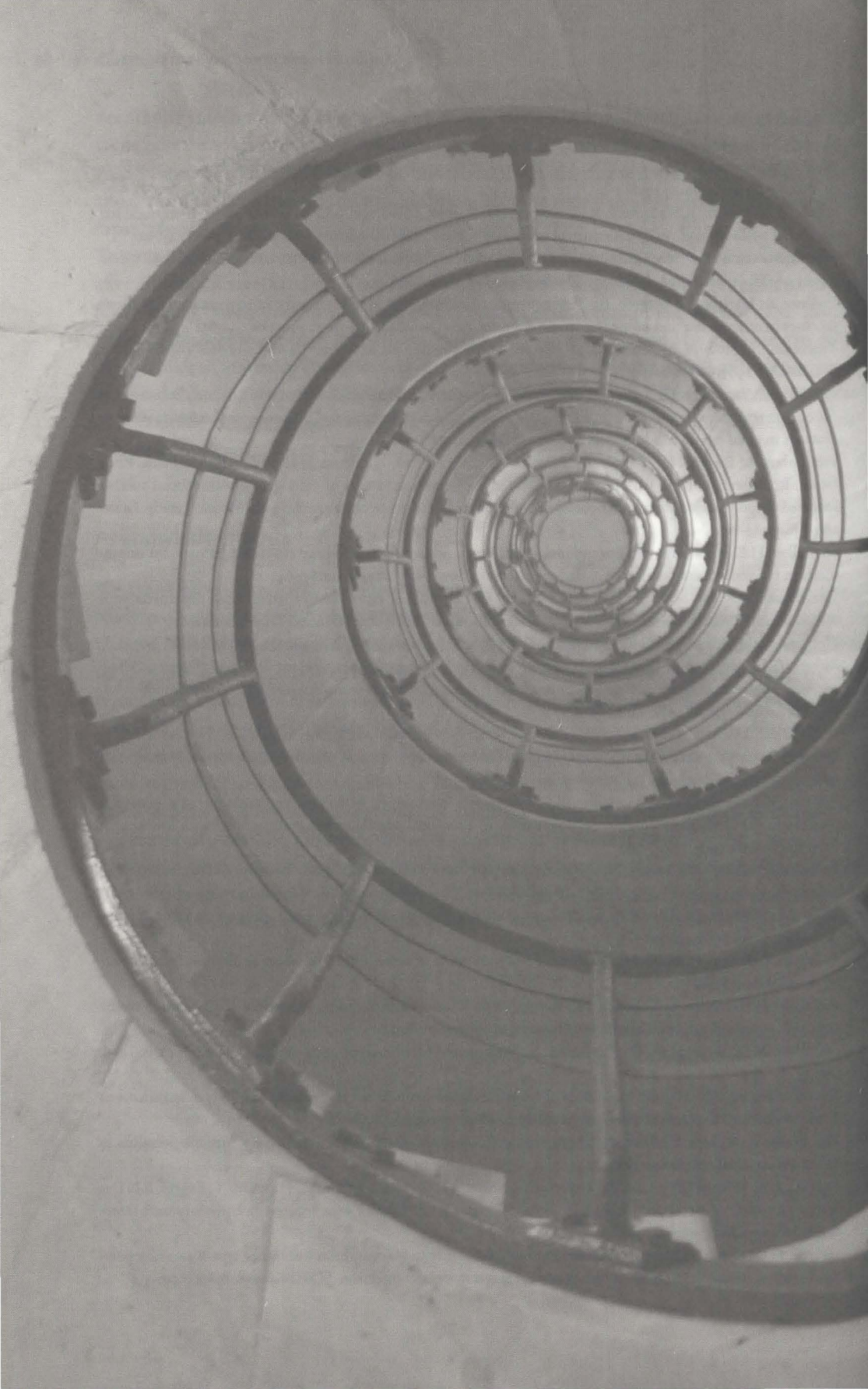
To summarize, our present study indicates that antibody levels determined in older children after childhood vaccinations of the NIP do not confirm the presence of a generalized immunodeficiency in response to vaccinations in children with recurrent episodes of OM. Instead, rather high specific antibody levels to diphtheria, tetanus, measles, and Hib are found in otitis-prone children, probably due to an activated immune system. Determining antibody responses at a fixed time point after vaccination with a polysaccharide vaccine, like the 23-valent PPS vaccine, possibly is more indicative for the patient's immunological status and proneness to develop recurrent episodes of OM.

Acknowledgements

We thank all children and parents that participated in the described studies. For the OME study, patients were recruited by ENT specialists from the Wilhelmina Children's Hospital in Utrecht, Hospital Gooi Noord in Blaricum, the Radboud Hospital and the Canisius Wilhelmina Hospital in Nijmegen, Twee Steden Hospital and the Elizabeth Hospital in Tilburg, the Deventer Hospital in Deventer and Hospital Koningin Beatrix in Winterswijk, who we are all very thankful for their efforts. Pieter van Gageldonk was of great assistance with the determination of anti-diphtheria and anti-tetanus antibodies at the Laboratory for Vaccine preventable Diseases at the National Institute for Public Health and the Environment. We thank Cuno Uiterwaal and Maroeska Rovers for help with the statistical analyses. The AOM study was supported by grants from ZonMw, the Netherlands Organization for Health, Research and Development (grant number 002828480), and the Health Insurance Company Zilveren Kruis-Achmea. The OME study was supported by a grant from ZonMw, the Netherlands Organization for Health, Research and Development (grant number 904–61–092).

References

1. Freid, V.M.; Makuc, D.M.; Rooks, R.N.: Ambulatory health care visits by children: principal diagnosis and place of visit. Vital Health Stat.13 **1998**; 1-23
2. Saxon, A.; Kobayashi, R.H.; Stevens, R.H.; Singer, A.D.; Stiehm, E.R.; Siegel, S.C.: In vitro analysis of humoral immunity in antibody deficiency with normal immunoglobulins. Clin.Immunol.Immunopathol. **1980**; 17:235-244
3. French, M.A. and Harrison, G.: Systemic antibody deficiency in patients without serum immunoglobulin deficiency or with selective IgA deficiency. Clin.Exp.Immunol. **1984**; 56:18-22
4. Prellner, K.; Harsten, G.; Lofgren, B.; Christenson, B.; Heldrup, J.: Responses to rubella, tetanus, and diphtheria vaccines in otitis-prone and non-otitis-prone children. Ann.Otol.Rhinol.Laryngol. **1990**; 99:628-632
5. Yamanaka, N.; Hotomi, M.; Shimada, J.; Togawa, A.: Immunological deficiency in "otitis-prone" children. Ann. N.Y.Acad.Sci. **1997**; 830:70-81.:70-81
6. Hotomi, M.; Yamanaka, N.; Saito, T.; Shimada, J.; Suzumoto, M.; Suetake, M.; Faden, H.: Antibody responses to the outer membrane protein P6 of non-typeable Haemophilus influenzae and pneumococcal capsular polysaccharides in otitis-prone children. Acta Otolaryngol. **1999**; 119:703-707
7. Veenhoven, R.; Bogaert, D.; Uiterwaal, C.; Brouwer, C.; Kiezebrink, H.; Bruin, J.; IJzerman, E.; Hermans, P.; de Groot, R.; Zegers, B.; Kuis, W.; Rijkers, G.; Schilder, A.; Sanders, E.: Effect of conjugate pneumococcal vaccine followed by polysaccharide pneumococcal vaccine on recurrent acute otitis media: a randomised study. Lancet **2003**; 361:2189-2195
8. de Melker, H.E. and Conyn-van Spaendonck, M.A.: Immunosurveillance and the evaluation of national immunization programmes: a population-based approach. Epidemiol.Infect. **1998**; 121:637-643
9. Hendriksen, C.F.; van der Gun, J.W.; Kreeftenberg, J.G.: Combined estimation of tetanus and diphtheria antitoxin in human sera by the in vitro Toxin-Binding Inhibition (ToBI) test. J.Biol.Stand. **1989**; 17:191-200
10. Herrmann, D.J.; Hamilton, R.G.; Barington, T.; Frasch, C.E.; Arakere, G.; Makela, O.; Mitchell, L.A.; Nagel, J.; Rijkers, G.T.; Zegers, B.; .: Quantitation of human IgG subclass antibodies to Haemophilus influenzae type b capsular polysaccharide. Results of an international collaborative study using enzyme immunoassay methodology. J.Immunol.Methods **1992**; 148:101-114
11. Mariani, M.; Luzzi, E.; Proietti, D.; Mancianti, S.; Casini, D.; Costantino, P.; van Gageldonk, P.; Berbers, G.: A competitive enzyme-linked immunosorbent assay for measuring the levels of serum antibody to Haemophilus influenzae type b. Clin.Diagn.Lab Immunol. **1998**; 5:667-674
12. van Den, H.S.; Berbers, G.A.; de Melker, H.E.; Conyn-van Spaendonck, M.A.: Sero-epidemiology of measles antibodies in the Netherlands, a cross-sectional study in a national sample and in communities with low vaccine coverage. Vaccine **1999**; 18:931-940
13. Teele, D.W.; Klein, J.O.; Rosner, B.A.: Epidemiology of otitis media in children. Ann.Otol.Rhinol.Laryngol.Suppl **1980**; 89:5-6
14. Sipila, M.; Pukander, J.; Karma, P.: Incidence of acute otitis media up to the age of 1 1/2 years in urban infants. Acta Otolaryngol. **1987**; 104:138-145
15. Pukander, J.; Karma, P.; Sipila, M.: Occurrence and recurrence of acute otitis media among children. Acta Otolaryngol. **1982**; 94:479-486
16. Pukander, J.; Luotonen, J.; Timonen, M.; Karma, P.: Risk factors affecting the occurrence of acute otitis media among 2-3-year-old urban children. Acta Otolaryngol. **1985**; 100:260-265
17. Stahlberg, M.R.; Ruuskanen, O.; Virolainen, E.: Risk factors for recurrent otitis media. Pediatr.Infect.Dis. **1986**; 5:30-32
18. Gross, S.; Blaiss, M.S.; Herrod, H.G.: Role of immunoglobulin subclasses and specific antibody determinations in the evaluation of recurrent infection in children. J.Pediatr. **1992**; 121:516-522
19. Veenhoven, R.; Rijkers, G.; Schilder, A.; Adelmeijer, J.; Uiterwaal, C.; Kuis, W.; Sanders, E.: Immunoglobulins in Otitis-Prone Children. Pediatr.Res. **2003**;
20. Sanders, L.A.; Rijkers, G.T.; Kuis, W.; Tenbergen-Meekes, A.J.; Graeff-Meeder, B.R.; Hiemstra, I.; Zegers, B.J.: Defective antipneumococcal polysaccharide antibody response in children with recurrent respiratory tract infections. J.Allergy Clin.Immunol. **1993**; 91:110-119
21. Herrod, H.G.; Gross, S.; Insel, R.: Selective antibody deficiency to Haemophilus influenzae type B capsular polysaccharide vaccination in children with recurrent respiratory tract infection. J.Clin.Immunol. **1989**; 9:429-434



5

Pneumococcal polysaccharide booster vaccination increases antibody functionality in toddlers with acute otitis media

Selma P. Wiertsema[1], Reinier H. Veenhoven[2], Anne G.M. Schilder[3], Vanessa Walraven[1], Ger T. Rijkers[1], Elisabeth A.M. Sanders[1]

1 Department of Pediatric Immunology, University Medical Center Utrecht, the Netherlands
2 Department of Pediatrics, Spaarne Hospital, Hoofddorp, the Netherlands
3 Department of Otorhinolaryngology, University Medical Center Utrecht, the Netherlands

Submitted

Summary

The objective of this study was to evaluate whether pneumococcal polysaccharide boosting after pneumococcal conjugate vaccine priming leads to enhanced antibody functionality against pneumococcal serotypes included in both vaccines.

Avidity and opsonophagocytic capacities of antibodies against pneumococcal serotypes 6B, 18C, 19F and 23F after a single or a double conjugate vaccine priming dose and after polysaccharide boosting, in children aged 1-7 years with a history of recurrent acute otitis media, were studied.

Polysaccharide boosting significantly increased antibody avidity and opsonophagocytic capacity. However, the increases in antibody functionality varied between the individual pneumococcal serotypes. For serotypes 6B and 18C, priming with two instead of one dose of conjugate vaccine, led to better avidity maturation upon polysaccharide boosting.

Avidity maturation upon a pure polysaccharide booster vaccination after conjugate vaccine priming is induced and polysaccharide boosting thus contributes to optimal protection against pneumococcal infection. Apart from serotype specific antibody titers, the increase in antibody avidity should be considered when evaluating vaccine immunogenicity.

Introduction

Otitis media (OM) is one of the most common childhood infections with *Streptococcus pneumoniae* as the main bacterial cause.[1-3] Prevention of pneumococcal infections by vaccination is the strategy of choice because of the rapid emergence of multi-drug resistant *S. pneumoniae* and the limited benefit of antibiotics in OM.[4]

The 23-valent pneumococcal polysaccharide vaccine (PPV23) includes capsular polysaccharides from the most prevalent pneumococci and aims to induce protective serotype-specific IgG antibodies. However, the immunogenicity of this T-cell independent polysaccharide vaccine proved to be low in children under 18 to 24 months of age, who are most at risk for mucosal infections like acute otitis media (AOM).[5-9] Furthermore, the T-cell independent polysaccharide vaccine alone fails to induce memory and affinity maturation of antibodies.[10-13]

To overcome the poor immunogenicity of polysaccharide vaccines, capsular antigens are covalently conjugated to a protein carrier. Pneumococcal conjugate vaccines (PCV) with T-cell dependent characteristics are immunogenic in infants, induce antibodies in serum and mucosal secretions, provoke immunologic memory and induce a progressive increase in avidity and opsonophagocytic capacity of antibodies upon repeated immunizations.[14]

Despite over 95% clinical efficacy against vaccine serotype invasive pneumococcal disease,[15,16] the efficacy of conjugate vaccines in preventing mucosal colonization and infection is at best 50%.[14,17] Furthermore, the current conjugate vaccines cover a limited number (7-11) of pneumococcal serotypes. A vaccine schedule including PPV23 booster vaccination after primary PCV7 vaccination would potentially increase serotype coverage, particularly in children after 2 years of age. Moreover, pneumococcal polysaccharide boosting after priming with a conjugate vaccine leads to higher serum IgG levels as compared to a conjugate vaccine booster.[18] Whether polysaccharide booster vaccination after conjugate vaccine priming would lead to functional maturation of antibodies against serotypes included in both vaccines is unknown.

We investigated avidity and opsonophagocytic capacity of antibodies against pneumococcal serotypes 6B, 18C, 19F and 23F after one or two PCV7 priming vaccinations and again after PPV23 boosting, in 40 children with a history of recurrent AOM who participated in a randomized controlled trial on the efficacy of combined PCV7/PPV23 vaccination.[17] Our data show that polysaccharide boosting improves the functional characteristics of pneumococcal serotype-specific antibodies, but large differences were observed between individual serotypes. Furthermore, the number of PCV7 priming doses (one or two) influences functional antibody maturation after PPV23 boosting.

Methods

Study population and vaccinations

Forty patients were randomly selected from 191 patients immunized with pneumococcal conjugate and polysaccharide vaccine, who participated in a randomized controlled trial of the clinical efficacy of pneumococcal vaccines in prevention of AOM. All children had a history of physician diagnosed recurrent AOM.[17] The ethical committees of the participating hospitals previously approved the study. Written informed parental consent was obtained from all subjects.

All children were between 1 and 7 years of age and had a history of 2 or more AOM episodes in the year before study entry. Twenty children aged between 12-24 months were randomly selected from the group who received the 7-valent pneumococcal conjugate vaccine twice with a one-month interval. Another 20 children aged 24-84 months were randomly selected from the group that received one dose of pneumococcal conjugate vaccine. All 40 patients of both age groups received a 23-valent pneumococcal polysaccharide booster vaccination 6 months after the last conjugate vaccination. Blood was drawn and serum was isolated 4 weeks after the last PCV7 vaccination and again 4 weeks after PPV23 boosting. Serum was stored at –20 °C until analyses.

The pneumococcal conjugate vaccine (Prevnar, Wyeth, Rochester, NY, USA) consists of 2 μg each of capsular polysaccharides of pneumococcal serotypes 4, 9V, 14, 19F, and 23F, 4 μg of serotype 6B polysaccharide and 2 μg of serotype 18C oligosaccharide, each conjugated individually to the CRM197 protein.

The pneumococcal polysaccharide vaccine (Pneumune, Wyeth, Rochester, NY, USA) consists of 25 μg of capsular polysaccharides of each of the pneumococcal serotypes 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19F, 19A, 20, 22F, 23F and 33F.

IgG antibody avidity

Avidity indices of IgG anti-pneumococcal antibodies against serotypes 6B, 18C, 19F and 23F polysaccharides were determined after PCV7 vaccination and after PPV23 booster vaccination in the same 40 patients. All serum samples were diluted to an IgG antibody concentration of 0.05 μg/ml for the specific serotype, which corresponds with an OD value of approximately 1.0 and allow accurate measurement in the most dynamic range.

A modification of the sodium thiocyanate (NaSCN, Fluka, Steinheim, Germany) elution ELISA as described previously,[19] was applied to the anti-pneumococcal antibody ELISA. In short, microtiter plates (Greiner Bio-One, Frickenhausen, Germany) were coated with pneumococcal polysaccharide (Statens Serum Institute, Copenhagen, Denmark). After 2 hours incubation with serum, increasing concentrations of NaSCN were added to individual wells, ranging from 0 to 3.5 M (0, 0.1, 0.3, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, and 3.5). After 15 minutes incubation at 37 °C plates were washed and incubated for 2 hours at 37°C with alkaline-phosphatase conjugated goat-anti-human IgG (Sanbio Biosource, Camarillo, CA, USA). After washing, p-nitrophenyl phosphate in DEAE buffer (diethanolamine, $MgCl_2.6H_2O$ in distilled H_2O) was added as substrate. Color intensity was measured at 405 nm in an MRX Revelation ELISA reader (Dynex Technology,

Chantilly, VA, USA).
Avidity index is indicated as the NaSCN concentration that elutes 50% of bound antibodies at a fixed antibody concentration.
To minimize technical variation, the post-PCV7 and post-PPV23 samples from one patient were run on the same plate and additionally, avidity indices of all 40 patients against a particular pneumococcal serotype were determined on a single day. Pre- immunization antibody levels were too low for reliable measurement of avidity indices.

IgG antibody opsonophagocytic capacity

Opsonophagocytic capacity of sera was determined against pneumococcal serotypes 6B, 18C, 19F and 23F after PCV7 vaccination (one or two doses) and after booster vaccination with the 23-valent polysaccharide vaccine according to a modification of the method described by Busetto et al.[20] We first established which incubation-time and amount of antibody yielded the best reproducible results in our assay (figure 1). Incubation times were varied from 10 to 30 minutes. Because within this time frame little variation in overall phagocytosis was observed, 15 minutes incubation time was applied in following experiments. Experiments were performed with a predetermined antibody concentration. For each serotype the lowest concentration of specific IgG antibody that yielded plateau level of phagocytosis was used for all further experiments. This was 0.5 μg/ml for antibodies against serotypes 6B, 18C and 19F, and 1.0 μg/ml for anti-23F antibodies (figure 1).
For opsonophagocytic experiments, individual clinical strains of 6B, 18C, 19F and 23F serotyped *S. pneumoniae*, cultured from the middle ear of patients with otitis media with effusion, were heat inactivated (56°C, 30 minutes) and labeled with Fluorescein Isothiocyanate (FITC la-

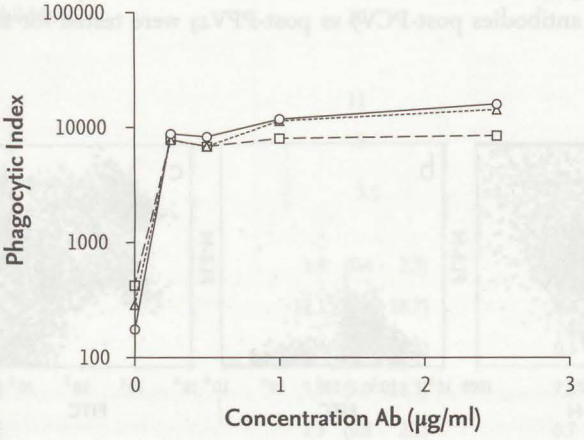


Figure 1 Optimization of opsonophagocytic assay.
Incubation of leukocytes, pneumococcal serotype 6B and patient serum for 10 minutes (circles, drawn line), 20 minutes (triangles, dotted line) or 30 minutes (squares, dashed line) and various amounts of pneumococcal serotype specific IgG antibody (0 to 2.5 μg/ml).
Graph shows a representative result, as was observed for all studied serotypes.

beling kit, Pierce, Rockford, IL, USA). Whole blood as a source of granulocytes was drawn from one volunteer donor. For assay standardization the same donor, with the optimal IgG binding FcγRIIa-131HH genotype,[21,22] was used for all experiments. Erythrocytes were lysed using hypotonic shock buffer (0.8% NH_4Cl; 0.1% $KHCO_3$; 0.003% EDTA in PBS). Fifty thousand leukocytes were mixed with $10x10^6$ bacteria in a total volume of 150 µl. Fifty µl patient serum with the pre-defined serum antibody concentration was then added and the mixture was incubated for 15 minutes at 37°C while shaking to allow attachment and phagocytosis. Samples were washed twice and FITC intensity was measured using a FACSCalibur (Becton Dickinson, Cal, USA). Cell-bound green fluorescence intensity of granulocytes corresponds with the amount of attached and ingested bacteria. After this first measurement, 100 µl trypan blue (TB) (Sigma-Aldrich Company, Irvine, UK; 250 µg/ml in 0.1M citrate buffer pH 4.0) was added to the sample and FITC intensity was measured again. TB quenches the FITC label of bacteria that are attached but not ingested, which shifts the emission wavelength to above 590 nm. The TB quenched signal was measured at 675/20 nm (FL-3 channel). Residual FITC fluorescence intensity corresponds with bacteria that are really phagocytosed (figure 2). Opsonophagocytic capacity was calculated as the product of the percentage of green fluorescent cells after adding TB and the geometric mean fluorescence intensity of these cells.
To minimize technical variation, opsonophagocytic capacities of antibodies against all 4 pneumococcal serotypes in the post-PCV7 and post-PPV23 samples from an individual patient were determined in a single experiment. A maximum of 10 patients were tested on a single day.

Statistical analyses

Differences in number of AOM episodes and immunoglobulin levels were tested for significance using Mann-Whitney U and t-tests, respectively. IgG antibody avidity and opsonophagocytic capacity were normally distributed. Therefore, differences in avidity indices and opsonophagocytic capacity of antibodies post-PCV7 vs post-PPV23 were tested for significance

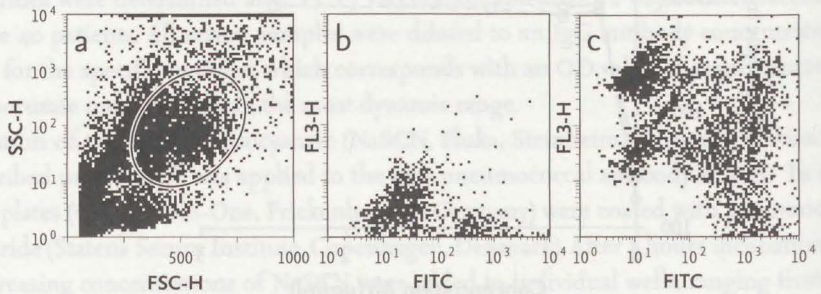


Figure 2 Representative result of FACS analysis in opsonophagocytic assay.
Forward scatter-side scatter dotplot gated on granulocytes is depicted in panel a.
Before addition of trypan blue (b), FITC positive cells represent granulocytes with both attached and ingested fluorescent bacteria. After addition of trypan blue (c), bacteria that are on the outside of the cell and therefore only attached and not ingested, become negative for FITC and positive in the FL3 channel.

using a paired t-test. Differences in post-PPV23 antibody functionality between groups receiving PCV7 priming once or twice were compared using t-test. Statistical analyses were performed using SPSS 12.0 for Windows (SPSS Inc., Chicago, IL, USA). P-values below 0.05 were considered significant.

Results

The questions in our study were whether polysaccharide booster vaccination would lead to a further increase in antibody functionality (avidity and opsonophagocytic capacity), and whether the number of conjugate vaccine priming doses influences antibody functionality after the polysaccharide booster. Characteristics of the 40 children studied for pneumococcal antibody functionality are described in Table 1. Children aged between 12 and 24 months had, because of their younger age, lower IgA, IgG, IgG_1 and IgG_2 immunoglobulin levels compared to children aged 2-7 years. Because of the significant variation in antibody titers, a fixed amount of pneumococcal serotype specific antibody was added in the antibody avidity and opsonophagocytic capacity measurements.

Figure 3 shows the increase in avidity of IgG antibodies against pneumococcal serotypes 6B, 18C, 19F and 23F after polysaccharide booster vaccination compared with conjugate vaccinations alone. For all serotypes, a significant increase in antibody avidity after pneumococcal polysac-

Table 1 General and immunological characteristics of the 40 children with recurrent AOM in the cohort

	1xPCV7	2xPCV7	p-value
Number of children	20	20	
Gender			
Boys	11	12	
Girls	9	8	
Median AOM episodes	3.5	3.5	0.6
Mean Ig (range)			
IgM (g/l)	1.4 (0.6 - 2.3)	1.3 (0.9 - 1.6)	0.3
IgG (g/l)	12.1 (6.2 - 18.7)	8.3 (3.4 - 12.2)	<0.001
IgA (g/l)	1.4 (0.4 - 5.5)	0.5 (0.1 - 1.0)	0.002
IgG_1 (g/l)	9.9 (5.0 - 18.3)	7.3 (4.5 - 10.8)	0.007
IgG_2 (g/l)	1.3 (0.8 - 2.1)	0.7 (0.2 - 2.0)	<0.001
IgG_3 (g/l)	0.5 (0.2 - 1.3)	0.5 (0.1 - 1.5)	0.7
IgG_4 (g/l)	0.3 (0.1 - 1.2)	0.2 (0.1 - 1.0)	0.2

Note. Differences in number of AOM episodes and immunoglobulin levels were tested for significance using Mann-Whitney U and t-tests, respectively.

charide boosting was found irrespective of whether one or two conjugate vaccine priming doses were given. Only for serotype 6B, the increase in antibody avidity failed to reach significance in children primed with PCV7 once. Anti-6B and anti-18C antibody avidity after polysaccharide boosting was significantly higher after priming with two doses of PCV7, compared with only one previous PCV7 vaccination (figure 3).

As a second in-vitro functional read-out system, the opsonophagocytic capacities of antibodies against serotypes 6B, 18C, 19F and 23F were evaluated (figure 4).

At a fixed antibody concentration, significantly higher opsonophagocytic capacities after polysaccharide boosting were observed for anti-6B and anti-23F antibodies after two conju-

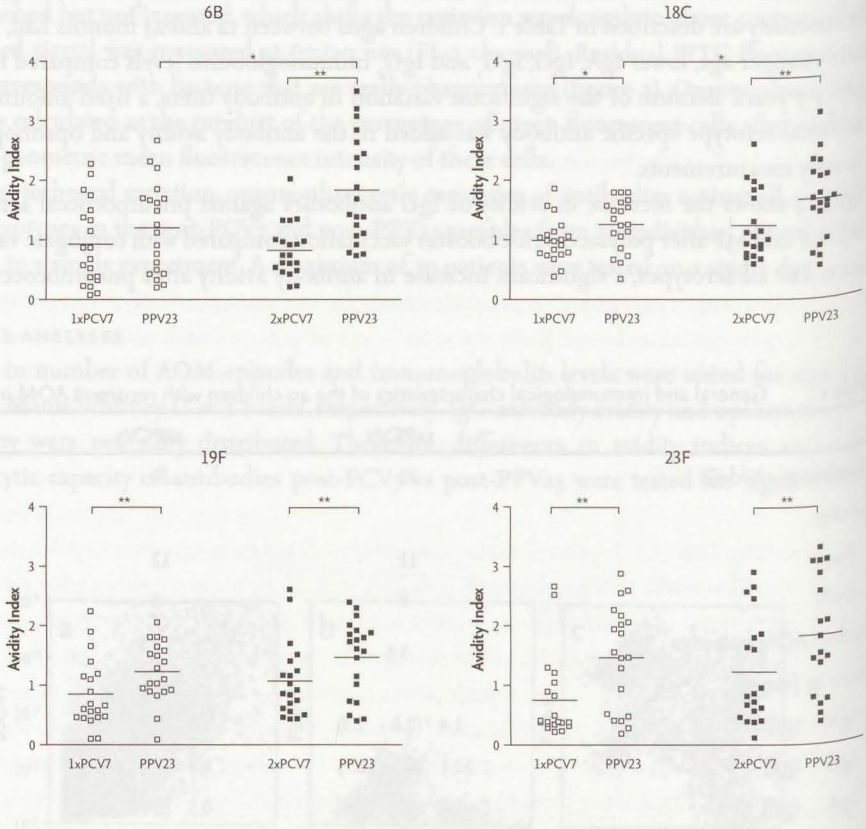


Figure 3 Avidity of IgG antibodies against serotypes 6B, 18C, 19F and 23F post-PCV7 vaccination and post-PPV23 booster vaccination after PCV7 priming once (open squares) and after PCV7 priming twice (closed squares).

Differences in avidity indices of antibodies post-PCV7 vs post-PPV23 were tested for significance using a paired t-test. Differences in post-PPV23 antibody avidity between groups receiving PCV7 priming once or twice were compared using t-test.

* $p<0.05$

** $p<0.001$

gate vaccinations. For antibodies against serotype 19F the increase was significant upon PPV23 boosting in the older group of children receiving only one PCV7 dose. Higher opsonophagocytic capacities of IgG antibodies after PPV23 boosting were observed for anti-19F and anti-23F antibodies after priming with PCV7 twice compared with only one conjugate vaccine priming dose. Remarkably, serotype 18C showed no increase in opsonophagocytic capacity with either one or two PCV7 priming doses at a fixed antibody concentration, despite the increase in antibody avidity.

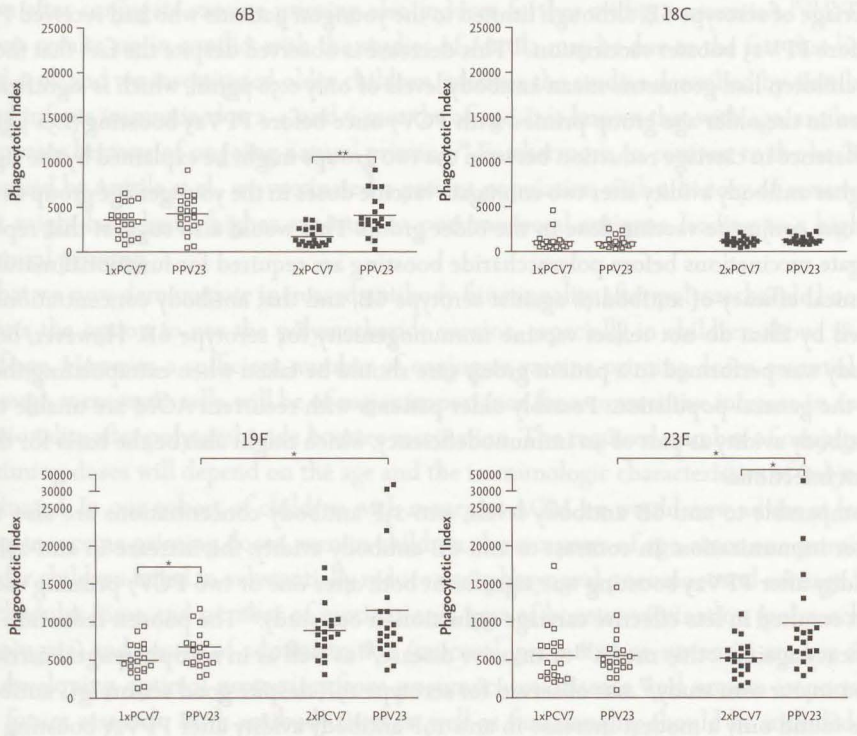


Figure 4 Opsonophagocytic capacity of IgG antibodies against serotypes 6B, 18C, 19F and 23F post-PCV7 vaccination and post-PPV23 booster vaccination after PCV7 priming once (open squares) and after PCV7 priming twice (closed squares).

Differences in opsonophagocytic capacity of antibodies post-PCV7 vs post-PPV23 were tested for significance using a paired t-test. Differences in post-PPV23 antibody avidity between groups receiving PCV7 priming once or twice were compared using t-test.

* $p<0.05$

** $p<0.001$

Discussion

Our data show that pneumococcal polysaccharide booster vaccination after conjugate vaccine priming in children with recurrent AOM, next to an increase in serotype specific IgG antibody titers, also results in a further increase in antibody avidity. This indicates that ongoing affinity maturation is not only achieved by repeated conjugate vaccinations, but also by polysaccharide booster vaccination after conjugate vaccine priming. However, marked differences in serotype-specific antibody avidity maturation and dependency upon the number of primary vaccinations with conjugate vaccine occur.

The capsular polysaccharide of serotype 6B is known to be a weak immunogenic antigen when Elisa IgG levels are considered.[23-25] However, despite these relatively low antibody titers, several studies observed the highest vaccine efficacy against AOM for serotype 6B.[14,26] In our randomized controlled vaccination study, we also observed a marked decrease in nasopharyngeal carriage of serotype 6B, although limited to the youngest patients who had received PCV7 twice before PPV23 booster vaccination.[27] This decrease is observed despite the fact that these younger children had geometric mean antibody levels of only 0.56 μg/ml, which is significantly lower then in the older age group primed with PCV7 once before PPV23 boosting (1.52 μg/ml).[27] The difference in carriage reduction between the two groups might be explained by the significantly higher antibody avidity after two conjugate vaccine doses in the younger age group as compared to one conjugate vaccine dose in the older group. This would also suggest that repeated conjugate vaccinations before polysaccharide boosting are required for functional maturation and clinical efficacy of antibodies against serotype 6B, and that antibody concentrations as measured by Elisa do not reflect vaccine immunogenicity for serotype 6B. However, because our study was performed in a patient group care should be taken when extrapolating these results to the general population. Possibly older patients with recurrent AOM are unable to increase antibody avidity as part of an immunodeficiency, which might also be the basis for their recurrent infections.

Comparable to anti-6B antibody levels, anti-23F antibody concentrations are also rather low after immunization. In contrast to anti-6B antibody avidity, the increase in anti-23F antibody avidity after PPV23 boosting was significant both after one or two PCV7 priming vaccinations, but resulted in less effective carriage reduction in our study.[27] The poorest reduction in clinical efficacy against otitis media[14] or invasive disease,[15] as well as in nasopharyngeal carriage reduction in our own study,[27] was observed for serotype 19F, despite good serum IgG antibody levels. We found only a modest increase in anti-19F antibody avidity after PPV23 boosting with both PCV7 priming schedules. Likewise, low anti-19F antibody avidity was seen by others.[28] For serotype 18C, both significantly increased antibody titers, antibody avidity and optimal carriage reduction was observed.[27] Despite the wide difference in clinical efficacy against individual serotypes, the overall data suggests that apart from Elisa determined antibody titers, antibody avidity also should be taken into account in evaluating vaccines immunogenicity.[29] However, to define standardized correlates of protection after pneumococcal vaccination remains complex and is probably not fully covered by antibody titers and avidity, particularly in mucosal disease. Next to these parameters, the opsonophagocytic activity might serve as a potential correlate

of protection. However, after polysaccharide boosting we observed only modest increases in opsonophagocytic capacities of antibodies as compared to the increase in antibody avidity and no strong correlation between opsonophagocytosis and antibody avidity maturation. This suggests that opsonophagocytosis might not be primarily or solely dependent on antibody avidity: In-vivo, both IgG concentrations and avidity, together with factors like complement and Fc receptor expression on granulocytes, will play a role in determining the opsonophagocytic capacity. Since opsonophagocytic capacity has been shown to correlate with serum IgG antipolysaccharide antibody levels in adults and in infants,[30-32] the fixed antibody concentration in our opsonophagocytic test might not be optimal and therefore adds little extra information as a correlate of protection.

A vaccine schedule including polysaccharide boosting would offer several benefits, like broader serotype coverage, reduced costs and optimal IgG antibody levels.[18] In contrast to earlier reports by Anttila *et al.*, data by Richmond *et al.* and our results now show that polysaccharide booster vaccination after conjugate vaccine priming also induces further avidity maturation.[10,11,33] The fact that our results are in conflict with the studies of Anttila may be due to the fact that both Richmond *et al.* and we investigated older children, whereas the studies described by Anttila *et al.* involved infants immunized at 2, 4, and 6 months of age. It is known that with age, antibody avidity increases because of ongoing natural priming.[34] Furthermore, in contrast to the healthy infants studied by Anttila *et al.*, we vaccinated a patient population with a history of recurrent AOM that might have had a higher exposure to pneumococcal antigens, leading to a higher level of natural priming.

The fact that we now demonstrate increased antibody functionality after polysaccharide boosting supports the option to use the polysaccharide vaccine, especially in children above 18-24 months of age. However, a sufficient number of conjugate vaccine priming doses, essential to induce enough memory B-cells, will be of major importance for a consecutive increase in antibody functionality after polysaccharide booster vaccination. The required number of conjugate vaccine priming doses will depend on the age and the immunologic characteristics of subjects to be vaccinated. In our cohort of children with recurrent AOM we would now advise at least two conjugate vaccine priming doses, even in children above 2 years of age, since one priming dose in older children failed to substantially reduce nasopharyngeal pneumococcal carriage.[17,27]

Vaccine schedules (time and number of vaccinations), type of booster vaccination (polysaccharide vs conjugate) and routes of administration (mucosal, parenteral or systemic) are key elements in developing optimal protection from pneumococcal disease, and remain important issues for future research. Both antibody titers as well as functionality should be included as parameters in studies on vaccine immunogenicity.

Acknowledgements

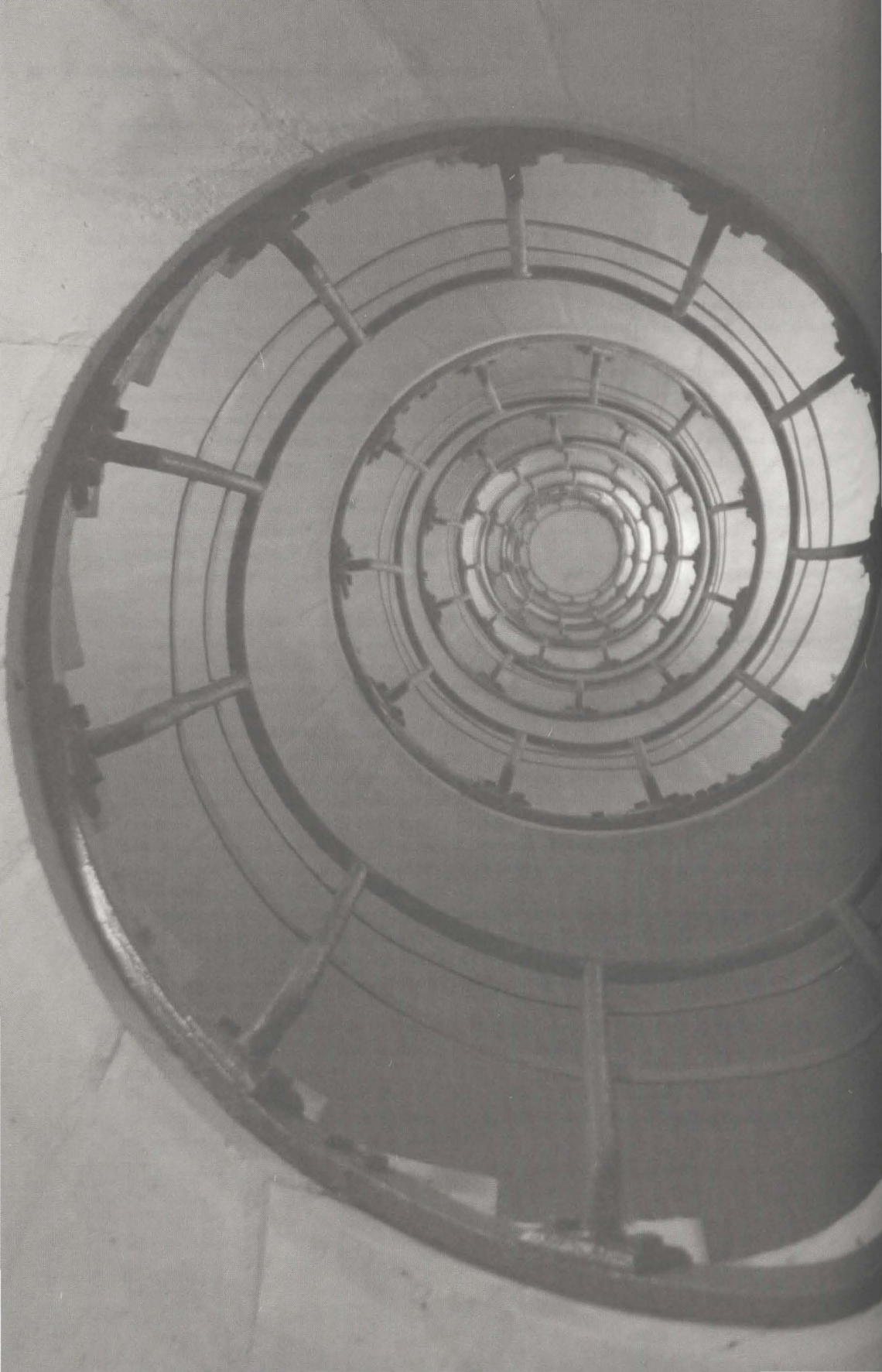
Pneumococcal culturing and serotyping of the clinical strains used in the opsonophagocytic assay was performed by Dr. J. Meis (Canisius Wilhelmina Hospital, Nijmegen, the Netherlands) and Dr. L. Spanjaard (The Netherlands Reference Laboratory for Bacterial Meningitis, Amsterdam, the Netherlands).

The work described in this paper was financially supported by the Netherlands Organization for Health Research and Development ZonMW (grant numbers 002828480 and 90461092), and the Health Insurance Company Zilveren Kruis-Achmea.

References

1. Luotonen, J.; Herva, E.; Karma, P.; Timonen, M.; Leinonen, M.; Makela, P.H.: The bacteriology of acute otitis media in children with special reference to Streptococcus pneumoniae as studied by bacteriological and antigen detection methods. Scand.J.Infect.Dis. 1981; 13:177-183
2. Bluestone, C.D.; Stephenson, J.S.; Martin, L.M.: Ten-year review of otitis media pathogens. Pediatr.Infect.Dis.J. 1992; 11:S7-11
3. Block, S.L.: Causative pathogens, antibiotic resistance and therapeutic considerations in acute otitis media. Pediatr.Infect.Dis.J. 1997; 16:449-456
4. Cripps, A.W.; Otczyk, D.C.; Kyd, J.M.: Bacterial otitis media: a vaccine preventable disease? Vaccine 2005; 23:2304-2310
5. Makela, P.H.; Sibakov, M.; Herva, E.; Henrichsen, J.; Luotonen, J.; Timonen, M.; Leinonen, M.; Koskela, M.; Pukander, J.; Pontynen, S.; Gronroos, P.; Karma, P.: Pneumococcal vaccine and otitis media. Lancet 1980; 2:547-551
6. Teele, D.W.; Klein, J.O.; Bratton, L.; Fisch, G.R.; Mathieu, O.R.; Porter, P.J.; Starobin, S.G.; Tarlin, L.D.; Younes, R.P.: Use of pneumococcal vaccine for prevention of recurrent acute otitis media in infants in Boston. The Greater Boston Collaborative otitis media Study Group. Rev.Infect.Dis. 1981; 3 Suppl:S113-S118
7. Douglas, R.M. and Miles, H.B.: Vaccination against Streptococcus pneumoniae in childhood: lack of demonstrable benefit in young Australian children. J.Infect.Dis. 1984; 149:861-869
8. Ambrosino, D.M.; Siber, G.R.; Chilmonczyk, B.A.; Jernberg, J.B.; Finberg, R.W.: An immunodeficiency characterized by impaired antibody responses to polysaccharides. N.Engl.J.Med. 1987; 316:790-793
9. Sanders, L.A.; Rijkers, G.T.; Kuis, W.; Tenbergen-Meekes, A.J.; Graeff-Meeder, B.R.; Hiemstra, I.; Zegers, B.J.: Defective antipneumococcal polysaccharide antibody response in children with recurrent respiratory tract infections. J.Allergy Clin.Immunol. 1993; 91:110-119
10. Anttila, M.; Eskola, J.; Ahman, H.; Kayhty, H.: Differences in the avidity of antibodies evoked by four different pneumococcal conjugate vaccines in early childhood. Vaccine 1999; 17:1970-1977
11. Anttila, M.; Eskola, J.; Ahman, H.; Kayhty, H.: Avidity of IgG for Streptococcus pneumoniae type 6B and 23F polysaccharides in infants primed with pneumococcal conjugates and boosted with polysaccharide or conjugate vaccines. J.Infect.Dis. 1998; 177:1614-1621
12. Kayhty, H.; Karanko, V.; Peltola, H.; Makela, P.H.: Serum antibodies after vaccination with Haemophilus influenzae type b capsular polysaccharide and responses to reimmunization: no evidence of immunologic tolerance or memory. Pediatrics 1984; 74:857-865
13. Insel, R.A.; Kittelberger, A.; Anderson, P.: Isoelectric focusing of human antibody to the Haemophilus influenzae b capsular polysaccharide: restricted and identical spectrotypes in adults. J.Immunol. 1985; 135:2810-2816
14. Eskola, J.; Kilpi, T.; Palmu, A.; Jokinen, J.; Haapakoski, J.; Herva, E.; Takala, A.; Kayhty, H.; Karma, P.; Kohberger, R.; Siber, G.; Makela, P.H.: Efficacy of a pneumococcal conjugate vaccine against acute otitis media. N.Engl.J Med. 2001; 344:403-409
15. Black, S.; Shinefield, H.; Fireman, B.; Lewis, E.; Ray, P.; Hansen, J.R.; Elvin, L.; Ensor, K.M.; Hackell, J.; Siber, G.; Malinoski, F.; Madore, D.; Chang, I.; Kohberger, R.; Watson, W.; Austrian, R.; Edwards, K.: Efficacy, safety and immunogenicity of heptavalent pneumococcal conjugate vaccine in children. Northern California Kaiser Permanente Vaccine Study Center Group. Pediatr.Infect.Dis.J 2000; 19:187-195
16. Shinefield, H.R. and Black, S.: Efficacy of pneumococcal conjugate vaccines in large scale field trials. Pediatr. Infect.Dis.J. 2000; 19:394-397
17. Veenhoven, R.; Bogaert, D.; Uiterwaal, C.; Brouwer, C.; Kiezebrink, H.; Bruin, J.; IJzerman, E.; Hermans, P.; de Groot, R.; Zegers, B.; Kuis, W.; Rijkers, G.; Schilder, A.; Sanders, E.: Effect of conjugate pneumococcal vaccine followed by polysaccharide pneumococcal vaccine on recurrent acute otitis media: a randomised study. Lancet 2003; 361:2189-2195
18. Kilpi, T.; Ahman, H.; Jokinen, J.; Lankinen, K.S.; Palmu, A.; Savolainen, H.; Gronholm, M.; Leinonen, M.; Hovi, T.; Eskola, J.; Kayhty, H.; Bohidar, N.; Sadoff, J.C.; Makela, P.H.: Protective efficacy of a second pneumococcal conjugate vaccine against pneumococcal acute otitis media in infants and children: randomized, controlled trial of a 7-valent pneumococcal polysaccharide-meningococcal outer membrane protein complex conjugate vaccine in 1666 children. Clin.Infect.Dis. 2003; 37:1155-1164

19. Pullen, G.R.; Fitzgerald, M.G.; Hosking, C.S.: Antibody avidity determination by ELISA using thiocyanate elution. J.Immunol.Methods 1986; 86:83-87
20. Busetto, S.; Trevisan, E.; Patriarca, P.; Menegazzi, R.: A single-step, sensitive flow cytofluorometric assay for the simultaneous assessment of membrane-bound and ingested Candida albicans in phagocytosing neutrophils. Cytometry A 2004; 58:201-206
21. Jansen, W.T.; Breukels, M.A.; Snippe, H.; Sanders, L.A.; Verheul, A.F.; Rijkers, G.T.: Fcgamma receptor polymorphisms determine the magnitude of in vitro phagocytosis of Streptococcus pneumoniae mediated by pneumococcal conjugate sera. J Infect.Dis. 1999; 180:888-891
22. Rodriguez, M.E.; van der Pol, W.L.; Sanders, L.A.; van de Winkel, J.G.: Crucial role of FcgammaRIIa (CD32) in assessment of functional anti-Streptococcus pneumoniae antibody activity in human sera. J Infect.Dis. 1999; 179:423-433
23. Sorensen, R.U.; Leiva, L.E.; Giangrosso, P.A.; Butler, B.; Javier, F.C., III; Sacerdote, D.M.; Bradford, N.; Moore, C.: Response to a heptavalent conjugate Streptococcus pneumoniae vaccine in children with recurrent infections who are unresponsive to the polysaccharide vaccine. Pediatr.Infect.Dis.J 1998; 17:685-691
24. Zielen, S.; Buhring, I.; Strnad, N.; Reichenbach, J.; Hofmann, D.: Immunogenicity and tolerance of a 7-valent pneumococcal conjugate vaccine in nonresponders to the 23-valent pneumococcal vaccine. Infect.Immun. 2000; 68:1435-1440
25. Freijd, A.; Hammarstrom, L.; Persson, M.A.; Smith, C.I.: Plasma anti-pneumococcal antibody activity of the IgG class and subclasses in otitis prone children. Clin.Exp.Immunol. 1984; 56:233-238
26. Jokinen, J.T.; Ahman, H.; Kilpi, T.M.; Makela, P.H.; Kayhty, M.H.: Concentration of antipneumococcal antibodies as a serological correlate of protection: an application to acute otitis media. J.Infect.Dis. 2004; 190:545-550
27. Veenhoven, R.H.; Bogaert, D.; Schilder, A.G.; Rijkers, G.T.; Uiterwaal, C.S.; Kiezebrink, H.H.; van Kempen, M.J.; Dhooge, I.J.; Bruin, J.; IJzerman, E.P.; de Groot, R.; Kuis, W.; Hermans, P.W.; Sanders, E.A.: Nasopharyngeal pneumococcal carriage after combined pneumococcal conjugate and polysaccharide vaccination in children with a history of recurrent acute otitis media. Clin.Infect.Dis. 2004; 39:911-919
28. Ekstrom, N.; Ahman, H.; Verho, J.; Jokinen, J.; Vakevainen, M.; Kilpi, T.; Kayhty, H.: Kinetics and avidity of antibodies evoked by heptavalent pneumococcal conjugate vaccines PncCRM and PncOMPC in the Finnish otitis media Vaccine Trial. Infect.Immun. 2005; 73:369-377
29. Usinger, W.R. and Lucas, A.H.: Avidity as a determinant of the protective efficacy of human antibodies to pneumococcal capsular polysaccharides. Infect.Immun. 1999; 67:2366-2370
30. Vidarsson, G.; Sigurdardottir, S.T.; Gudnason, T.; Kjartansson, S.; Kristinsson, K.G.; Ingolfsdottir, G.; Jonsson, S.; Valdimarsson, H.; Schiffman, G.; Schneerson, R.; Jonsdottir, I.: Isotypes and opsonophagocytosis of pneumococcus type 6B antibodies elicited in infants and adults by an experimental pneumococcus type 6B-tetanus toxoid vaccine. Infect.Immun. 1998; 66:2866-2870
31. Bardardottir, E.; Jonsson, S.; Jonsdottir, I.; Sigfusson, A.; Valdimarsson, H.: IgG subclass response and opsonization of Streptococcus pneumoniae after vaccination of healthy adults. J.Infect.Dis. 1990; 162:482-488
32. Puumalainen, T.; Ekstrom, N.; Zeta-Capeding, R.; Ollgren, J.; Jousimies, K.; Lucero, M.; Nohynek, H.; Kayhty, H.: Functional antibodies elicited by an 11-valent diphtheria-tetanus toxoid-conjugated pneumococcal vaccine. J.Infect.Dis. 2003; 187:1704-1708
33. Richmond, P.; Borrow, R.; Goldblatt, D.; Findlow, J.; Martin, S.; Morris, R.; Cartwright, K.; Miller, E.: Ability of 3 different meningococcal C conjugate vaccines to induce immunologic memory after a single dose in UK toddlers. J.Infect.Dis. 2001; 183:160-163
34. Wuorimaa, T.; Dagan, R.; Vakevainen, M.; Bailleux, F.; Haikala, R.; Yaich, M.; Eskola, J.; Kayhty, H.: Avidity and subclasses of IgG after immunization of infants with an 11-valent pneumococcal conjugate vaccine with or without aluminum adjuvant. J.Infect.Dis. 2001; 184:1211-1215



6

Pneumococcal vaccine efficacy for mucosal pneumococcal infections depends on Fcγ receptor IIa polymorphism

Selma P. Wiertsema[1], Reinier H. Veenhoven[2], Vanessa Walraven[1], Cuno S.P.M. Uiterwaal[3], Anne G.M. Schilder[4], Ger T. Rijkers[1], Elisabeth A.M. Sanders[1]

1 Department of Pediatric Immunology, University Medical Center Utrecht, the Netherlands
2 Department of Pediatrics, Spaarne Hospital, Hoofddorp, the Netherlands
3 Julius Center for Health Sciences and Primary Care, Utrecht, the Netherlands
4 Department of Otorhinolaryngology, University Medical Center Utrecht, the Netherlands

Vaccine, 2005 (in press)

Summary

IgG_2 antibodies are the main antibody subclass produced after pneumococcal polysaccharide vaccination. For these antibodies to be effective, interaction with FcγIIa receptors on phagocytic cells is necessary. FcγRIIa displays a functional polymorphism with either a histidine (H) or arginine (R) at position 131. Interaction of IgG_2 antibodies depends on the H131 allele, whereas this interaction is low to absent with the R131 allele.

We tested the clinical efficacy of combined pneumococcal conjugate and pneumococcal polysaccharide vaccination according to FcγIIa-H/R131 genotype in a randomized double blind placebo controlled vaccination trial in children with a history of acute otitis media.

We found a decisive role for the FcγIIa-H/R131 polymorphism on the clinical vaccine efficacy of combined pneumococcal conjugate and polysaccharide vaccinations. RR homozygotes showed a significant increase in recurrence of acute otitis media after pneumococcal vaccinations. This cannot be explained by differences in the pneumococcal specific antibody response or differences in nasopharyngeal pneumococcal carriage, but may be explained by less efficient interaction of FcγRIIa with polysaccharide-induced IgG_2 anti-pneumococcal antibodies in RR homozygotes. Our data show that the genetic make-up of individuals or populations under study should be considered while evaluating vaccine efficacy trials.

Introduction

The recently developed pneumococcal polysaccharide-protein conjugate vaccines (PCVs) have offered new possibilities for prevention of invasive and mucosal pneumococcal infections, most notably in young children. While the efficacy for vaccine serotype invasive pneumococcal disease has proven to be consistently high (>95%),[1,2] even in immune-compromised target groups such as HIV+ children (vaccine efficacy of 65% with 95% CI 24% to 86%),[3] successes in preventing mucosal pneumococcal infection, i.e. otitis media, are much lower, with a vaccine efficacy against conjugate vaccine serotypes of only 57% (95% CI 44% to 67%).[4] Apart from less effective protection against PCV serotypes, pneumococcal conjugate vaccination resulted in a 33% increase in acute otitis media episodes due to non-PCV serotypes.[4] This phenomenon of replacement of PCV type pneumococci by non-PCV type pneumococci after vaccination with pneumococcal conjugate vaccine has been described for carriage on the nasopharyngeal level in infants and toddlers as well.[5,6] Furthermore, a small but significant increase in non-PCV serotype invasive disease has been observed after widespread immunization with PCV7 in Massachusetts.[7] Because the increase in invasive pneumococcal disease caused by non-PCV serotypes is small, the overall effect of vaccination is a decline in invasive pneumococcal disease. Replacement by non-conjugate vaccine serotypes at the nasopharyngeal level causing invasive and mucosal pneumococcal disease will be an important public health factor to keep track of the coming years. To prevent an increase in carriage and disease caused by non-vaccine type pneumococci after vaccination, vaccines including a wider range of pneumococcal serotypes will be an important step forward. However, the number of pneumococcal serotypes that can be included in a conjugate vaccine is limited. Moreover, conjugate vaccines are expensive and are therefore not readily accessible to major populations with a presumed high risk, especially in developing countries. Use of the 23-valent pneumococcal polysaccharide vaccine (PPV23) as a booster after primary pneumococcal conjugate vaccination might offer broader pneumococcal serotype coverage and possibly prevent replacement by frequently circulating serotypes included in the 23-valent pneumococcal polysaccharide vaccine. At the nasopharyngeal level, polysaccharide vaccination could not prevent replacement with PPV23 serotypes, despite PPV23 booster vaccination after conjugate priming.[8] However, polysaccharide vaccine induced IgG antibodies might offer broader protection, particular against invasive disease, where lower antibody levels may already be sufficient.[9,10]

Opsonisation by IgG antibodies plays a decisive role in the immune defense against polysaccharide encapsulated bacteria such as *Streptococcus pneumoniae*. In adults the antibodies induced by pneumococcal polysaccharide antigens are mainly of the IgG_2 subclass,[11,12] whereas in children both IgG_1 and IgG_2 anti-polysaccharide antibodies are formed.[13] For these antibodies to be effective, an interaction of the constant part of the antibody with receptors for IgG on phagocytic cells, i.e. Fcγ receptors, is necessary.[14,15] IgG_2 antibodies exclusively interact with FcγRIIa, which has 2 genetically determined and functionally different allotypes, with either a histidine (H) or an arginine (R) at position 131 of the receptor. While both FcγRIIa allotypes interact with IgG_1 and IgG_3, interaction with IgG_2 is mainly restricted to the FcγRIIa-H131 allele, whereas the interaction of IgG_2 with the FcγRIIa-R131 allele is as good as absent.[16] Clinical relevance of

this functional polymorphism in FcγRIIa has been previously described in bacterial respiratory tract infections and meningococcal disease.[17,18]

For this reason we hypothesized that the genetic polymorphism in FcγRIIa might influence pneumococcal vaccine efficacy against otitis media. Our data indeed show that clinical vaccine efficacy of polyvalent pneumococcal vaccines is influenced by the genetic make-up of the host or population under study.

Materials and Methods

Patients and vaccinations

From April 1998 to December 2001, 383 children, aged 1-7 years, who had experienced two or more episodes of AOM in the previous year, were enrolled in a double-blind randomized vaccination trial.[19] Hundred-ninety children received a 7-valent pneumococcal conjugate vaccine (Prevnar®, Wyeth). Children were immunized twice, with one-month interval, when between 12 and 24 months of age, and only once when older than 2 years of age. Children in both age groups received the 23-valent pneumococcal polysaccharide vaccine 6 months after (the final) PCV7 vaccination (Pneumune®, Wyeth).

PCV7 consisted of 2 μg each of capsular polysaccharides of pneumococcal serotypes 4, 9V, 14, 19F, and 23F, 4 μg of serotype 6B polysaccharide, and 2 μg of serotype 18C oligosaccharide, each conjugated individually to the CRM197 protein. PPV23 consisted of 25 μg of capsular polysaccharides of each of the pneumococcal serotypes 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19F, 19A, 20, 22F, 23F, and 33F.

The 193 control children received, depending on age, three doses of Hepatitis B (EngerixB=AE Junior®, GlaxoSmithkline) or two doses Hepatitis A vaccine (Havrix=AE Junior®, GlaxoSmithkline).

Parents who were willing to participate, signed a consent form to enroll their child in the study. The ethical committees of the participating institutions approved the study.

Fc receptor genotyping

Genomic DNA was extracted from whole blood using a QIAamp DNA Blood Kit (Qiagen, Westburg, the Netherlands). FcγRIIa-H/R131 genotype was determined by means of allele-specific PCR amplification methods as described in detail previously.[20] DNA samples from sequence confirmed homozygous and heterozygous genotypes were included as controls. PCR products were loaded on a 2% agarose gel containing ethidium bromide, visualized under UV light and photographed (Gel Doc 1000, Bio Rad).

Antibody determination

IgG antibodies to capsular polysaccharides of *S. pneumoniae* serotypes 4, 6B, 9V, 14, 18C, 19F, 23F in 4 weeks post-immunization serum samples of 86 PCV7/PPV23 vaccinated subjects were measured by ELISA.[21-23] Additionally, we measured antibodies against serotypes 1, 7F, 11A and 15B, which are only included in PPV23, in 4 weeks post-immunization serum samples of 48 vac-

cinated children. Minimal detection levels for these pneumococcal serotypes in our assay are 0.03, 0.09, 0.12, 0.61, 0.05, 0.11, 0.08, 0.006, 0.04, 0.008 and 0.02 μg/ml, respectively. Serum samples were pre-incubated with excess (50 μg/ml) pneumococcal common cell wall polysaccharide (CPS) overnight at 4 °C to block anti-CPS antibodies.[24,25] The pneumococcal antibody reference serum (lot 89-SF) was used for assay standardization.[26]

Nasopharyngeal swabs and microbiology

At study entry, directly before booster vaccination with PPV23 and at 7, 13, and 19 months after PPV23 vaccination, nasopharyngeal samples were taken trans-nasally with a flexible, sterile, dry cotton-wool swab. After sampling, swabs were placed immediately in Stuart's transport medium. Samples were plated within 6 hours onto 5% sheep blood agar plates (with or w/o 5 mg/L gentamicin) to culture for S. *pneumoniae*. Identification of S. *pneumoniae* was based on colony morphology and conventional methods of determination. When S. *pneumoniae* was isolated, serotyping was performed with the capsular swelling method (Quellung reaction) by microscopy with commercially available antisera (Statens Seruminstitut, Copenhagen, Denmark).

Statistics

The primary endpoint of the study was the efficacy of pneumococcal vaccination against clinical episodes of AOM according to FcγRIIa-H/R131 genotype during a follow-up period of 18 months, starting 1 month after completion of the vaccination scheme. Cox-type proportional hazards regression models, including a frailty term allowing for differences between individuals in numbers of recurrent AOM episodes were used. Both groups were matched for age, gender, number of previous AOM episodes and environmental factors.[19] This analysis was done in S-plus, version 2000. Results were considered significant when the CI did not include 1.

Geometric mean IgG antibody titers (GMT) according to FcγRIIa-H/R131 genotype were compared using a general linear model, correcting for age, gender and number of AOM episodes in the year prior to study, using SPSS 12.0 for windows.

Pneumococcal carriage data were analyzed by combining all pneumococcal positive cultures at the three time-points after complete vaccination (time-point 7, 13 and 19 months after PPV23 vaccination). If the same pneumococcal serotype was cultured consecutively, the result was included in the analysis only once. Cultures were classified as being either a 7-valent pneumococcal conjugate vaccine serotype (PCV7-serotype), being a 23-valent pneumococcal polysaccharide vaccine serotype (PPV23-serotype) or being an other pneumococcal serotype or negative for S. *pneumoniae*, and compared between FcγRIIa131 –H/H and –R/R individuals in the pneumococcal vaccine and control group using chi-square analysis in SPSS 12.0 for windows.

Results

Of the 383 enrolled children, 367 children completed the vaccine schedule. Of these 367 children, DNA was available from 342. The overall FcγRIIa-H/R131 genotype distribution in children with recurrent AOM was found to be similar to the genotype distribution in a group of

239 healthy unrelated Dutch blood donors.[18] In our study population age, gender and treatment assignment were similarly distributed across the three FcγRIIa-H/R131 genotype groups (Table 1).

Our main study outcome was clinical vaccine efficacy, measured as recurrence of AOM after completion of the vaccination scheme, according to FcγRIIa-H/R131 genotype. We found that in HH homozygotes, pneumococcal vaccination had no effect on AOM recurrence as compared to the control vaccine group (rate ratio 0.97, 95% CI 0.63-1.5, p=0.89) (figure 1). In contrast, children carrying the poor IgG_2 binding RR genotype, who received pneumococcal vaccines, had a statistically significant increased risk of 77% to develop AOM recurrences compared to children vaccinated with control vaccines (rate ratio 1.77, 95% CI 1.07-2.9, p=0.03). For children with the heterozygous HR genotype an intermediate risk to develop AOM recurrences after pneumococcal vaccination compared to control vaccinated children was observed (rate ratio 1.35, 95% CI 0.96-1.9, p=0.09) (figure 1).

This difference in clinical vaccine efficacy was not caused by a difference in magnitude of the polysaccharide specific antibody response between carriers of the different FcγRIIa-H/R131 genotypes. Similar geometric mean antibody levels against serotypes included in PCV7 or only in PPV23 were observed after vaccination in individuals with different FcγRIIa-H/R131 genotypes (Table 2).

Next, nasopharyngeal pneumococcal carriage according to FcγRIIa-H/R131 genotype was studied to explain the vaccine efficacy results. In both HH and RR homozygote individuals we found a similar statistically significant reduction in carriage of PCV7 serotypes in children vaccinated with pneumococcal vaccines compared to those vaccinated with control vaccines (Table 3). RR homozygotes, vaccinated with pneumococcal vaccines, were found to be colonized more often with PPV23 serotypes (20.5%) compared to HH homozygotes (14.2%), although this was not statistically significant (p=0.2). Such a difference in carriage of PPV23 serotypes based on FcγRIIa-H/R131 genotype was not observed in controls, with a carriage of 14.6% in RR homozygotes and 15.1% in HH homozygotes.

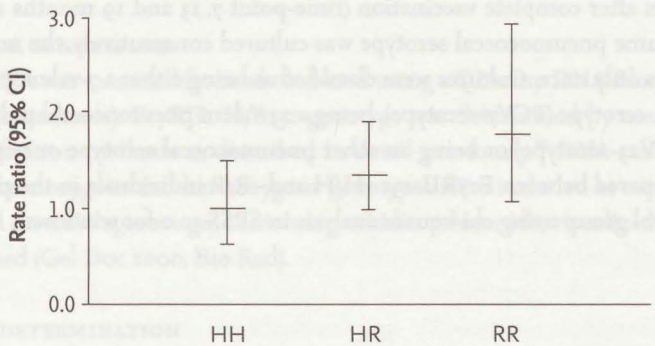


Figure 1 Risk (rate ratio) to develop recurrence of AOM after vaccination with pneumococcal vaccines according to FcγRIIa-H/R131 genotype.
Values in the graph represent rate ratio in pneumococcal vaccinated subjects, with 95% CI indicated with error bars. Rate ratio is 1 for control vaccinated children.

Table 1 FcγRIIa-H/R131 genotype distribution in the total AOM study population and according to gender and number of AOM episodes the child experienced in the year prior to study

		FcγRIIa-H/H131 (%)	FcγRIIa-H/R131 (%)	FcγRIIa-R/R131 (%)	p-value
Total AOM population		96 (28.1)	169 (49.4)	77 (22.5)	
Mean age (year)		2.9	2.8	2.6	0.6
Gender	Boy	65 (30.5)	97 (45.5)	51 (23.9)	0.2
	Girl	31 (24.0)	72 (55.8)	26 (20.2)	
Treatment	Pnc.vacc	51 (30.0)	83 (48.8)	36 (21.2)	0.7
	Control	45 (26.2)	86 (50.0)	41 (23.8)	
AOM episodes	2/3	36 (28.8)	66 (52.8)	23 (18.4)	0.4
	>4	60 (27.6)	103 (47.5)	54 (24.9)	

Table 2 Geometric mean (95% CI) IgG antibody titers (μg/ml) against the serotypes included in the 7-valent pneumococcal conjugate vaccine and against 4 serotypes only included in PPV23 according to FcγRIIa-H/R131 genotype

	FcγRIIa-H/H131	FcγRIIa-H/R131	FcγRIIa-R/R131	p-value
N	25	40	21	
PS 4	4.3 (3.0-6.2)	4.6 (3.5-6.2)	3.6 (2.4-5.3)	0.6
PS 6B	0.9 (0.4-2.0)	1.0 (0.6-1.9)	1.5 (0.7-3.6)	0.7
PS 9V	31.4 (19.8-49.6)	31.7 (21.9-46.0)	19.3 (11.5-32.2)	0.2
PS 14	94.0 (66.3-133.4)	92.2 (69.4-122.5)	59.5 (40.2-88.1)	0.1
PS 18C	8.5 (6.1-11.7)	9.1 (7.0-11.9)	12.3 (8.5-17.7)	0.3
PS 19F	12.6 (7.8-20.3)	15.2 (10.3-22.5)	13.8 (8.1-23.6)	0.8
PS 23F	3.4 (1.8-6.4)	2.9 (1.7-4.8)	3.5 (1.7-7.1)	0.9
N	13	23	12	
PS 1	1.2 (0.7-2.1)	1.0 (0.7-1.5)	0.9 (0.5-1.7)	0.8
PS 7F	1.9 (1.0-3.5)	1.1 (0.7-1.5)	1.0 (0.5-2.0)	0.3
PS 11A	1.5 (0.9-3.5)	1.0 (0.7-1.4)	0.9 (0.5-1.6)	0.4
PS 15B	1.2 (0.6-2.4)	2.1 (1.3-3.5)	1.2 (0.6-2.5)	0.3

Discussion

We here describe the influence of a genetic polymorphism in Fc receptors on pneumococcal vaccine efficacy. We have reported previously that, overall, pneumococcal vaccination did not prevent recurrence of AOM in our study population of children of 1 year of age and older with a history of recurrent AOM (rate ratio 1.25, 95% CI 0.99-1.57).[19] Analysis of vaccine efficacy according to FcγRIIa-H/R131 genotype now showed that pneumococcal vaccinated children with the poor IgG_2 binding RR genotype even had a significantly increased risk to develop AOM recurrences (rate ratio 1.77, 95% CI 1.07-2.9) whereas in children with the HH genotype, pneumococcal vaccinations had no effect on AOM recurrence, and heterozygotes showed an intermediate risk to develop recurrence of otitis media compared to controls.

We hypothesized that the difference in vaccine efficacy between carriers of the different FcγRIIa-H/R131 genotypes might be related to a difference in the magnitude of the pneumococcal specific antibody response, since Fc receptors are described to play an augmenting role in the initiation of the immune response, i.e. by facilitating antigen presentation.[15] However, an immune-complex-mediated feedback inhibition of antibody production, which depends on co-ligation of the B-cell receptor with FcγRIIb, might counteract this effect.[27,28] We did not observe any difference in the overall IgG pneumococcal polysaccharide specific antibody response between carriers of the different FcγRIIa-H/R131 genotypes. Since FcγRIIa is not expressed on B-cells, and since FcγRIIa, in contrast to FcγRIIb, carries an activation signaling motif, the lack of an inhibitory effect of the FcγRIIa-H/R131 polymorphism on the humoral immune response is not totally unexpected.

The difference in clinical vaccine efficacy might also be brought about by a difference in nasopharyngeal pneumococcal carriage according to FcγRIIa-H/R131 genotype. We previously showed that vaccination with combined pneumococcal conjugate and polysaccharide vaccines induces a 50% reduction of nasopharyngeal carriage of PCV7 type pneumococci with a concurrent increase in non-PCV7 type pneumococci, including serotypes of the 23-valent polysaccharide vaccine.[8,19] This shift was found to be mainly due to true replacement and not to capsular switch or unmasking.[29] We found the significant reduction in carriage of PCV7 serotypes to be

Table 3 Pneumococcal carriage data in children vaccinated with pneumococcal vaccines or control vaccines according to homozygous FcγRIIa-H/R131 genotypes

	FcγRIIa-H/H131			FcγRIIa-R/R131		
	Vaccinated	Control	p-value	Vaccinated	Control	p-value
Children	51	45		36	41	
Swabs (n)	120	107		88	103	
PCV7	15 (12.5%)	27 (25.2%)	0.02	9 (10.2%)	34 (33.0%)	< 0.001
PPV23	17 (14.2%)	17 (15.9%)	0.7	18 (20.5%)	15 (14.6%)	0.3
Other/neg	88 (73.3%)	63 (58.9%)		61 (69.3%)	54 (52.4%)	

similar in both HH and RR genotyped subjects who received pneumococcal vaccines. This is likely to occur since PCV7 vaccination mainly elicits IgG_1 antibodies, which bind equally well to the genetically and functionally different FcγIIa receptors. In contrast, polysaccharide vaccines mainly induce IgG_2 antibodies that can only interact with the HH genotype. Therefore, PPV23 vaccine induced IgG_2 antibodies might prevent nasopharyngeal acquisition of PPV23 serotypes particularly in HH homozygotes and subsequently decrease the risk for new AOM episodes in these subjects since the risk of AOM is increased after the recent acquirement of new pneumococcal serotypes.[30] Nasopharyngeal replacement by PPV23 serotypes after elimination of the PCV7 serotype pneumococci in RR homozygotes might not be prevented as efficiently as in HH carriers. Indeed we observed a higher percentage of vaccinated RR subjects to nasopharyngeally carry PPV23 serotypes compared to vaccinated HH children. However, this non-significant difference in PPV23 serotype pneumococcal carriage can only partly explain the observed significant higher rate of AOM after pneumococcal vaccinations in RR homozygotes. Bacterial colonization of the nasopharynx is a very dynamic process and bacterial appearance and subsequent disappearance can occur within days. Taking nasopharyngeal swabs with six months intervals as was done in our study might lead to under-representation of both the true PCV7 and the true PPV23 serotype pneumococcal carriage. Therefore more detailed study of nasopharyngeal pneumococcal colonization after pneumococcal vaccinations is necessary. Furthermore, the fact that we find no significant difference in nasopharyngeal carriage but do find a significant clinical association might imply that the FcγRIIa polymorphism mainly affects systemic but not mucosal immune responses. Presumably serum IgG and phagocytic cells will be of major importance in the clinical protection once there is an infection, like otitis media. In this regard, our group has already extensively shown that effective phagocytosis of bacteria, either *S. pneumoniae* or group B streptococci type III, depends on FcγRIIa, with significantly higher phagocytosis levels in HH homozygotes compared to RR carriers.[31-34] Therefore, an explanation for the difference in clinical protection against AOM after pneumococcal vaccination according to FcγRIIa genotype might be more efficient local, in the case of AOM locally in the middle ear, IgG_2 mediated phagocytosis of PPV23 serotype pneumococci in HH homozygotes. Our current study provides insufficient bacteriological data on middle ear fluids during acute otitis media episodes to statistically support this concept.[19] This concept might be explored by pooling data of the various pneumococcal vaccine studies where FcγRIIa genotyping can be performed.

In conclusion, FcγRIIa genotype does not explain the otitis-prone condition in general. However, after combined pneumococcal conjugate and polysaccharide vaccinations, the RR homozygotes do show a significant increased risk of new otitis media episodes as compared to HH homozygotes, indicating this polymorphism plays a role in determining clinical vaccine efficacy. This increased recurrence rate of acute otitis media after pneumococcal vaccinations in RR homozygotes cannot be explained by differences in the pneumococcal specific antibody response or differences in nasopharyngeal pneumococcal carriage, but is possibly caused by less effective IgG_2 mediated phagocytosis.
Although we can only speculate on the mechanisms behind the differences in clinical vaccine efficacy, our data do show that genetic differences between individuals or between populations should be carefully considered while evaluating vaccination trials and when optimizing vaccine schedules, especially in high-risk groups.

Acknowledgements

The work described in this paper was financially supported by the Netherlands Organization for Health Research and Development ZonMW (grant numbers 002828480 and 90461092), and the Health Insurance Company Zilveren Kruis-Achmea.

References

1. Black, S.; Shinefield, H.; Fireman, B.; Lewis, E.; Ray, P.; Hansen, J.R.; Elvin, L.; Ensor, K.M.; Hackell, J.; Siber, G.; Malinoski, F.; Madore, D.; Chang, I.; Kohberger, R.; Watson, W.; Austrian, R.; Edwards, K.: Efficacy, safety and immunogenicity of heptavalent pneumococcal conjugate vaccine in children. Northern California Kaiser Permanente Vaccine Study Center Group. Pediatr.Infect.Dis.J 2000; 19:187-195
2. Shinefield, H.R. and Black, S.: Efficacy of pneumococcal conjugate vaccines in large scale field trials. Pediatr. Infect.Dis.J. 2000; 19:394-397
3. Klugman, K.P.; Madhi, S.A.; Huebner, R.E.; Kohberger, R.; Mbelle, N.; Pierce, N.: A trial of a 9-valent pneumococcal conjugate vaccine in children with and those without HIV infection. N.Engl.J.Med. 2003; 349:1341-1348
4. Eskola, J.; Kilpi, T.; Palmu, A.; Jokinen, J.; Haapakoski, J.; Herva, E.; Takala, A.; Kayhty, H.; Karma, P.; Kohberger, R.; Siber, G.; Makela, P.H.: Efficacy of a pneumococcal conjugate vaccine against acute otitis media. N.Engl.J Med. 2001; 344:403-409
5. Mbelle, N.; Huebner, R.E.; Wasas, A.D.; Kimura, A.; Chang, I.; Klugman, K.P.: Immunogenicity and impact on nasopharyngeal carriage of a nonavalent pneumococcal conjugate vaccine. J.Infect.Dis. 1999; 180:1171-1176
6. Dagan, R.; Givon-Lavi, N.; Zamir, O.; Sikuler-Cohen, M.; Guy, L.; Janco, J.; Yagupsky, P.; Fraser, D.: Reduction of nasopharyngeal carriage of Streptococcus pneumoniae after administration of a 9-valent pneumococcal conjugate vaccine to toddlers attending day care centers. J.Infect.Dis. 2002; 185:927-936
7. Hsu, K.; Pelton, S.; Karumuri, S.; Heisey-Grove, D.; Klein, J.: Population-based surveillance for childhood invasive pneumococcal disease in the era of conjugate vaccine. Pediatr.Infect.Dis.J. 2005; 24:17-23
8. Veenhoven, R.H.; Bogaert, D.; Schilder, A.G.; Rijkers, G.T.; Uiterwaal, C.S.; Kiezebrink, H.H.; van Kempen, M.J.; Dhooge, I.J.; Bruin, J.; IJzerman, E.P.; de Groot, R.; Kuis, W.; Hermans, P.W.; Sanders, E.A.: Nasopharyngeal pneumococcal carriage after combined pneumococcal conjugate and polysaccharide vaccination in children with a history of recurrent acute otitis media. Clin.Infect.Dis. 2004; 39:911-919
9. Jodar, L.; Butler, J.; Carlone, G.; Dagan, R.; Goldblatt, D.; Kayhty, H.; Klugman, K.; Plikaytis, B.; Siber, G.; Kohberger, R.; Chang, I.; Cherian, T.: Serological criteria for evaluation and licensure of new pneumococcal conjugate vaccine formulations for use in infants. Vaccine 2003; 21:3265-3272
10. Lee, L.H.; Frasch, C.E.; Falk, L.A.; Klein, D.L.; Deal, C.D.: Correlates of immunity for pneumococcal conjugate vaccines. Vaccine 2003; 21:2190-2196
11. Barrett, D.J. and Ayoub, E.M.: IgG2 subclass restriction of antibody to pneumococcal polysaccharides. Clin.Exp. Immunol. 1986; 63:127-134
12. Soininen, A.; Seppala, I.; Nieminen, T.; Eskola, J.; Kayhty, H.: IgG subclass distribution of antibodies after vaccination of adults with pneumococcal conjugate vaccines. Vaccine 1999; 17:1889-1897
13. Lottenbach, K.R.; Mink, C.M.; Barenkamp, S.J.; Anderson, E.L.; Homan, S.M.; Powers, D.C.: Age-associated differences in immunoglobulin G1 (IgG1) and IgG2 subclass antibodies to pneumococcal polysaccharides following vaccination. Infect.Immun. 1999; 67:4935-4938
14. Ravetch, J.V. and Bolland, S.: IgG Fc receptors. Annu.Rev.Immunol. 2001; 19:275-290
15. van de Winkel, J.G. and Capel, P.J.: Human IgG Fc receptor heterogeneity: molecular aspects and clinical implications. Immunol.Today 1993; 14:215-221
16. Warmerdam, P.A.; van de Winkel, J.G.; Vlug, A.; Westerdaal, N.A.; Capel, P.J.: A single amino acid in the second Ig-like domain of the human Fc gamma receptor II is critical for human IgG2 binding. J.Immunol. 1991; 147:1338-1343
17. Sanders, L.A.; van de Winkel, J.G.; Rijkers, G.T.; Voorhorst-Ogink, M.M.; De Haas, M.; Capel, P.J.; Zegers, B.J.: Fc gamma receptor IIa (CD32) heterogeneity in patients with recurrent bacterial respiratory tract infections. J.Infect.Dis. 1994; 170:854-861
18. van der Pol, W.L.; Huizinga, T.W.; Vidarsson, G.; van der Linden, M.W.; Jansen, M.D.; Keijsers, V.; de Straat, F.G.; Westerdaal, N.A.; De Winkel, J.G.; Westendorp, R.G.: Relevance of Fcgamma receptor and interleukin-10 polymorphisms for meningococcal disease. J.Infect.Dis. 2001; 184:1548-1555
19. Veenhoven, R.; Bogaert, D.; Uiterwaal, C.; Brouwer, C.; Kiezebrink, H.; Bruin, J.; IJzerman, E.; Hermans, P.; de Groot, R.; Zegers, B.; Kuis, W.; Rijkers, G.; Schilder, A.; Sanders, E.: Effect of conjugate pneumococcal vaccine followed by polysaccharide pneumococcal vaccine on recurrent acute otitis media: a randomised study. Lancet

2003; 361:2189-2195

20. Breij, E.C.; van der Pol, W.L.; van Winsen, L.; Jansen, M.D.; Dijkstra, C.D.; van de Winkel, J.G.; Uitdehaag, B.M.: No association of FcgammaRIIa, FcgammaRIIIa and FcgammaRIIIb polymorphisms with MS. J.Neuroimmunol. 2003; 140:210-215
21. Sanders, L.A.; Rijkers, G.T.; Kuis, W.; Tenbergen-Meekes, A.J.; Graeff-Meeder, B.R.; Hiemstra, I.; Zegers, B.J.: Defective antipneumococcal polysaccharide antibody response in children with recurrent respiratory tract infections. J.Allergy Clin.Immunol. 1993; 91:110-119
22. Sanders, L.; Rijkers, G.; Tenbergen-Meekes, A.; Voorhorst-Ogink, M.; Zegers, B.: Immunoglobulin isotype-specific antibody responses to pneumococcal polysaccharide vaccine in patients with recurrent bacterial respiratory tract infections. Pediatr.Res. 1995; 37:812-819
23. Gordon, J. and Murgita, R.A.: Suppression and augmentation of the primary in vitro immune response by different classes of antibodies. Cell Immunol. 1975; 15:392-402
24. Musher, D.M.; Luchi, M.J.; Watson, D.A.; Hamilton, R.; Baughn, R.E.: Pneumococcal polysaccharide vaccine in young adults and older bronchitics: determination of IgG responses by ELISA and the effect of adsorption of serum with non-type-specific cell wall polysaccharide. J.Infect.Dis. 1990; 161:728-735
25. Goldblatt, D.; Levinsky, R.J.; Turner, M.W.: Role of cell wall polysaccharide in the assessment of IgG antibodies to the capsular polysaccharides of Streptococcus pneumoniae in childhood. J.Infect.Dis. 1992; 166:632-634
26. Quataert, S.A.; Kirch, C.S.; Wiedl, L.J.; Phipps, D.C.; Strohmeyer, S.; Cimino, C.O.; Skuse, J.; Madore, D.V.: Assignment of weight-based antibody units to a human antipneumococcal standard reference serum, lot 89-S. Clin. Diagn.Lab Immunol. 1995; 2:590-597
27. Takai, T.; Ono, M.; Hikida, M.; Ohmori, H.; Ravetch, J.V.: Augmented humoral and anaphylactic responses in Fc gamma RII-deficient mice. Nature 1996; 379:346-349
28. Ravetch, J.V.: Fc receptors. Curr.Opin.Immunol. 1997; 9:121-125
29. Bogaert, D.; Veenhoven, R.H.; Sluijter, M.; Sanders, E.A.; de Groot, R.; Hermans, P.W.: Colony blot assay: a useful method to detect multiple pneumococcal serotypes within clinical specimens. FEMS Immunol.Med.Microbiol. 2004; 41:259-264
30. Faden, H.; Duffy, L.; Wasielewski, R.; Wolf, J.; Krystofik, D.; Tung, Y.: Relationship between nasopharyngeal colonization and the development of otitis media in children. Tonawanda/Williamsville Pediatrics. J.Infect.Dis. 1997; 175:1440-1445
31. Sanders, L.A.; Feldman, R.G.; Voorhorst-Ogink, M.M.; De Haas, M.; Rijkers, G.T.; Capel, P.J.; Zegers, B.J.; van de Winkel, J.G.: Human immunoglobulin G (IgG) Fc receptor IIA (CD32) polymorphism and IgG2-mediated bacterial phagocytosis by neutrophils. Infect.Immun. 1995; 63:73-81
32. Rodriguez, M.E.; van der Pol, W.L.; Sanders, L.A.; van de Winkel, J.G.: Crucial role of FcgammaRIIa (CD32) in assessment of functional anti-Streptococcus pneumoniae antibody activity in human sera. J Infect.Dis. 1999; 179:423-433
33. Sanders, E.A.; van de Winkel, J.G.; Feldman, R.G.; Voorhorst-Ogink, M.M.; Rijkers, G.T.; Capel, P.J.; Zegers, B.J.: Fc gamma IIA receptor phenotype and opsonophagocytosis in two patients with recurrent bacterial infections. Immunodeficiency 1993; 4:163-165
34. Jansen, W.T.; Breukels, M.A.; Snippe, H.; Sanders, L.A.; Verheul, A.F.; Rijkers, G.T.: Fcgamma receptor polymorphisms determine the magnitude of in vitro phagocytosis of Streptococcus pneumoniae mediated by pneumococcal conjugate sera. J Infect.Dis. 1999; 180:888-891

7

Association of genetic variants in IL-4, IL-4 RA and IL-13 with the anti-pneumococcal antibody response

Selma P. Wiertsema[1], Gareth Baynam[2], Siew-Kim Khoo[2], Reinier H. Veenhoven[3], Niels van Heerbeek[4], Guicheng Zhang[2], Ingrid A. Laing[2], Ger T. Rijkers[1], Jack Goldblatt[2,5], Elisabeth A.M. Sanders[1], Peter N. LeSouëf[2]

1 Department of Paediatric Immunology, University Medical Center Utrecht, the Netherlands
2 School of Paediatrics and Child Health, University of Western Australia, Perth, Australia
3 Department of Paediatrics, Spaarne Hospital, Hoofddorp, the Netherlands
4 Department of Otorhinolaryngology, Radboud University Nijmegen Medical Centre, the Netherlands
5 Genetic Services of Western Australia, Perth, Australia

Submitted

Summary

Significant differences in immune responses upon vaccination between individuals and different ethnic groups have been described, suggesting genetic influences are important in determining the magnitude of vaccine responses. The IL-4 pathway, including IL-4, IL-13 and the IL-4 receptor α chain, could potentially have impact on vaccine responses, since this pathway is known to stimulate B cells.

The objective of this study was to investigate whether single nucleotide polymorphisms (SNPs) in IL-4, IL-13 and IL-4 RA influence pneumococcal serotype specific IgG antibody responses. SNPs in the IL-4 gene (C–589T, G2979T), the IL-13 gene (A–1112G, Arg130Gln) and in the IL-4 RA gene (Ile50Val, Gln551Arg) were investigated in isolation and in combination, for their influence on serotype specific IgG antibody responses upon combined pneumococcal conjugate and polysaccharide vaccinations in children with a history of recurrent otitis media.

Depending on the pneumococcal serotype under study, an association between single SNPs and the serotype specific antibody response was observed for the IL-4 C–599T, IL-4 G2979T, IL-4 Rα Gln551Arg, and for the IL-13 A–1112G SNP. Effects were stronger when investigating haplotypes and haplotype-haplotype combinations.

This study highlights the importance of host genetic factors in modulating responses to vaccination. The additive effect of haplotype and haplotype-haplotype combination analyses, compared to single locus and single haplotype analyses respectively, supports the approach of studying the effect of combinations of multiple loci, both within and between genes, on complex phenotypes.

Introduction

Streptococcus pneumoniae may cause severe invasive infections including sepsis, and meningitis or lower respiratory tract infections like pneumonia. It is also the most commonly reported bacterial cause of upper respiratory tract infections such as otitis media.[1,2] Host defense against S. pneumoniae depends largely on opsonization by antibodies and complement,[3] followed by phagocytosis and intracellular killing by leukocytes and macrophages.[4,5] Prevention by vaccination is the favored strategy against pneumococcal infection because of the persisting mortality and morbidity associated with pneumococcal diseases despite antibiotic treatment, and the rapid emergence of multi-drug resistant S. pneumoniae.[6] Current pneumococcal vaccines contain capsular polysaccharides from the most prevalent pneumococci and aim to induce protective anti-capsular serotype-specific opsonizing IgG antibodies.

The optimal design of vaccines requires understanding of the factors controlling disease and immune pathways. Significant differences in immune responses upon vaccination between individuals and different ethnic groups have been described, suggesting genetic influences are important in determining the magnitude of vaccine responses.[7,8] The relative contributions of genetic and environmental factors to vaccine responses are yet to be determined, but associations between specific genes and vaccine antibody responses have been described.[8,9] To date, research on genetic influences on vaccine responses has mainly focused on human leukocyte antigen (HLA) alleles[10,11] and the immunoglobulin allotype genes.[12,13] Clearly, many additional genes may be involved and therefore a systematic approach is needed to prioritize which genes to examine. We investigated genetic variants in interleukin 4 (IL-4), IL-13 and the IL-4 receptor α chain (IL-4 RA), as they are components of the pathway regulating antibody responses by B-cells. IL-4 and IL-13 are pleiotropic cytokines produced by mast-cells, basophils, and T-cells.[14] They are T helper 2 cytokines that trigger isotype switching from IgM to IgE in B-cells.[15] Furthermore, they enhance the expression of surface molecules, such as the IL-4Rα chain, the low affinity receptor for IgE (FcεRII, CD23) and MHC class II, and down-regulate the IgG type I receptor (FcγRI). Moreover, IL-4 is necessary for the promotion of its own production.[16] IL-13 shares several biological functions with IL-4,[17,18] but also has unique roles in mediating immune responses.[19,20] The receptors for IL-4 and IL-13 share a common α chain: IL-4 Rα. IL-4 Rα dimerizes with the common γ chain in the IL-4 receptor, which is expressed on T- and B-cells, and with the IL-13 Rα1 chain in the IL-13 receptor, which is expressed on B-cells only.[14,21] Functional polymorphisms in both cytokines and in the IL-4Rα chain have been associated with atopy, asthma and associated phenotypes.[22-24]

This study investigated single nucleotide polymorphisms (SNPs) in the IL-4 gene (C-589T, G2979T), the IL-13 gene (A-1112G, Arg130Gln) and in the IL-4 RA gene (Ile50Val, Gln551Arg), in isolation and in combination, for their influence on antibody responses upon combined pneumococcal conjugate and polysaccharide vaccinations.

Materials and Methods

Patients and vaccinations

Serum and DNA was available from 121 randomly selected children with recurrent otitis media participating in one of two randomized controlled vaccination trials investigating prevention of recurrence of otitis media by pneumococcal vaccinations in the Netherlands.[25,26] Both studies were approved by the ethical committees of participating hospitals and institutions. Written informed parental consent was obtained from all subjects.

Pneumococcal polysaccharide specific IgG antibody responses against the 7 conjugate vaccine capsular polysaccharide 4, 6B, 9V, 14, 18C, 19F, and 23F were ascertained.

The first group consisted of 89 children aged 1 to 7 years who had a history of 2 or more physician diagnosed episodes of acute otitis media (AOM) in the previous 12 months. These children received the 7-valent pneumococcal conjugate vaccine (Prevnar®, Wyeth Pharmaceuticals, Philadelphia, PA, PCV7). Children below 24 months of age received a second dose of PCV7 4 weeks later. After pneumococcal conjugate vaccination all children received a 23-valent pneumococcal polysaccharide booster vaccination 6 months later (Pneumune®, Wyeth Pharmaceuticals, Philadelphia, PA, PPV23).

The second group consisted of 32 children aged 2 to 7 years who had a history of at least two prolonged periods of bilateral otitis media with effusion (OME), each lasting 3 months or longer and documented by an ENT specialist. These children were vaccinated with PCV7 once followed by a PPV23 booster vaccination 4 months later.

Since we have previously shown that both groups of children display similar antibody responses after vaccination, these groups were pooled for data analysis.[27]

PCV7 consists of 2 μg each of capsular polysaccharides of pneumococcal serotypes 4, 9V, 14, 19F, and 23F, 4 μg of serotype 6B polysaccharide, and 2 μg of serotype 18C oligosaccharide, each conjugated individually to the CRM197 protein. PPV23 consists of 25 μg of capsular polysaccharides of each of the pneumococcal serotypes 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19F, 19A, 20, 22F, 23F, and 33F.

Genotyping and haplotyping

Genomic DNA was extracted from whole blood using a QIAamp DNA Blood Kit (Qiagen, Hilden, Germany). Patients were genotyped for single nucleotide polymorphisms in IL-4 (C–589T, G2979T), IL-4 Rα (Gln551Arg) and IL-13 (A–1112G) using polymerase chain reaction (PCR) and restriction enzyme digestion. The IL-4 Rα Ile50Val and IL-13 Arg130Gln SNPs were determined using denaturing high-performance liquid chromatography (dHPLC) using the method developed by Oefner and Underhill[28] using an automated Varian HPLC system (Varian Helix-System®). See Table 1 for SNP, primer and restriction enzyme details.

Haplotypes were inferred using PHASE2.1 (http://www.stat.washington.edu/stephens/software.html).[29,30]

Pneumococcal antibody responses

Blood samples for determination of pneumococcal antibodies were obtained 4 weeks after the

Table 1 Investigated SNPs and used primers

SNP*	dbSNP identifier	Primer	Restriction Enzyme
IL-4 C-589T	rs2243250	F-ACTAGGCCTCACCTGATACG	BsmF I
		R-GTTGTAATGCAGTCCTCCTG	
IL-4 G2979T	rs2227284	F-TAGGTCCTGGGCTTCACAG	Hinf I
		R-TTAGCTCTCTTTGGTAAATAGGGAA	
IL-4 Rα Ile50Val	rs1805010	F-GCAAGAGAGGCAACCCTA	dHPLC
		R-GCCTCCGTTGTTCTCAG	
IL-4 Rα Gln551Arg	rs1801275	F-GCCCCCGTCTCGGCCCCCACCAGTGGCTACC	Msp I
		R-GCCCCAAACCCACATTTCTCTGG	
IL-13 A-1112G	rs1800925	F-CGAGGACAGGACGGAGGGAGCCT	BstU I
		R-GTCGCCTTTTCCTGCTCTTCCCG	
IL-13 Arg130Gln	rs20541	F-CTTCCGTGAGGACTGAATGAGACAGTC	dHPLC
		R-GCAAATAATGATGCTTTCGAAGTTTCAGTGGA	

Notes. Underlined base in primers indicates a base change to create restriction enzyme site
* Amino acids numbered from the beginning of the mature protein

pneumococcal polysaccharide booster vaccination. Serum was isolated and stored at –20°C until analysis. Post vaccination IgG antibody levels to all PCV7 serotypes, 4, 6B, 9V, 14, 18C, 19F and 23F, were measured by ELISA as described previously.[31] Minimal detection levels for these pneumococcal serotypes in our assay were 0.03, 0.09, 0.12, 0.61, 0.05, 0.11, and 0.08 μg/ml, respectively. All sera were pre-incubated overnight at 4°C with pneumococcal cell wall polysaccharide (CPS) in diluting buffer for blocking of non-specific anti-CPS antibodies (50 μg/ml; Statens Serum Institute, Copenhagen, Denmark).[32] The pneumococcal antibody reference serum (lot 89-SF) was used for assay standardisation.[33]

Statistical analyses

The IgG antibody levels appeared to be positively skewed. Consequently, their geometric means (GM) were calculated after applying a logarithmic transformation. A general linear model was used to compare the geometric means between the genotypes and haplotypes, adjusted for the covariates of interest, namely age, gender, type of ear disease, number of PCV vaccinations and total IgE levels.

When one of the genotype frequencies was less than 10%, this genotype was pooled with the respective heterozygotes before testing.

P-values <0.05 were considered statistically significant. SPSS 12.0.1 for windows (SPSS Inc., Chicago, IL) was used for all statistical analyses.

Results

SNPs in 3 genes in a pathway known to influence antibody production by B-cells were studied for their influence on antibody production (Table 1). All genotype frequencies were in Hardy-Weinberg equilibrium. As expected, given their collocation on chromosome 5q31, significant linkage was observed between SNPs in IL-4 and IL-13 (Table 2).

Analyses by genotype

Pneumococcal specific IgG antibody responses were analysed according to the six individual SNPs (Table 3). For the two IL-4 SNPs, IL-4 C–599T and IL-4 G2979T, antibody levels against all seven pneumococcal serotypes were higher in CC and GG homozygotes, respectively. For the IL-4 C–599T SNP these differences were significant when using a model recessive for the C allele for antibodies against serotypes 4 and 23F (p=0.002 and p=0.05). For the intronic SNP a trend was observed for antibodies against these same serotypes when using a model recessive for the G allele (p=0.07 and p=0.06).

For the IL-4 Rα Ile50Val SNP, antibody levels were not consistently higher for a specific genotype. For the IL-4 Rα Gln551Arg SNP, antibodies against all seven serotypes were lowest in Gln/Gln homozygotes compared to those with Gln/Arg and Arg/Arg genotypes. These differences were significant for antibodies against serotypes 4, 18C and 14 (p=0.001, 0.02 and 0.04, respectively).

For the IL-13 A–1112G SNP, higher antibody levels were found in GG homozygotes compared to GA/AA genotypes, except for antibodies against serotype 23F. Differences did not reach significance. For the IL-13 Arg130Gln SNP, antibody levels were not consistently higher for a specific genotype, and no trend or significant difference was observed.

Analyses by haplotype

Geometric mean (GM) antibody levels according to haplotypes of each gene were investigated in a linear model (Table 4). Given the results of single locus analyses, it was predicted that those haplotypes composed of two alleles associated with high antibody responses would be associated with the greatest antibody responses. Similarly it was predicted that haplotypes composed of two alleles associated with low antibody responses, would be associated with lowest antibody

Table 2 D' scores indicating linkage between SNPs on the same chromosome

	IL-4 G2979T	IL-13 A–1112G	IL-13 Arg130Gln	IL-4 Rα Gln551Arg
IL-4 C–589T	0.89	0.30	0.46	
IL-4 G2979T		0.49	0.76	
IL-13 A–1112G			0.62	
IL-4 Rα Ile50Val				0.36*

Note. P<0.0001, except for * p=0.004

Table 3 *Geometric mean antibody concentration according to the six investigated SNPs*

	IL-4 C–589T			*IL-4 G2979T*			*IL-4 Rα Ile50Val*				*IL-4 Rα Gln551Arg*			*IL-13 A–1112G*			*IL-13 Arg130Gln*		
	CC	CT/TT	p	GG	GT/TT	p	Ile/Ile	Ile/Val	Val/Val	p	Gln/Gln	Gln/Arg Arg/Arg	p	GG	GA/AA	p	Arg/Arg	Arg/Gln Gln/Gln	p
N	87	31/2		70	41/6		35	64	22		77	36/3		80	35/5		86	30/4	
6B	1.3	1.0	0.5	1.3	1.1	0.5	1.0	1.4	1.0	0.6	1.1	1.5	0.3	1.4	0.9	0.2	1.2	1.1	0.7
4	4.4	2.8	0.002	4.5	3.2	0.07	4.1	4.2	2.7	0.1	3.2	5.8	0.001	4.3	3.1	0.09	4.1	3.5	0.5
23F	4.8	3.0	0.05	4.9	3.2	0.06	3.3	4.6	4.8	0.3	4.0	5.0	0.3	4.2	4.2	0.9	4.2	4.3	0.9
18C	9.1	8.5	0.7	9.4	8.3	0.5	8.2	9.9	7.5	0.4	7.7	12.1	0.02	9.4	8.1	0.5	9.1	8.5	0.7
19F	10.4	10.3	0.9	10.2	10.2	0.9	11.0	9.9	11.0	0.9	9.9	12.1	0.5	10.9	9.3	0.6	10.2	10.8	0.9
9V	27.0	17.8	0.1	26.2	21.4	0.4	19.0	27.3	25.3	0.4	22.4	30.5	0.2	26.6	19.9	0.3	25.4	21.6	0.6
14	73.5	65.8	0.7	71.1	70.1	0.9	73.1	69.0	72.8	0.9	59.5	99.6	0.04	69.6	71.0	0.9	65.4	87.8	0.3

Note. When one of the genotype frequencies is <10%, data for heterozygotes and least common homozygotes were pooled.

responses. For the purposes of this study these were designated as "high responder" and "low responder" haplotypes, respectively.

In accordance with this the IL-4 C*G high responder haplotype, composed of the IL-4 C–599T and IL-4 G2979T SNPs, was associated with the highest antibody responses against pneumococcal serotypes 4, 6B, 9V, 19F and 23F serotypes. These differences were significant for antibodies against serotypes 4 and 23F compared with the T*T low responder haplotype (p=0.04 and 0.05, respectively) (Table 4).

For the IL-4 RA gene the high responder haplotype was Ile*Arg, whereas the low responder haplotype was Val*Gln. Antibody levels against all pneumococcal serotypes were highest in Ile*Arg carriers, except for antibodies against serotype 23F. Differences in antibody levels between Ile*Arg and Val*Gln haplotype carriers were significant for antibodies against serotypes 4 and 18C (p=<0.001 and 0.03, respectively) and a trend was observed for serotype 14 (p=0.07).

For the IL-13 Arg130Gln SNP no clear high or low responder genotype was observed. No consistently higher antibody levels against the seven pneumococcal serotypes were observed with a specific haplotype.

Since significant differences within IL-4 and IL-4 RA haplotypes were observed for antibodies against pneumococcal serotype 4, we investigated combinations of these two haplotypes (figure 1). An additive effect was observed, with significantly higher antibody levels in carriers of both high responder haplotypes compared to levels in carriers of both low responder haplotypes (p=0.004).

Table 4 Geometric mean IgG antibody concentration according to IL-4, IL-4 Rα and IL-13 haplotypes

	IL-4			IL-4 Rα			IL-13		
	C–589T * G2979T			Ile50Val * Gln551Arg			A–1112G * Arg130Gln		
	C*G	T*T	p	Ile*Arg	Val*Gln	p	G*Arg	A*Gln	p
N	185	33		15	82		185	27	
6B	1.3	1.0	0.4	1.5	1.2	0.5	1.2	1.0	0.5
4	4.1	2.9	0.04	7.9	3.2	< 0.001	4.0	3.5	0.8
23F	4.4	2.9	0.05	4.0	4.6	0.6	4.2	4.5	0.7
18C	9.1	8.1	0.5	14.4	8.1	0.03	9.1	8.0	0.5
19F	10.5	10.3	0.9	12.9	10.3	0.6	10.8	13.0	0.5
9V	25.3	20.2	0.4	32.3	25.5	0.5	25.1	22.0	0.6
14	71.4	66.0	0.8	127.4	66.1	0.07	69.7	84.4	0.5

Note. P-values are determined using pairwise comparisons in a general linear model.

Discussion

This study demonstrates associations of SNPs and haplotypes in IL-4 and IL-4 RA with pneumococcal antibody responses. The IL-4 –589T, IL-4 2979T and IL-4 RA 551Gln alleles were associated with lower pneumococcal antibody levels. Additionally, haplotypes and haplotype combinations of IL-4 and IL-4 RA, composed of alleles associated with decreased antibody responses in single locus analyses, were associated with lower vaccination responses compared to these single locus analyses. Thus, for the first time, additive genetic effects of combinations of haplotypes on antibody response to vaccination are shown.

Several lines of evidence suggest that the IL-4 –589T allele may be associated with dys-regulated immune responses. The IL-4 –589T allele has previously been associated with increased IgE levels,[34-36] an increased frequency and rate of progression of disease in HIV infected patients[37] and increased susceptibility to Kawasaki's disease.[38] Furthermore, it was shown that amongst children with severe malaria, total IgE levels were significantly elevated in those carrying the IL-4 –589T allele, suggesting the possibility that there is a relationship between susceptibility to severe malaria, IgE production and genetic variation in IL-4.[39]

IL-4 induces B-cell activation and modifies humoral B-cell responses to both T-cell dependent and T-cell independent stimuli.[40] The IL-4 –589 T allele enhances IL-4 transcription in vitro.[41] Therefore, prima facie, the T allele might be expected to be associated with increased humoral responses to vaccines. However, Vos and colleagues showed T-cell independent hu-

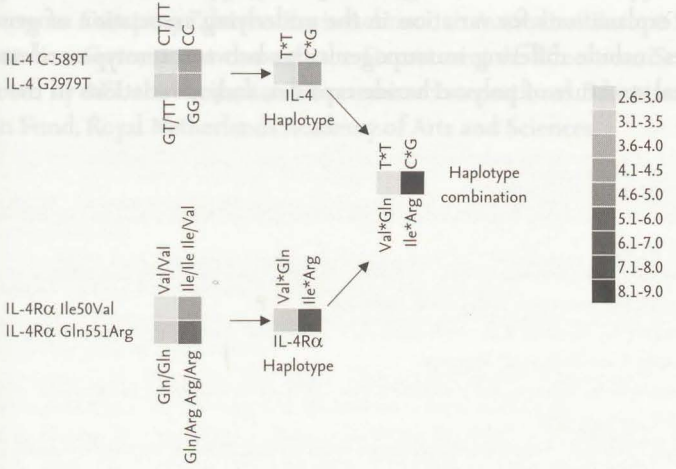


Figure 1 Schematic overview of geometric mean antibody level against pneumococcal serotype 4 according to IL-4 and IL-4Rα genotype, haplotype and haplotype combination.
An additive effect of haplotype and haplotypes combined on antibody titers is shown.

moral responses to be enhanced by short-term exposure to IL-4, in the absence of IFNγ, but suppressed by persistent IL-4 exposure.[42] Thus, humoral responses may be dependent upon the cytokine microenvironment and the length of exposure to this microenvironment. Therefore, under certain conditions an IL-4 allele associated with increased IL-4 transcription might be associated with decreased humoral responses. These effects may be further modified by the coexistence of genetically mediated alterations of IL-4 Rα function. Furthermore a gene-environment interaction between day-care attendance in the first 6 months of life and the IL-4 RA Ile50Val locus on lipopolysaccharide induced IFNγ production has been shown.[43] Therefore, relationships between individual alleles or haplotypes of those genes and vaccination responses may be further dependent on microenvironmental, macroenvironmental and developmental influences.

As for the IL-4 –589T allele, the IL-4 2979T allele has been associated with asthma.[44] However, no functional work on this SNP has been reported. Functional data on the IL-4 RA Arg551Gln SNP is conflicting.[45,46] However, the 551Gln allele has been associated with asthma and atopic phenotypes.[47-49] Interestingly, the IL-4 and IL-4 RA alleles that have been associated with atopy show a diminished antibody response in our study. Furthermore, atopic eczema has been associated with delayed maturation of the antibody response to pneumococcal vaccine.[50] This might suggest a relationship between atopy, genotype and the responses upon pneumococcal vaccination, possibly brought about by an altered cytokine milieu secondary to an altered Th1/Th2 balance.

The antibody response against pneumococcal serotype 4 was most often associated with polymorphic genotypes and haplotypes. Significant associations with genotype or haplotype were not demonstrated for antibody responses to all serotypes. Potential reasons for lack of consistent replication across all serotypes in this study include: true variation in the underlying association between genotype and outcome (effect heterogeneity), type 1 error or lack of power.[51] Possible biological explanations for variation in the underlying association of genotype with individual serotypes include differing immunogenicities between serotypes, related to variations in the chemical structure of polysaccharide capsules, and/or variations in the magnitude

of polysaccharide specific response dependent on the prevalence of specific serotypes, and thus the presence or absence of natural priming. We would expect the effect of genotype to be most apparent in antibody responses against serotypes of intermediate immunogenicity and/or prevalence. It is possible that the effect of the studied SNPs might be insufficient to significantly bolster IgG antibody responses against poorly immunogenic and/or low prevalent serotypes. Conversely, with high immunogenic and/or high prevalent serotypes the relative contribution of genetic variation may be small.

Studying haplotypes of multiple polymorphisms in one gene maybe more informative than analysis of single SNPs in genetic association studies of complex diseases.[52] The importance of investigating gene-gene interactions in complex biological pathways has also been demonstrated.[49] The additive effect of haplotype and haplotype-haplotype combination analyses, compared to single locus and single haplotype analyses respectively, reported in this study supports the approach of studying the effect of combinations of multiple loci, both within and between genes, on complex phenotypes. Furthermore, this study highlights the importance of host genetic factors in modulating responses to vaccination.

Further studies are required to investigate the role of variation in immunomodulatory genes in vaccine response, within and across populations, to this and other vaccines. Understanding of these genetic influences is likely to be important for the development of improved and novel vaccines.

Acknowledgements

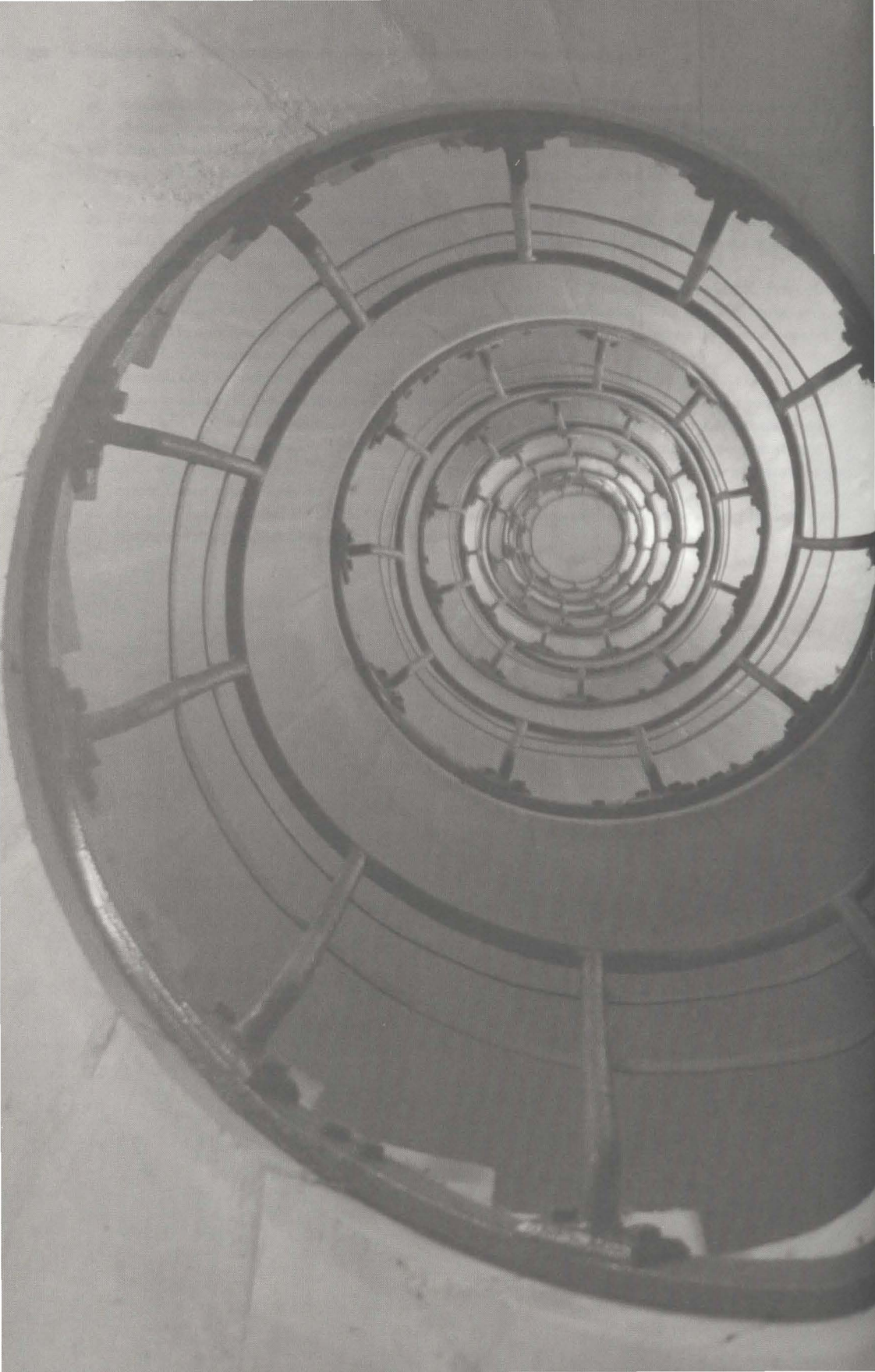
The work described in this paper was financially supported by the Netherlands Organization for Health Research and Development ZonMW (grant numbers 002828480 and 90461092), the Health Insurance Company Zilveren Kruis-Achmea, an Australian-Europe Scholarship funded by the Australian Government through the Department of Education, Science and Training and promoted by AEI, the Australian Government International Education Network, and the Ter Meulen Fund, Royal Netherlands Academy of Arts and Sciences.

References

1. Luotonen, J.; Herva, E.; Karma, P.; Timonen, M.; Leinonen, M.; Makela, P.H.: The bacteriology of acute otitis media in children with special reference to Streptococcus pneumoniae as studied by bacteriological and antigen detection methods. Scand.J.Infect.Dis. 1981; 13:177-183
2. Fedson, D.S. and Scott, J.A.: The burden of pneumococcal disease among adults in developed and developing countries: what is and is not known. Vaccine 1999; 17 Suppl 1:S11-S18
3. Vitharsson, G.; Jonsdottir, I.; Jonsson, S.; Valdimarsson, H.: Opsonization and antibodies to capsular and cell wall polysaccharides of Streptococcus pneumoniae. J Infect.Dis. 1994; 170:592-599
4. Tuomanen, E.I.; Austrian, R.; Masure, H.R.: Pathogenesis of pneumococcal infection. N.Engl.J.Med. 1995; 332:1280-1284
5. Watson, D.A.; Musher, D.M.; Verhoef, J.: Pneumococcal virulence factors and host immune responses to them. Eur.J.Clin.Microbiol.Infect.Dis. 1995; 14:479-490
6. Harwell, J.I. and Brown, R.B.: The drug-resistant pneumococcus: clinical relevance, therapy, and prevention. Chest 2000; 117:530-541
7. Le Souef, P.N.; Goldblatt, J.; Lynch, N.R.: Evolutionary adaptation of inflammatory immune responses in human beings. Lancet 2000; 356:242-244
8. Poland, G.A. and Jacobson, R.M.: The genetic basis for variation in antibody response to vaccines. Curr.Opin. Pediatr. 1998; 10:208-215
9. Newport, M.J.; Goetghebuer, T.; Weiss, H.A.; Whittle, H.; Siegrist, C.A.; Marchant, A.: Genetic regulation of immune responses to vaccines in early life. Genes Immun. 2004; 5:122-129
10. Kruskall, M.S.; Alper, C.A.; Awdeh, Z.; Yunis, E.J.; Marcus-Bagley, D.: The immune response to hepatitis B vaccine in humans: inheritance patterns in families. J.Exp.Med. 1992; 175:495-502
11. Poland, G.A.; Jacobson, R.M.; Schaid, D.; Moore, S.B.; Jacobsen, S.J.: The association between HLA class I alleles and measles vaccine-induced antibody response: evidence of a significant association. Vaccine 1998; 16:1869-1871
12. Allansmith, M.; McClellan, B.; Butterworth, M.: The influence of heredity and environment on human immunoglobulin levels. J.Immunol. 1969; 102:1504-1510
13. Pandey, J.P.; Zollinger, W.D.; Fudenberg, H.H.; Loadholt, C.B.: Immunoglobulin allotypes and immune response to meningococcal group B polysaccharide. J.Clin.Invest 1981; 68:1378-1380
14. Shirakawa, I.; Deichmann, K.A.; Izuhara, I.; Mao, I.; Adra, C.N.; Hopkin, J.M.: Atopy and asthma: genetic variants of IL-4 and IL-13 signalling. Immunol.Today 2000; 21:60-64
15. Punnonen, J.; Yssel, H.; de Vries, J.E.: The relative contribution of IL-4 and IL-13 to human IgE synthesis induced by activated CD4+ or CD8+ T cells. J.Allergy Clin.Immunol. 1997; 100:792-801
16. Kabesch, M.; Tzotcheva, I.; Carr, D.; Hofler, C.; Weiland, S.K.; Fritzsch, C.; von Mutius, E.; Martinez, F.D.: A complete screening of the IL4 gene: novel polymorphisms and their association with asthma and IgE in childhood. J.Allergy Clin.Immunol. 2003; 112:893-898
17. Izuhara, K. and Shirakawa, T.: Signal transduction via the interleukin-4 receptor and its correlation with atopy. Int.J.Mol.Med. 1999; 3:3-10
18. Shirakawa, I.; Deichmann, K.A.; Izuhara, I.; Mao, I.; Adra, C.N.; Hopkin, J.M.: Atopy and asthma: genetic variants of IL-4 and IL-13 signalling. Immunol.Today 2000; 21:60-64
19. McKenzie, G.J.; Bancroft, A.; Grencis, R.K.; McKenzie, A.N.: A distinct role for interleukin-13 in Th2-cell-mediated immune responses. Curr.Biol. 1998; 8:339-342
20. Bancroft, A.J.; McKenzie, A.N.; Grencis, R.K.: A critical role for IL-13 in resistance to intestinal nematode infection. J.Immunol. 1998; 160:3453-3461
21. Murata, T.; Obiri, N.I.; Puri, R.K.: Structure of and signal transduction through interleukin-4 and interleukin-13 receptors (review). Int.J.Mol.Med. 1998; 1:551-557
22. LeSouef, P.: Genetics of asthma: what do we need to know? Pediatr.Pulmonol.Suppl 1997; 15:3-8
23. Halapi, E. and Hakonarson, H.: Recent development in genomic and proteomic research for asthma. Curr.Opin. Pulm.Med. 2004; 10:22-30
24. Hoffjan, S. and Ober, C.: Present status on the genetic studies of asthma. Curr.Opin.Immunol. 2002; 14:709-717

25. Veenhoven, R.; Bogaert, D.; Uiterwaal, C.; Brouwer, C.; Kiezebrink, H.; Bruin, J.; IJzerman, E.; Hermans, P.; de Groot, R.; Zegers, B.; Kuis, W.; Rijkers, G.; Schilder, A.; Sanders, E.: Effect of conjugate pneumococcal vaccine followed by polysaccharide pneumococcal vaccine on recurrent acute otitis media: a randomised study. Lancet 2003; 361:2189-2195
26. Straetemans, M.; van Heerbeek, N.; Tonnaer, E.; Ingels, K.J.; Rijkers, G.T.; Zielhuis, G.A.: A comprehensive model for the aetiology of otitis media with effusion. Med.Hypotheses 2001; 57:784-791
27. Wiertsema, S.P.; Sanders, E.A.; Veenhoven, R.H.; van Heerbeek, N.; van Den, H.S.; Berbers, G.A.; Rijkers, G.T.: Antibody levels after regular childhood vaccinations in the immunological screening of children with recurrent otitis media. J.Clin.Immunol. 2004; 24:354-360
28. Oefner, P. and Underhill, P.: DNA mutation detection used denaturating high-performance liquid chromatography (DHPLC).2005; 7.10.1-7.10.12
29. Stephens, M. and Donnelly, P.: A comparison of bayesian methods for haplotype reconstruction from population genotype data. Am.J.Hum.Genet. 2003; 73:1162-1169
30. Stephens, M.; Smith, N.J.; Donnelly, P.: A new statistical method for haplotype reconstruction from population data. Am.J.Hum.Genet. 2001; 68:978-989
31. Sanders, L.A.; Rijkers, G.T.; Tenbergen-Meekes, A.M.; Voorhorst-Ogink, M.M.; Zegers, B.J.: Immunoglobulin isotype-specific antibody responses to pneumococcal polysaccharide vaccine in patients with recurrent bacterial respiratory tract infections. Pediatr.Res. 1995; 37:812-819
32. Musher, D.M.; Luchi, M.J.; Watson, D.A.; Hamilton, R.; Baughn, R.E.: Pneumococcal polysaccharide vaccine in young adults and older bronchitics: determination of IgG responses by ELISA and the effect of adsorption of serum with non-type-specific cell wall polysaccharide. J.Infect.Dis. 1990; 161:728-735
33. Quataert, S.A.; Kirch, C.S.; Wiedl, L.J.; Phipps, D.C.; Strohmeyer, S.; Cimino, C.O.; Skuse, J.; Madore, D.V.: Assignment of weight-based antibody units to a human antipneumococcal standard reference serum, lot 89-S. Clin. Diagn.Lab Immunol. 1995; 2:590-597
34. Rosenwasser, L.J.; Klemm, D.J.; Dresback, J.K.; Inamura, H.; Mascali, J.J.; Klinnert, M.; Borish, L.: Promoter polymorphisms in the chromosome 5 gene cluster in asthma and atopy. Clin.Exp.Allergy 1995; 25 Suppl 2:74-78
35. Rosenwasser, L.J. and Borish, L.: Genetics of atopy and asthma: the rationale behind promoter-based candidate gene studies (IL-4 and IL-10). Am.J.Respir.Crit Care Med. 1997; 156:S152-S155
36. Noguchi, E.; Shibasaki, M.; Arinami, T.; Takeda, K.; Maki, T.; Miyamoto, T.; Kawashima, T.; Kobayashi, K.; Hamaguchi, H.: Evidence for linkage between asthma/atopy in childhood and chromosome 5q31-q33 in a Japanese population. Am.J.Respir.Crit Care Med. 1997; 156:1390-1393
37. Smolnikova, M.V. and Konenkov, V.I.: Association of IL2, TNFA, IL4 and IL10 Promoter Gene Polymorphisms with the Rate of Progression of the HIV Infection. Russ.J.Immunol. 2002; 7:349-356
38. Burns, J.C.; Shimizu, C.; Shike, H.; Newburger, J.W.; Sundel, R.P.; Baker, A.L.; Matsubara, T.; Ishikawa, Y.; Brophy, V.A.; Cheng, S.; Grow, M.A.; Steiner, L.L.; Kono, N.; Cantor, R.M.: Family-based association analysis implicates IL-4 in susceptibility to Kawasaki disease. Genes Immun. 2005;
39. Verra, F.; Luoni, G.; Calissano, C.; Troye-Blomberg, M.; Perlmann, P.; Perlmann, H.; Arca, B.; Sirima, B.S.; Konate, A.; Coluzzi, M.; Kwiatkowski, D.; Modiano, D.: IL4-589C/T polymorphism and IgE levels in severe malaria. Acta Trop. 2004; 90:205-209
40. Jordan, M.B.; Mills, D.M.; Kappler, J.; Marrack, P.; Cambier, J.C.: Promotion of B cell immune responses via an alum-induced myeloid cell population. Science 2004; 304:1808-1810
41. Rosenwasser, L.J.: Structure/function variants as candidate genes in asthma: linkage vs. association for relevance. Clin.Exp.Allergy 1998; 28 Suppl 1:90-92
42. Vos, Q.; Snapper, C.M.; Mond, J.J.: T(h)1 versus T(h)2 cytokine profile determines the modulation of in vitro T cell-independent type 2 responses by IL-4. Int.Immunol. 2000; 12:1337-1345
43. Hoffjan, S.; Nicolae, D.; Ostrovnaya, I.; Roberg, K.; Evans, M.; Mirel, D.B.; Steiner, L.; Walker, K.; Shult, P.; Gangnon, R.E.; Gern, J.E.; Martinez, F.D.; Lemanske, R.F.; Ober, C.: Gene-environment interaction effects on the development of immune responses in the 1st year of life. Am.J.Hum.Genet. 2005; 76:696-704
44. Basehore, M.J.; Howard, T.D.; Lange, L.A.; Moore, W.C.; Hawkins, G.A.; Marshik, P.L.; Harkins, M.S.; Meyers, D.A.; Bleecker, E.R.: A comprehensive evaluation of IL4 variants in ethnically diverse populations: association of total serum IgE levels and asthma in white subjects. J.Allergy Clin.Immunol. 2004; 114:80-87

45. Hershey, G.K.; Friedrich, M.F.; Esswein, L.A.; Thomas, M.L.; Chatila, T.A.: The association of atopy with a gain-of-function mutation in the alpha subunit of the interleukin-4 receptor. N.Engl.J.Med. 1997; 337:1720-1725
46. Wang, H.Y.; Shelburne, C.P.; Zamorano, J.; Kelly, A.E.; Ryan, J.J.; Keegan, A.D.: Cutting edge: effects of an allergy-associated mutation in the human IL-4R alpha (Q576R) on human IL-4-induced signal transduction. J.Immunol. 1999; 162:4385-4389
47. Kruse, S.; Japha, T.; Tedner, M.; Sparholt, S.H.; Forster, J.; Kuehr, J.; Deichmann, K.A.: The polymorphisms S503P and Q576R in the interleukin-4 receptor alpha gene are associated with atopy and influence the signal transduction. Immunology 1999; 96:365-371
48. Ober, C.; Leavitt, S.A.; Tsalenko, A.; Howard, T.D.; Hoki, D.M.; Daniel, R.; Newman, D.L.; Wu, X.; Parry, R.; Lester, L.A.; Solway, J.; Blumenthal, M.; King, R.A.; Xu, J.; Meyers, D.A.; Bleecker, E.R.; Cox, N.J.: Variation in the interleukin 4-receptor alpha gene confers susceptibility to asthma and atopy in ethnically diverse populations. Am.J.Hum.Genet. 2000; 66:517-526
49. Howard, T.D.; Koppelman, G.H.; Xu, J.; Zheng, S.L.; Postma, D.S.; Meyers, D.A.; Bleecker, E.R.: Gene-gene interaction in asthma: IL4RA and IL13 in a Dutch population with asthma. Am.J.Hum.Genet. 2002; 70:230-236
50. Arkwright, P.D.; Patel, L.; Moran, A.; Haeney, M.R.; Ewing, C.I.; David, T.J.: Atopic eczema is associated with delayed maturation of the antibody response to pneumococcal vaccine. Clin.Exp.Immunol. 2000; 122:16-19
51. Colhoun, H.M.; McKeigue, P.M.; Davey, S.G.: Problems of reporting genetic associations with complex outcomes. Lancet 2003; 361:865-872
52. Lee, J.E.; Choi, J.H.; Lee, J.H.; Lee, M.G.: Gene SNPs and mutations in clinical genetic testing: haplotype-based testing and analysis. Mutat.Res. 2005; 573:195-204



8

Immunological status in the aetiology of recurrent otitis media with effusion: serum immunoglobulin levels, functional mannose- binding lectin and Fc receptor polymorphisms for IgG

Masja Straetemans[1], Selma P. Wiertsema[2], Elisabeth A.M. Sanders[2], Ger T. Rijkers[2], Kees Graamans[3], Bert van der Baan[4], Gerhard A. Zielhuis[1]

1 Department of Epidemiology and Biostatistics, University Medical Centre Nijmegen, the Netherlands
2 Department of Paediatric Immunology, University Medical Center Utrecht, the Netherlands
3 Department of Otorhinolaryngology, University Medical Centre Nijmegen, the Netherlands
4 Departments of Otorhinolaryngology, Hospital Gooi Noord, Blaricum, the Netherlands

J Clin Immunol. 2005 Jan;25(1): 78-86.

Summary

The objective was to study the role of serum immunoglobulin levels, mannose-binding lectin and Fc gamma receptor (FcγR) polymorphisms on the development of recurrent otitis media with effusion (OME). Children aged between two and seven years with persisting OME received bilateral tympanostomy tubes and immunological parameters were investigated in relation with OME recurrence within six months after tube extrusion. No statistically significant differences in serum immunoglobulin levels were present between children with and without OME recurrence. In children with bilateral recurrence (n=56), median levels of mannose-binding lectin were 1.39 mg/l compared to 2.48 mg/l in children with OME recurrence (n=17) (p value 0.29). In addition, 34% of the children with bilateral recurrence were homozygous for the genotype FcγRIIa-R/R131, whereas less than 20% of the children with unilateral recurrence or those without recurrence were homozygous for this Fcγ receptor (p value 0.26).
Serum mannose-binding lectin and FcγRIIa-R/R131 polymorphism may play a role in the aetio-pathogenesis of recurrent OME.

Introduction

Otitis media with effusion (OME) is a highly prevalent ear disorder in young children; at least 80% of children experience one or more episodes of OME by the age of four years.[1,2] OME is characterised by a high rate of spontaneous recovery, but also by a high rate of recurrence.[3] As adverse effects of OME mainly occur in the group of children with a history of recurrent or chronic OME, it is necessary to distinguish these children from the total group of OME children early in the course of the disease to focus intervention measures such as tympanostomy tubes on this particular group.[4-8] Predisposing factors for the recurrence of OME are believed to be related to its aetiology. OME is a multifactorial-generated condition in which the inflammatory response to respiratory pathogens, both bacterial and viral, seems to be a crucial element.[9] In order to distinguish children with transient OME from those with recurrent OME, we studied three aspects of the immune response to respiratory tract pathogens in a group of children with chronic OME. These children were referred for bilateral tympanostomy tube insertion. Serum immunoglobulin levels, functional mannose-binding lectin serum levels (MBL) and Fcγ receptor polymorphisms were assessed.

Low serum immunoglobulin levels, despite infections or chronic inflammatory processes, may be indicative for a subtle immunodeficiency.[10,11] This phenomenon was the basis of our first hypothesis: children with low IgA, IgG (and subclasses) or IgM levels early in the course of the disease are more likely to develop recurrent OME.[12-14]

Independent of antibodies, MBL is able to initiate the complement pathway by directly opsonizing pathogens by binding to specific oligosaccharides.[15,16] Increased susceptibility to bacterial and viral respiratory infections in early childhood is associated with low or absent serum MBL levels.[17,18] This phenomenon led us to the second hypothesis: children who have lower functional MBL serum levels early in the course of OME are more likely to develop recurrent disease.

Leukocyte antibody receptors for IgG (FcγR) play an important role in IgG-facilitated phagocytosis of bacteria. Genetically determined functional polymorphisms for three classes of human FcγR have been described.[19,20] In FcγRIIA, the bi-allelic polymorphism consists of the presence of either Arginine (R) or Histidine (H) at position 131. Only the H131-allotype is capable of binding IgG2 opsonized bacteria, such as *Streptococcus pneumoniae*. In FcγRIIIA, the allotype with Phenylalanine (F) at position 158 has been shown to bind complexed IgG1, IgG3 and IgG4 less avidly than the Valine (V) allotype. FcγRIIIb bears the neutrophil antigen (NA) polymorphism. NA1 homozygotes have a higher phagocytotic capacity for IgG1 and IgG3 opsonized bacteria than NA2 homozygotes. Recently, it has become evident that some of these genetically determined functional polymorphisms may contribute to susceptibility to infections.[21-25] Thus our third hypothesis was: children with recurrent OME are more likely to be homozygous for the Fcγ receptors with the lower binding affinity for IgG subclasses IgG1, IgG2 and IgG3 (FcγRIIa-R/R131) and IgG1 and IgG3 (FcγRIIIb-NA2/NA2).

To test these three hypotheses, we conducted a cohort study on a group of children with their first clinical episode of OME. Follow-up of these children enabled us to study the role of immunological status on the subsequent probability of developing recurrent OME.

Methods

Patients and study design

In the Netherlands, health insurance companies require formal referral by a general practitioner (GP) before refunding specialist care. Therefore, nearly all patients with OME are initially seen by their GP. According to the guidelines of the Dutch College of General Practitioners, children with chronic OME should only be referred to an otologist after repeated observations of middle ear effusion over a period of at least three months. Children were eligible for the study if they were aged between two and seven years, their first clinical episode of bilateral OME had persisted for at least three months as documented by their GP and they had been referred for the first time to the Departments of Otorhinolaryngology of one of the three participating hospitals in Nijmegen or Winterswijk (the Netherlands) between December 1999 and March 2002. Children with Down's syndrome, cleft palate, or daily treatment with inhalation or topical corticosteroids for at least one month per year were excluded, as were children with proven immunodeficiency or previous adenoidectomy. The Medical Ethical Committees of the participating hospitals approved the study protocol. Signed informed consent was obtained from the parents or legal guardians. At study entry, all the children received bilateral tympanostomy tubes for OME under general anaesthesia. Blood samples were collected during this surgical intervention. Serum and DNA were isolated and stored at -20°C until required for analysis. To monitor OME recurrence, an otologist examined the ear status of each child once every three months until six months after documentation of spontaneous tube extrusion. The date of tube extrusion was taken as the date of the first check-up during which extrusion had been observed (per ear).

Definition of OME recurrence

OME was defined in accordance with an algorithm, which is primarily based on tympanometry.[26] Tympanograms were classified in accordance with Jerger.[27] OME was considered to be present when tympanometry resulted in a type B tympanogram or a type C2 tympanogram with otoscopic findings that suggested the presence of effusion in the middle ear and the absence of an acute ear infection.[26] If tympanometry (Rexton Danplex TYMP 87 A/S Copenhagen, Denmark) could not be performed, otoscopic findings that suggested effusion in the middle ear were considered to diagnose OME. Children were categorised into those who developed bilateral OME (bil_rOME), children with unilateral OME recurrence (uni_rOME) and children who did not develop OME in the follow-up period (no_rOME).

Immunological tests

Total serum immunoglobulin concentrations of IgA, IgM and IgG as well as IgG1, IgG2, IgG3 and IgG4 subclass concentrations were determined by radial immunodiffusion (Behring Werke, Mannheim, Germany and Central Laboratory of the Red Cross Blood Transfusion Service, Amsterdam, the Netherlands).[28] To determine functional mannose-binding lectin levels in serum, the assay described by Kuipers *et al.* was used.[29] This assay is based on the principle of yeast-induced bystander lysis of chicken erythrocytes. In short, serum samples were diluted and in-

cubated with a standardised amount of freshly cultured baker's yeast (*S. cerevisiae*) to activate the MBL pathway. Chicken erythrocytes were used as the target for haemolysis due to MBL pathway activation. Haemoglobin release was measured in an ELISA reader at 405 nm. Percentages of haemolysis were calculated using controls for 100% (water lysed) and 0% (buffer control) haemolysis. Genomic DNA was extracted from whole blood using a QIAamp DNA Blood Kit (Qiagen, Westburg, the Netherlands). FcγRIIa and FcγRIIIb genotypes were determined by means of PCR amplification methods as described previously.[30]

Statistical analyses

As most of the immunoglobulin and MBL levels were not normally distributed, non-parametric Kruskal Wallis tests were performed to compare distributions of immunoglobulins and MBL levels between OME groups. To study the possible confounding effect of age, gender and day care attendance, stratified analyses were performed as these parameters are likely to be associated with immune status and constitute risk factors for the recurrence of OME.[32] The distributions (of combinations) of Fcγ-receptor polymorphisms in OME groups were compared using Fisher's exact test.

All analyses were performed with the Statistical Analysis Systems (SAS version 8.0).

Results

Study population

A total of 186 children met the inclusion criteria; 136 of them (73%) participated in the study. The median age of the children at study entry was 5.3 years (range, 2.1 to 7.5 years). Owing to mostly practical problems during the study period 15 children were lost to follow-up. In the 90 children with complete follow-up data at the end of the study (August 2003), OME recurrence was high: 56 children (62%) developed bilateral OME, 17 children (19%) developed unilateral OME and only 17 children (19%) did not develop OME. The remaining 31 children were not included in the analyses because of unkown clinical outcome due to incomplete follow-up in either 1 or 2 ears at the predetermined end of the study.

The groups were comparable except for age, gender and exposure to smoking at home (Table 1). The group of children without OME recurrence contained fewer boys and fewer children had been exposed to smoking at home. Children who developed bilateral OME were slightly younger, both at study entry and when extrusion of the last tube was documented. Tympanostomy tubes appeared to be extruded a median of 9 months after insertion. The documentation date of tube extrusion ranged from 3 to 27 months (Table 1). Due to missing serum samples, immunological data were not always complete in the 90 children who completed follow-up.

Immunoglobulins and IgG subclasses

Median concentrations of all immunoglobulins except for IgM were similar in the no_rOME group, the uni_rOME group and the bil_rOME group (figure 1, Table 2). After stratification by gender, the differences in IgM levels between the uni_rOME and bil_rOME became smaller,

Table 1 Characteristics of the study population

	No_rOME	Uni_rOME	Bil_rOME
N	17	17	56
Male gender	35%	59%	54%
Median age (range) at study entry	5.4 (2.7-7.2)	5.7 (2.4-7.5)	5.0 (2.8-7.2)
Median age (range) at start of follow-up	6.2 (3.6-9.1)	6.9 (3.4-8.9)	6.0 (3.5-8.3)
Children aged between 2-4 years at study entry	41%	29%	48%
Children aged between 5-7 years at study entry	59%	71%	52%
Attending day care at study entry	18%	24%	18%
Children aged 2-4 years and attending day care at study entry	12%	18%	18%
Siblings present	94%	94%	93%
Exposure to smoking at home	18%	35%	36%
Father born in the Netherlands	94%	100%	93%
Mother born in the Netherlands	94%	95%	88%
Median check-up (range) tube extrusion:			
From left ear	9 (3-27)	12 (3-27)	9 (3-27)
From right ear	9 (3-24)	9 (6-18)	9 (3-21)

while the differences with the no_rOME group remained the same. Adjustment for age and day care attendance did not affect the overall results. However, IgM levels and all the other immunoglobulin levels were within the normal range of age-matched healthy children (Table 2).

Mannose-binding lectin

Children in the bil_rOME group tended to have lower functional MBL serum levels at baseline (median 1.38 mg/l) compared to the children in the no_rOME group (median 2.48 mg/l) and the uni_rOME group (median 1.88 mg/l) (Kruskal Wallis p = 0.29) (figure 2, Table 2). In the bil_rOME group and the uni_rOME group, about 33% of the children had MBL levels of below 1.0 mg/l, compared to 24% in the no_rOME group (Chi^2 p=0.48). Stratified analyses for age, gender and day care attendance showed the same, but non-significant, trend towards lower MBL levels in the bil_rOME group. The largest difference in MBL between the bil_rOME group and the other two groups was seen in the youngest age category (p value 0.09, data not shown).

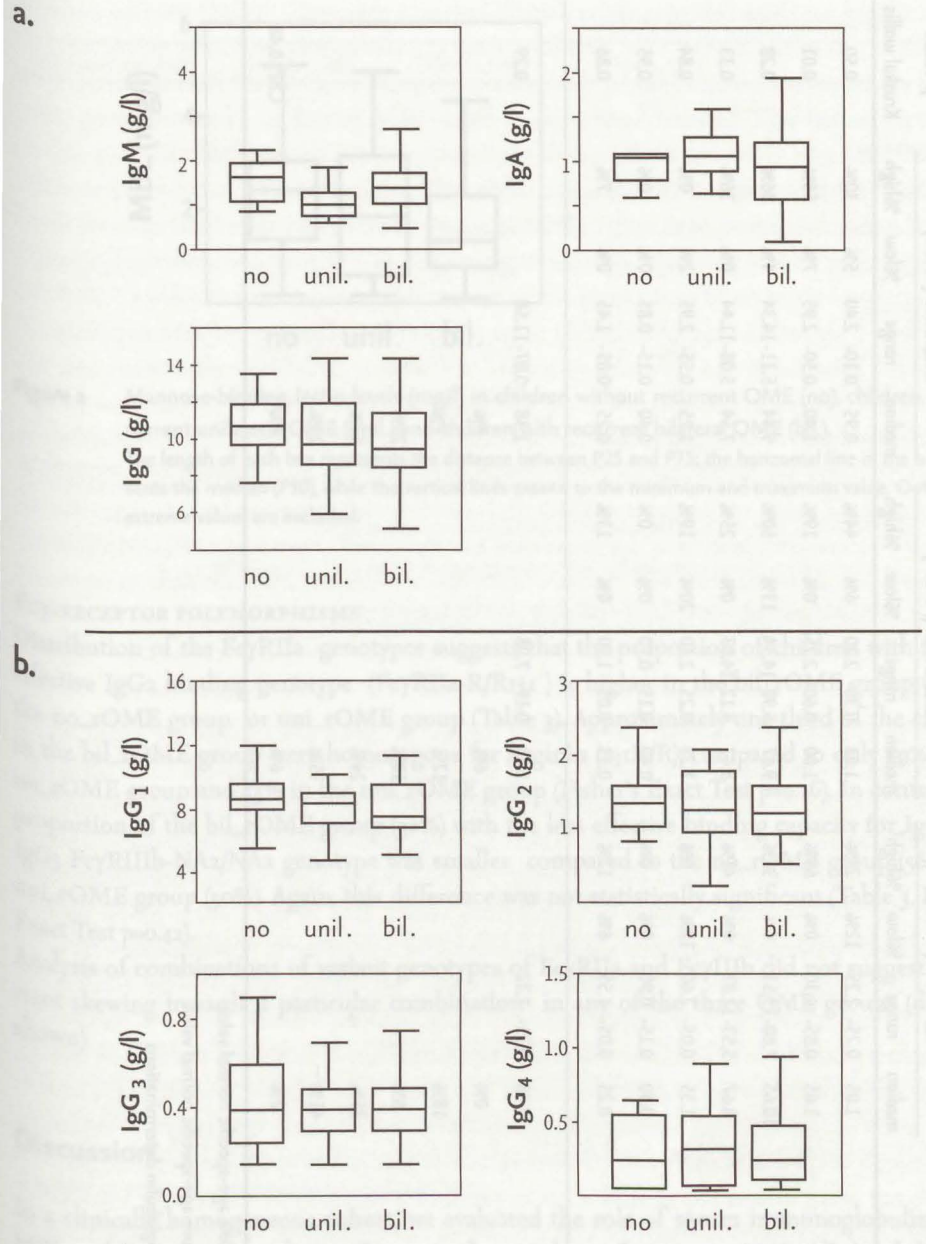


Figure 1 (a) Serum immunoglobulin levels (g/l) in children without recurrent OME (no), children with recurrent unilateral OME (unil.) and children with recurrent bilateral OME (bil.).

(b) IgG subclass levels (g/l) in children without recurrent OME (no), children with recurrent unilateral OME (unil.) and children with recurrent bilateral OME (bil.).

The length of each box represents the distance between P25 and P75; the horizontal line in the box represents the median (P50), while the vertical lines extend to the minimum and maximum value. Outliers and extreme values are excluded.

Table 2 Serum immunoglobulin and mannose-binding lectin levels in each of the OME groups

	No_rOME (N=17)				Uni_rOME (N=17‡)				Bil_rome (N=56)				p value
Ig level (g/L)	median	range	%low*	%high†	median	range	%low	%high	median	range	%low	%high	Kruskal wallis
IgA	1.05	0.25- 2.25	12%	24%	1.08	0.30- 2.20	6%	44%	0.95	0.10- 2.40	5%	30%	0.50
IgM	1.65	0.85- 4.30	0%	65%	1.00	0.60- 2.25	0%	19%	1.40	0.50- 2.95	7%	55%	0.01
IgG	10.65	7.60-13.51	6%	35%	9.67	5.90-14.34	13%	50%	9.44	5.11-14.34	4%	36%	0.28
IgG_1	8.67	5.53-12.87	0%	41%	8.47	4.12-14.03	0%	25%	7.54	5.08-11.44	0%	36%	0.33
IgG_2	1.35	0.05- 2.60	12%	0%	1.28	0.25- 2.10	20%	19%	1.25	0.55- 2.95	2%	0%	0.84
IgG_3	0.40	0.15- 0.90	0%	0%	0.40	0.15- 0.70	0%	0%	0.40	0.15- 0.85	0%	0%	0.95
IgG_4	0.25	0.05- 1.55	6%	12%	0.33	0.05- 1.90	0%	13%	0.25	0.05- 1.45	0%	7%	0.86
MBL-level (mg/L)	2.48	0.21- 7.32			1.88	0.19- 7.98			1.38	0.07-11.58			0.29
<0.20	0%				6%				7%				
0.20 -0.42	18%				12%				7%				
0.42 -1.0	6%				18%				20%				
1.0 - 2.5	35%				24%				43%				
2.5 - 7.5	41%				35%				20%				
>7.5	0%				6%				4%				Chi^2 0.48

Notes. * Concentration below age-specific normal value

† Concentration above age-specific normal value

‡ N=16 for immunoglobulin determinations

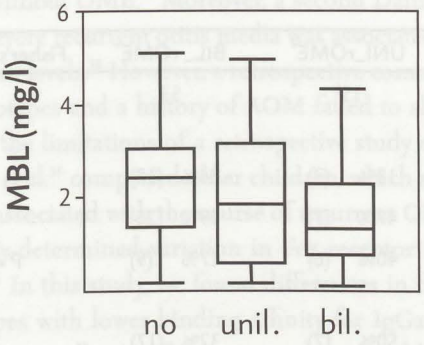


Figure 2 Mannose-binding lectin levels (mg/l) in children without recurrent OME (no), children with recurrent unilateral OME (unil.) and children with recurrent bilateral OME (bil.).
The length of each box represents the distance between P25 and P75; the horizontal line in the box represents the median (P50), while the vertical lines extend to the minimum and maximum value. Outliers and extreme values are excluded.

Fcγ-receptor polymorphisms

Distribution of the FcγRIIa genotypes suggests that the proportion of children with the less effective IgG2 binding genotype (FcγRIIa-R/R131) is higher in the bil_rOME group than in the no_rOME group or uni_rOME group (Table 3). Approximately one third of the children in the bil_rOME group were homozygous for Arginin (131R/R), compared to only 19% in the no_rOME group and 13% in the uni_rOME group (Fisher's Exact Test p=0.26). In contrast, the proportion of the bil_rOME group (32%) with the less effective binding capacity for IgG1 and IgG3 FcγRIIIb-NA2/NA2 genotype was smaller compared to the no_rOME group (56%) and uni_rOME group (50%). Again, this difference was not statistically significant (Table 3, Fisher's Exact Test p=0.42).
Analysis of combinations of variant genotypes of FcγRIIa and FcγIIIb did not suggest significant skewing towards a particular combination in any of the three OME groups (data not shown).

Discussion

In a clinically homogeneous cohort, we evaluated the role of serum immunoglobulin levels, MBL and Fcγ receptor polymorphisms in the aetiology of recurrent OME. All the children in this cohort had had OME for a period of at least three months and had been referred to the otologist for the first time for this indication. No statistically significant differences were found in immunoglobulin levels between the groups. In the majority of children, immunoglobulin levels were in the normal range of age-matched healthy children. The proportion of children with immunoglobulin levels of two standard deviations below the age-specific mean varied from 0% to 20%. In fact, a substantial proportion of children in all the OME groups had IgA,

Table 3 Distribution FcγR-polymorphisms

	NO_rOME*	UNI_rOME*	BIL_ rOME*	Fisher's Exact Test
N	16	15‡	53	
FcγRIIa genotype				
FcγRIIa-R/R131	19% (3)	13% (2)	34% (18)	
FcγRIIa-R/H131	63% (10)	47% (7)	49% (26)	
FcγRIIa-H/H131	19% (3)	40% (6)	17% (9)	P = 0.26
FcγRIIIb genotype				
FcγRIIIb-NA2/2	56% (9)	50% (7)	32% (17)	
FcγRIIIb-NA1/2	38% (6)	43% (6)	59% (31)	
FcγRIIIb-NA1/1	6% (1)	7% (1)	9% (5)	P = 0.42

Notes. The genotype distribution of FcγRIIa in healthy subjects is R/R131 24%, R/H131 50%, and H/H131 26%; for FcγRIIIb: NA2/2 40%, NA1/2 47%, and NA1/1 13% (data from reference 36)

* In six children, blood samples for extracting genomic DNA were unavailable

‡ One child with unilateral OME lacked the FcγRIIIb-genotype

IgG and IgM levels higher than two standard deviations above the age-specific mean, which has also been demonstrated in a large group of Dutch children with recurrent acute otitis media (AOM).[11] Recently, significant associations (Veenhoven *et al.*)[11] and non-significant associations (Berman *et al.*[33] and Drake-Lee *et al.*[10]) have been reported between otitis media and serum immunoglobulin levels. All three studies found a trend towards lower serum immunoglobulin levels in the children with frequent recurrent otitis media than in the control group without otitis media or only occasional otitis media episodes. Our results are in concordance with these findings. Non-significant lower serum levels of IgA, IgG, IgG1, IgG2, and IgM were measured at baseline in the children who developed recurrent bilateral OME within a period of six months after the first OME episode had been resolved. The consistency of this trend in so many studies, even after adjustment for age and gender, makes it unlikely to be solely due to chance. The lower IgM levels in children with unilateral recurrent otitis reported here may suggest a decreased IgM antibody response in this group. However, in our opinion total serum immunoglobulin levels do not seem to have any clinical value in predicting which child will recover from OME and which child will develop recurrent OME, based on our findings of only small differences in serum immunoglobulin levels.

Deficiencies in classical and alternative complement pathways are rare, but at least 20% of the Caucasian population is found to have decreased or absent MBL levels in association with respiratory tract infections.[17,18] We demonstrated a non-significant trend in functional MBL serum levels towards lower levels at baseline in children who subsequently developed bilateral OME. In a cross-sectional hospital-based study, a similar non-statistically significant, trend was shown towards lower median functional MBL levels in 22 Danish children with OME compared to 15

children without OME.[34] Moreover, a second Danish study provided evidence for the hypothesis that severe recurrent otitis media was associated with MBL variant alleles that result in low MBL serum levels.[18] However, a retrospective community-based study on associations between MBL genotypes and a history of AOM failed to show these trends.[35] This lack of trends may be due to the limitations of a retrospective study design. Both our study population and that of Garred *et al.*[34] comprised older children, which suggests that in older children, MBL serum levels are associated with the course of recurrent OME, as has been postulated earlier.[17]

Genetically-determined variation in Fcγ receptor function may play a role in the recurrence of OME.[25] In this study, we found differences in the proportion of children homozygous for the allotypes with lower binding affinity for IgG2 (FcγRIIa-R/R131). Although this difference was not statistically significant, a trend was visible in line with our hypothesis. Comparable figures were shown for the FcγRIIa-R/R131 genotype in a cross-sectional study on AOM: 31% of the children with frequent recurrent episodes of AOM or sinusitis were homozygous for the R-allotype, compared to 24% of the healthy controls.[25,36] Our results on the FcγRIIIb genotype do not support the hypothesis that children with OME recurrence are more likely to have the FcγRIIIb-NA2/NA2 genotype. Elsewhere, we also reported a normal frequency of the latter genotype in children with recurrent acute otitis media.[25] The proportion of children in our study with the FcγRIIIb-NA1/NA1 genotype was smaller (10%) than the proportions found in children with recurrent AOM or in healthy controls (20%).[25,36,38] Future studies should elucidate whether this difference reflects a characteristic of OME children or is merely due to chance.

This is the first prospective study in which baseline immunological parameters were evaluated in relation with the subsequent risk of OME recurrence in a homogeneous population of children treated for their first clinical episode of bilateral OME. By using this cohort design, we were able to exclude selection bias and avoid discussions about what is cause and what is effect. Our study results are based on 90 children with known clinical outcome. We feel that this population is representative of the full cohort of children with a history of at least three months of bilateral OME, because the predetermined end-point of the follow-up period and the reasons for loss to follow-up were unrelated to the clinical outcomes. Although the study size is limited and hence statistical significance was not reached, the results are not likely to be merely due to chance, as most of these accord with etiological hypotheses postulated *a priori*.

The various immunological parameters analysed in this study may be connected or act in conjuction. Polymorphisms of FcγR do not influence immunoglobulin levels,[37] nor MBL.[39,40] However, a low level of IgG2 combined with the FcγRIIa-R/R131 genotype or with low MBL does pose an increased risk for bacterial infections.

In conclusion, serum immunoglobulin levels only seemed to play a limited role in the aetiopathogenesis of recurrent OME, while MBL and FcγRIIa genotype may provide interesting clues towards the discovery of mechanisms to explain OME recurrence in children.

Acknowledgements

We thank the parents and the children who took part in this study, the ENT surgeons and staff at the Department of Otorhinolaryngology of the University Medical Centre Nijmegen, the Canisius-Wilhelmina Hospital Nijmegen and the District Hospital Queen Beatrix Winterswijk, P. Aerts and Prof. H. van Dijk from the Centre for Microbiology, Infectious Diseases and Inflammation of the University Medical Centre Utrecht for testing serum samples for MBL, and Sonja van Oosterhout from the Department of Epidemiology and Biostatistics of the University Medical Centre Nijmegen for study management and data entry. This study was supported by a grant from ZonMW, the Netherlands Organisation for Health Research and Development (no. 904 61 092).

References

1. Zielhuis, G.A.; Rach, G.H.; van den, B.P.: The occurrence of otitis media with effusion in Dutch pre-school children. Clin.Otolaryngol.Allied Sci. 1990; 15:147-153
2. Zielhuis, G.A.; Rach, G.H.; van den, B.A.; van den, B.P.: The prevalence of otitis media with effusion: a critical review of the literature. Clin.Otolaryngol.Allied Sci. 1990; 15:283-288
3. Gates, G.A.; Avery, C.A.; Prihoda, T.J.; Cooper, J.C., Jr.: Effectiveness of adenoidectomy and tympanostomy tubes in the treatment of chronic otitis media with effusion. N.Engl.J.Med. 1987; 317:1444-1451
4. Ryding, M.; Konradsson, K.; Kalm, O.; Prellner, K.: Sequelae of recurrent acute otitis media. Ten-year follow-up of a prospectively studied cohort of children. Acta Paediatr. 1997; 86:1208-1213
5. Schilder, A.G.; Snik, A.F.; Straatman, H.; van den, B.P.: The effect of otitis media with effusion at preschool age on some aspects of auditory perception at school age. Ear Hear. 1994; 15:224-231
6. Schilder, A.G.; Zielhuis, G.A.; Haggard, M.P.; van den, B.P.: Long-term effects of otitis media with effusion: otomicroscopic findings. Am.J.Otol. 1995; 16:365-372
7. Zielhuis, G.A.; Gerritsen, A.A.; Gorissen, W.H.; Dekker, L.J.; Rovers, M.M.; van der Wilt, G.J.; Ingels, K.: Hearing deficits at school age; the predictive value of otitis media in infants. Int.J.Pediatr.Otorhinolaryngol. 1998; 44:227-234
8. Daly, K.A.; Hunter, L.L.; Giebink, G.S.: Chronic otitis media with effusion. Pediatr.Rev. 1999; 20:85-93
9. Straetemans, M.; van Heerbeek, N.; Tonnaer, E.; Ingels, K.J.; Rijkers, G.T.; Zielhuis, G.A.: A comprehensive model for the aetiology of otitis media with effusion. Med.Hypotheses 2001; 57:784-791
10. Drake-Lee, A.B.; Hughes, R.G.; Dunn, C.: Serum IgA and IgG functional antibodies and their subclasses to Streptococcus pneumoniae capsular antigen found in two aged-matched cohorts of children with and without otitis media with effusion. Clin.Otolaryngol. 2003; 28:335-340
11. Veenhoven, R.; Rijkers, G.; Schilder, A.; Adelmeijer, J.; Uiterwaal, C.; Kuis, W.; Sanders, E.: Immunoglobulins in Otitis-Prone Children. Pediatr.Res. 2003;
12. Yamanaka, N.; Hotomi, M.; Shimada, J.; Togawa, A.: Immunological deficiency in "otitis-prone" children. Ann. N.Y.Acad.Sci. 1997; 830:70-81.:70-81
13. Prellner, K.; Kalm, O.; Pedersen, F.K.: Pneumococcal antibodies and complement during and after periods of recurrent otitis. Int.J.Pediatr.Otorhinolaryngol. 1984; 7:39-49
14. Freijd, A.; Hammarstrom, L.; Persson, M.A.; Smith, C.I.: Plasma anti-pneumococcal antibody activity of the IgG class and subclasses in otitis prone children. Clin.Exp.Immunol. 1984; 56:233-238
15. Roy, S.; Knox, K.; Segal, S.; Griffiths, D.; Moore, C.E.; Welsh, K.I.; Smarason, A.; Day, N.P.; McPheat, W.L.; Crook, D.W.; Hill, A.V.: MBL genotype and risk of invasive pneumococcal disease: a case-control study. Lancet 2002; 359:1569-1573
16. Sumiya, M.; Super, M.; Tabona, P.; Levinsky, R.J.; Arai, T.; Turner, M.W.; Summerfield, J.A.: Molecular basis of opsonic defect in immunodeficient children. Lancet 1991; 337:1569-1570
17. Koch, A.; Melbye, M.; Sorensen, P.; Homoe, P.; Madsen, H.O.; Molbak, K.; Hansen, C.H.; Andersen, L.H.; Hahn, G.W.; Garred, P.: Acute respiratory tract infections and mannose-binding lectin insufficiency during early childhood. JAMA 2001; 285:1316-1321
18. Garred, P.; Madsen, H.O.; Hofmann, B.; Svejgaard, A.: Increased frequency of homozygosity of abnormal mannan-binding-protein alleles in patients with suspected immunodeficiency. Lancet 1995; 346:941-943
19. van der, P.W. and van de Winkel, J.G.: IgG receptor polymorphisms: risk factors for disease. Immunogenetics 1998; 48:222-232
20. Lehrnbecher, T.; Foster, C.B.; Zhu, S.; Leitman, S.F.; Goldin, L.R.; Huppi, K.; Chanock, S.J.: Variant genotypes of the low-affinity Fcgamma receptors in two control populations and a review of low-affinity Fcgamma receptor polymorphisms in control and disease populations. Blood 1999; 94:4220-4232
21. De Haas, M.: IgG-Fc receptors and the clinical relevance of their polymorphisms. Wien.Klin.Wochenschr. 2001; 113:825-831
22. Platonov, A.E.; Shipulin, G.A.; Vershinina, I.V.; Dankert, J.; van de Winkel, J.G.; Kuijper, E.J.: Association of human Fc gamma RIIa (CD32) polymorphism with susceptibility to and severity of meningococcal disease. Clin. Infect.Dis. 1998; 27:746-750

23. Vedeler, C.A.; Raknes, G.; Myhr, K.M.; Nyland, H.: IgG Fc-receptor polymorphisms in Guillain-Barre syndrome. Neurology 2000; 55:705-707
24. Domingo, P.; Muniz-Diaz, E.; Baraldes, M.A.; Arilla, M.; Barquet, N.; Pericas, R.; Juarez, C.; Madoz, P.; Vazquez, G.: Associations between Fc gamma receptor IIA polymorphisms and the risk and prognosis of meningococcal disease. Am.J.Med. 2002; 112:19-25
25. Sanders, L.A.; van de Winkel, J.G.; Rijkers, G.T.; Voorhorst-Ogink, M.M.; De Haas, M.; Capel, P.J.; Zegers, B.J.: Fc gamma receptor IIa (CD32) heterogeneity in patients with recurrent bacterial respiratory tract infections. J.Infect.Dis. 1994; 170:854-861
26. Engel, J.; Anteunis, L.; Volovics, A.; Hendriks, J.; Marres, E.: Prevalence rates of otitis media with effusion from 0 to 2 years of age: healthy-born versus high-risk-born infants. Int.J.Pediatr.Otorhinolaryngol. 1999; 47:243-251
27. Jerger, J.: Clinical experience with impedance audiometry. Arch.Otolaryngol. 1970; 92:311-324
28. Mancini, G.; Carbonara, A.O.; Heremans, J.F.: Immunochemical quantitation of antigens by single radial immunodiffusion. Immunochemistry. 1965; 2:235-254
29. Kuipers, S.; Aerts, P.C.; Sjoholm, A.G.; Harmsen, T.; van Dijk, H.: A hemolytic assay for the estimation of functional mannose-binding lectin levels in human serum. J.Immunol.Methods 2002; 268:149-157
30. Breij, E.C.; van der Pol, W.L.; van Winsen, L.; Jansen, M.D.; Dijkstra, C.D.; van de Winkel, J.G.; Uitdehaag, B.M.: No association of FcgammaRIIa, FcgammaRIIIa and FcgammaRIIIb polymorphisms with MS. J.Neuroimmunol. 2003; 140:210-215
31. Matsuoka, A.; Shitara, T.; Okamoto, M.; Sano, H.: Immunological aspect of inflammatory middle ear diseases in children: sex differences.1994; 141-145
32. Berman, S.; Lee, B.; Nuss, R.; Roark, R.; Giclas, P.C.: Immunoglobulin G, total and subclass, in children with or without recurrent otitis media. J.Pediatr. 1992; 121:249-251
33. Garred, P.; Brygge, K.; Sorensen, C.H.; Madsen, H.O.; Thiel, S.; Svejgaard, A.: Mannan-binding protein--levels in plasma and upper-airways secretions and frequency of genotypes in children with recurrence of otitis media. Clin.Exp.Immunol. 1993; 94:99-104
34. Homoe, P.; Madsen, H.O.; Sandvej, K.; Koch, A.; Garred, P.: Lack of association between mannose-binding lectin, acute otitis media and early Epstein-Barr virus infection among children in Greenland. Scand.J.Infect.Dis. 1999; 31:363-366
35. van der Pol, W.L.; Jansen, M.D.; Sluiter, W.J.; van de, S.B.; Leppers-van de Straat FG; Kobayashi, T.; Westendorp, R.G.; Huizinga, T.W.; van de Winkel, J.G.: Evidence for non-random distribution of Fcgamma receptor genotype combinations. Immunogenetics 2003; 55:240-246
36. Huizinga, T.W.; Roos, D.; dem Borne, A.E.: Fc-gamma receptors: mediators, targets and markers of disease. Baillieres Clin.Haematol. 1991; 4:889-902
37. van der Pol, W.L.; Huizinga, T.W.; Vidarsson, G.; van der Linden, M.W.; Jansen, M.D.; Keijsers, V.; de Straat, F.G.; Westerdaal, N.A.; De Winkel, J.G.; Westendorp, R.G.: Relevance of Fcgamma receptor and interleukin-10 polymorphisms for meningococcal disease. J.Infect.Dis. 2001; 184:1548-1555
38. Aittoniemi, J.; Koskinen, S.; Laippala, P.; Laine, S.; Miettinen, A.: The significance of IgG subclasses and mannan-binding lectin (MBL) for susceptibility to infection in apparently healthy adults with IgA deficiency. Clin.Exp. Immunol. 1999; 116:505-508
39. Villarreal, J.; Crosdale, D.; Ollier, W.; Hajeer, A.; Thomson, W.; Ordi, J.; Balada, E.; Villardell, M.; Teh, L.S.; Poulton, K.: Mannose binding lectin and FcgammaRIIa (CD32) polymorphism in Spanish systemic lupus erythematosus patients. Rheumatology.(Oxford) 2001; 40:1009-1012

9

Summarizing discussion

Summarizing discussion

In this thesis various aspects of innate and adaptive immunity in children with recurrent acute otitis media (AOM) and persistent otitis media with effusion (OME) were evaluated. The children in the studies all participated in one of two large randomized controlled vaccination trials performed in the Netherlands on the efficacy of combined pneumococcal conjugate and polysaccharide vaccination to prevent recurrence of ear infection. The clinical efficacy results of these vaccination trials have been described elsewhere.[1,2] The children, aged 1-7 years, were all vaccinated with 7-valent pneumococcal conjugate vaccine (Prevnar®, Wyeth Pharmaceuticals) followed by the 23-valent pneumococcal polysaccharide vaccine (Pneumune®, Wyeth Pharmaceuticals). All children had a history of either recurrent acute otitis media or recurrent persisting otitis media with effusion. Despite the fact that vaccinations resulted in the induction of apparently adequate serum IgG antibody levels, these could not prevent recurrence of either AOM or OME. This may be due to replacement of conjugate-vaccine type pneumococci by non-conjugate vaccine serotypes or by other pathogens after vaccination.[3-9] The cause of otitis media however is multi-factorial and other aspects, like genetically determined variation in innate and adaptive immunity, may also contribute to the susceptibility to middle ear infections.
The first two chapters of this thesis have focused on innate immunity pathways in children with recurrent acute otitis media. In **chapter 2** we showed in a cohort of 204 children with a history of 2 or more episodes of AOM in the year before study entry, that single nucleotide polymorphisms (SNPs) in the MBL2 gene resulted in low functional serum MBL levels. Next to the 7 commonly recognized MBL haplotypes, derived from 3 SNPs in the promoter region and 3 exon 1 SNPs, we found that an SNP in exon 4 at position 3130 further determines serum MBL levels within one of the common haplotypes (LXPA). This finding underscores the importance to analyze the functional impact of all the 87 SNPs that have currently been identified across the MBL2 gene,[10] and not just to focus on the 7 common secretor haplotypes. We also found the genetically determined MBL2 polymorphisms to have clinical impact on acute otitis media in those children below 2 years of age. Non-wild type carriers had a history of significantly more AOM episodes compared with matched wild type carriers. We were not able to investigate all the 7 common haplotypes separately for their clinical impact, due to limited numbers of children in the various subgroups. However, in our opinion assessment of MBL genotypes should be extended with SNPs with to be identified functional implications. Since the 3130 SNP is regularly found in Caucasians, it should be one of the first candidates to add to the list of SNPs to be investigated.
The fact that the clinical association with respect to AOM was apparent only in children below 2 years of age and not in older ones is in agreement with the hypothesis that MBL may be of importance in the protection against OM particularly in children between 6 and 24 months of age, when maternally derived antibodies have waned and the adaptive immune response against polysaccharide antigens is not yet fully developed. Whether this is true and what the real impact of low MBL levels on susceptibility to ear infections is, should be investigated in a large prospectively followed birth cohort study, with documentation of infections, environmental factors and for instance the presence of atopy. Also the potential relevance in the development of OM

of other innate immune pathways, like Toll-like receptors (TLR), TLR associated molecules like CD14 and Myd88 and possibly DC-SIGN, are best investigated in such a birth cohort.[11-14]

We described in **chapter 3** that the CD14 C–159T promoter polymorphism, already shown to be associated with increased serum soluble CD14 (sCD14) levels, was associated with a decreased number of AOM episodes in the year prior to the study in children aged between 1-2 years. We also found that in children carrying the T allele, the serum IgG antibody response upon combined pneumococcal conjugate and polysaccharide vaccinations is higher as compared to wild-type C carriers. Preliminary data showed increased IL-6, TNFα and IL-10 cytokine levels in PBMC supernatants of TT homozygous donors. Also after adding recombinant sCD14 to PBMC cultures of CC homozygotes, higher levels of these cytokines and additionally increased levels of IL1α and IL1β were found. This data may indicate that different sCD14 levels could lead to a different cytokine environment in CD14 polymorphic TT and wild-type CC carriers. We therefore propose the following hypothesis to explain the association between the CD14 promoter polymorphism, pneumococcal specific IgG antibody production and the protection against infection in the youngest children: sCD14 might promote presentation of antigens (possibly including polysaccharides) to DCs and macrophages.[15,16] sCD14 might also stabilize and/or facilitate the formation of the CD14/TLR4 complex, with a subsequent increase in signal transduction.[17,18] In both models, higher levels of sCD14, as in carriers of the CD14 –159TT genotype, might result in enhanced cell activation, which augments production and secretion of pro-inflammatory cytokines, as was shown by Cauwels *et al.*[19] This cytokine environment could lead to the enhanced anti-pneumococcal antibody responses we observed after vaccination. An enhanced immune response upon natural exposure to pathogens able to cause AOM might explain the observed lower otitis media frequency in carriers of the TT genotype in the youngest children. As soon as the child has developed a more mature adaptive immune system, this innate immunity pathway has less impact on the infection susceptibility. Future in depth cellular research on the functional impact of this CD14 promoter polymorphism will be required to substantiate these models.

The observed association between a polymorphism in an innate immune pathway and pneumococcal specific IgG antibody responses, stresses the fact that innate pathways control acquired immune responses. This area of research has gained interest over the last years. Somehow the evolutionary success of the innate immune response has not been recognized for a long time, and "mainstream" immunologists only described innate defenses as a primitive measure to hold the fort before the arrival of sophisticated adaptive immune responses. With the discovery of the Toll-like receptors the innate immune system gained interest again, and with it came the realization that the innate immune response not only provides a first line of defense but also is critical for prodding the adaptive immune response into action.

In **chapter 4** we evaluated the adaptive immune response in children with a history of OM, by measuring antibody responses after viral (measles), protein (diphtheria and tetanus), and protein-polysaccharide conjugate (Haemophilus influenzae type B: Hib) vaccinations in children with recurrent AOM or persistent OME. We hypothesized that, apart from impaired antibody responses to polysaccharide vaccines,[20-24] these children might have a more generalized hypo-responsiveness which would be reflected in lower antibody responses to protein antigens, as was

reported in otitis-prone children for the antibody response after rubella vaccination by Prellner *et al.* and for responses against the outer membrane protein P6 of non-typeable Haemophilus influenzae by Hotomi *et al.*[25,26] To this aim antibody levels to 4 vaccinations included in the national immunization program (Rijksvaccinatieprogramma) were measured in children between 2-7 years of age with recurrent AOM or recurrent persisting OME. Antibody levels were compared to titers in 521 healthy age-matched controls without previous middle ear disease. We found no differences in IgG antibody levels after childhood vaccinations between the three groups, and if any, children with AOM showed even higher antibody levels. Therefore, IgG antibody levels upon protein-, polysaccharide-protein conjugate- and viral- vaccinations given in infancy are not indicative for otitis-proneness. Consequently, these antibody titers should not be used as a diagnostic tool for the immunological assessment of children presenting with OM. However, we did not evaluate antibody responses upon recent (booster) vaccinations. Possibly, antibody responses measured 4 weeks after administration of a protein or viral vaccine might be more informative. This however remains to be shown, especially since we and others already showed that patients with recurrent acute otitis media or other upper respiratory tract infections, despite failure to respond to pneumococcal polysaccharide vaccination, responded normally to pneumococcal conjugate vaccines.[1,27-29]

In our two randomized controlled vaccination studies we choose to prime with a 7-valent pneumococcal conjugate vaccine but to booster with the 23-valent pneumococcal polysaccharide vaccine. Those children who were below 2 years of age were primed with two doses of conjugate vaccine with a 4-weeks interval, whereas older children in both the AOM and the OME study received one conjugate priming vaccination followed by the 23-valent pneumococcal polysaccharide booster vaccination. It was previously shown that boosting with a pure polysaccharide instead of a conjugate vaccine leads to optimal IgG antibody levels as determined by ELISA.[30] Furthermore, boosting with the polysaccharide vaccine, especially after 18 to 24 months of age, might increase serotype coverage particularly against invasive pneumococcal disease.

Antibody responses after the combined pneumococcal conjugate and polysaccharide vaccinations in both studies were described in the clinical efficacy papers.[1,2] Despite their history of recurrent OM, all children showed adequate ELISA IgG antibody responses, similar or higher to those described in studies with the conjugate vaccine in infants,[5,31] except for serum IgG titers against serotype 6B, which were lower in our studies.

Antibody levels as such did not predict clinical efficacy, as was most apparent in the AOM cohort for serotype 6B: the highest decrease in nasopharyngeal pneumococcal carriage was induced in the youngest patients between 12 and 24 months, who had significantly lower serum anti-6B IgG antibody levels than the older children.[3] In **chapter 5** we therefore set out to explain these differences in carriage reduction by further investigating functional antibody characteristics, like antibody avidity and the opsonophagocytic capacity of antibodies. We observed a significantly higher increase in avidity of antibodies directed against serotype 6B in the younger children compared to the older age group, which might thus explain the better carriage reduction in the youngest patients. It is currently unknown which degree of avidity is necessary for optimal function of antibodies and which factors exactly determine antibody avidity. It was previously shown by our group that the avidity of anti-tetanus antibodies is significantly higher

than that of anti-Hib antibodies.[32] This might be caused by the limited variety of available Hib capsular polysaccharide epitopes, which is associated with an oligoclonality of the serum anti-Hib antibody response and a restricted use of V_H genes.[33-35] These factors make further avidity maturation of anti-Hib antibodies after repeated vaccinations less likely compared to the response to a complex protein antigen such as tetanus. Possibly similar mechanisms are involved in the antibody response and maturation against pneumococcal polysaccharide antigens.

We showed that the polysaccharide booster vaccination after conjugate vaccine priming further increased antibody avidity, which proves further affinity maturation after a plain polysaccharide booster vaccine. Polysaccharides are classified as T-cell independent type 2 antigens, meaning that T lymphocytes are not strictly necessary for, but can augment the B-cell response.[36,37] The exact mechanism of T-cell activation by polysaccharides however is unknown, particular since polysaccharide antigens do not associate with MHC.[38,39] Yet, the progressive affinity maturation after polysaccharide booster immunization that we observed is suggestive for involvement of T-cells. Whether cognate T-B cell interaction is required for affinity maturation (i.e. somatic hypermutation) is unknown. Theoretically, alternative CD40 ligation could substitute for direct T-B cell interaction.[40]

We also found that priming with two instead of one dose of conjugate vaccine led to better avidity maturation upon polysaccharide boosting. For this reason we would now advise to immunize all children with increased risk for invasive pneumococcal infections, even those above 2 years of age, with at least two conjugate vaccine doses. In addition, our observations indicate that polysaccharide booster after 2 conjugate vaccine priming vaccinations might be useful, since it not only induces a raise in serum antibody levels, but also further increases antibody avidity and potentially would improve serotype coverage against invasive pneumococcal disease. Last but not least use of the 23-valent polysaccharide vaccine is more cost-effective as compared with the conjugate vaccine.

We furthermore think that avidity measurements are essential in the evaluation of vaccine efficacy, and that both antibody levels and antibody avidity should be considered in the assessment of vaccination strategies. We also studied the opsonophagocytic capacity of antibodies, however no clear association with clinical efficacy (i.e. nasopharyngeal pneumococcal carriage reduction) was observed. Possibly the way we performed our assay, with a standardized amount of antibody added, is not optimal. Further research is necessary to develop a well-standardized assay to evaluate opsonophagocytic capacity, which would also contribute to the evaluation of vaccines.

The efficacy of phagocytosis, which is the principal route for antibody mediated elimination of encapsulated bacteria, is determined not only by the above described antibody characteristics, but also by the quality of Fc receptors for IgG (Fcγ receptors) expressed on phagocytic cells. Thus differences in expression or function of Fcγ receptors, particularly Fcγ receptor IIa that interacts with IgG_2, could predispose a child for recurrence of OM. The functional H/R131 polymorphism of FcγRIIa is known to influence the binding capacity and clearance of immune complexes with IgG_2 and IgG_3 and the phagocytosis of IgG_2 opsonized bacteria.[41,42] In the past, we found the homozygous FcγRIIa-H/H131 receptor with optimal binding of IgG_2-opsonized bacteria to be associated with less respiratory tract infections in young children, who have low IgG_2 antibody levels.[43] For this reason the FcγRIIa polymorphism was now investigated as an

additional risk factor for recurrence of AOM after pneumococcal vaccinations in a much larger cohort of children prospectively followed for 18 months. Children with the poorly IgG_2 binding FcγIIa-R/R131 genotype showed an increased number of AOM recurrences after pneumococcal conjugate and polysaccharide vaccinations, as described in **chapter 6**. Due to limited data on pathogens present in the middle ear in case of recurrence of AOM, we could not prove that indeed less effective clearance of either conjugate vaccine serotypes or replacing pneumococci caused the higher recurrence rate of middle ear infections in FcγRIIa-R/R131 children. We saw no differences in nasopharyngeal pneumococcal colonization, or in serum IgG anti-pneumococcal antibody levels after vaccinations. Future studies should elucidate whether indeed our model of decreased local phagocytosis depending of FcγRIIa genotype holds true and whether this explains the high AOM recurrence rate in FcγRIIa-R/R131 children. In our opinion Fc receptor polymorphisms and their role in the development of upper respiratory tract infections still deserve attention. Animal models could be of use, but since different Fc receptors and different IgG subclasses are expressed in experimental animals like mice, these studies remain difficult to extrapolate to clinical conditions in men.

The possible genetic regulation of pneumococcal specific IgG antibody response was investigated in **chapter 7**. Six single nucleotide polymorphisms (SNPs) in the genes encoding interleukin 4 (IL-4), IL-13 and the gene encoding the IL-4 receptor α chain (IL-4RA), were investigated for their influence on pneumococcal specific IgG antibody responses. We choose to study Il-4 and IL-13 and the IL-4 receptor α chain, which is involved in both the IL-4 and the IL-13 receptor, since it is know that these cytokines stimulate antibody production by B-cells. Furthermore, SNPs in these genes have been widely studied for their possible influence on asthma and atopy development,[44-46] and functional work on some of these SNPs has been performed.[47-49] In our study group of children with recurrent AOM or OME, we found an association between antibody responses and the SNPs in IL4 (IL4C–599T and IL4 G2979T) and IL4RA (Gln551Arg), depending on the pneumococcal serotype under investigation. Interestingly, the genotypes associated with decreased IgG antibody levels for several pneumococcal serotypes, were also associated with increased serum IgE levels. This might suggest an inverse relationship between atopic phenotypes and the response upon pneumococcal vaccination, possibly brought about by genotype and an altered Th1/Th2 balance. This model would fit with the inverse relationship between infection and atopy as proposed in the hygiene hypothesis where infections at early age are suggested to protect against development of atopy.[50] Conversely however, many patients with an atopic constitution seem to have an increased risk of respiratory tract infections.[51,52]

The additional value of investigating haplotypes or gene-gene interactions instead of single SNPs, especially when studying complex biological pathways, has been recognized.[53,54] We therefore also analyzed haplotypes of SNPs in IL-4, IL-13 and IL-4RA. This showed an additive effect above investigation of single SNPs. Furthermore, we made combinations of haplotypes, and despite limited numbers of children in the various subgroups, indeed antibody responses were found lowest in carriers of two low producing haplotypes, and highest in carriers of two high-producing haplotypes. This highlights the importance to study gene-gene interactions, haplotypes or even haplotype-haplotype combinations in relation with environmental factors. However, very large and well-defined cohorts are necessary for such genetic analyses, especially

when studying common disorders with multiple susceptibility genes, and when their interaction with the environment is considered. For genetic association studies, in general two approaches can be used: a whole genome linkage survey or the candidate-gene approach. For our study with limited numbers of patients we choose to only evaluate the specific hypothesis of IL-4 and IL-13 influencing vaccine responsiveness. To identify new disease associated genes and to gain better knowledge on the influence of genetic variations on vaccine responses, which will have great value for the development of novel and improved vaccines, further in-depth studies are required.

In **chapter 8** it was investigated whether several of the risk factors identified in the development of AOM may be recognized as risk factors predisposing for persistent OME as well. In this study serum immunoglobulin levels, serum MBL levels, and polymorphisms in FcγRIIa and FcγRIIIb were evaluated. Immunoglobulin levels were within the normal range and did not have any predictive value for the recurrence of a period of OME. Functional serum MBL levels seemed to have some association with OME recurrence. However, we think that before drawing conclusions, genetic MBL analyses should be performed in addition to the investigation of MBL serum levels alone. Furthermore, the OME cohort did not include children below 2 years of age, in whom the impact of MBL seems to be most outspoken as was shown in the in the AOM study cohort. Further studies on the true impact of MBL on OME are thus warranted. Fcγ receptor polymorphisms also did seem to have some influence on OME recurrence, but again differences were not statistically significant. Therefore, care should be taken to draw general conclusions, especially since the OME study cohort was rather small for the assessment of genetic risk factors.

OME is a common condition in childhood occurring in more than 50% of children at least once before their first birthday.[55] After a first recognized episode of OME the recurrence rate is high, up to 40%.[56] In contrast to AOM, the diagnosis of OME and the persistence of symptoms may often not be clinically recognized. No consensus has been reached on the question whether AOM and OME should be regarded as the same disease with varying clinical characteristics within one spectrum of middle ear infections, or as different disease entities at an identical site, the middle ear cavity. There is an ongoing debate on clinical distinction, differences in aetiology, and on the relative importance of several immunological parameters. Also from our studies, no generalized conclusions on the differences between OME and AOM can be made, especially since we cannot fully compare the two study cohorts. Both cohorts differed not only regarding age and baseline characteristics, but also in follow-up. Future research should elucidate whether potential risk factors for AOM also play a role in the development of persisting and recurrent OME. This knowledge might direct the future treatment strategies for both diseases.

General conclusions and future research

The work described in this thesis highlights the importance of adaptive immunity, especially the production of polysaccharide specific antibodies and functional characteristics of these antibodies, and the value of the innate immune system, especially in very young children when adaptive anti-polysaccharide specific immunity is still immature, in the protection from OM. Our work further indicates there is a dynamic but intricate balance between the innate and

adaptive immune systems.

The influence of genetic variation in several immune parameters of both the innate and adaptive immune systems was investigated. This area of genetic research is rapidly progressing, and future work will have to show us the true impact of variation in disease-associated genes and disease modifier genes, taking into consideration their interaction with environmental factors, in a multi-factorial infectious disease like otitis media.

An area of research that we did not touch on in this thesis is the role of local and mucosal immunity in the development of OM. Pneumococcal conjugate vaccines are described to elicit anti-capsular IgA, and to a lesser extent IgG antibody responses in saliva of children vaccinated in infancy.[57,58] Local mucosal immunity, either innate or acquired by natural exposition to pathogens like S. *pneumoniae*, possibly plays a larger role in the protection from mucosal diseases like OM as compared to vaccine-induced systemic antibodies. Animal models have shown that mucosal administration of conjugated vaccines induces a pronounced salivary IgA response, which offers protection against invasive disease and bacterial carriage.[59,60] Future research into this route of administration of vaccines potentially could lead to vaccines with higher efficacy against mucosal diseases like OM, and could also result in vaccines that will be better applicable and available for children.[61] Clinical trials however are warranted to prove mucosal vaccine efficacy and to make sure no tolerance is induced.

The current vaccination strategies in children based on polysaccharide antigens will most likely not solve the problem of OM, since replacing pneumococci and pathogens like H. *influenzae* and M. *catharralis* were shown to have increased as middle ear pathogens after the introduction of infant pneumococcal conjugate vaccinations.[4,9,62] The main future challenge thus remains the development of a successful and widely useable vaccine against OM. A pneumococcal vaccine including protein antigens that are conserved across the varying pneumococcal serotypes and antigens of H. *influenzae* and M. *catharralis* may be more likely to be successful (R. Prymula *et al.*, abstract ESPID, 2005). A number of lead protein antigens for all three bacteria have now been identified, which have the potential to protect across the range of serotypes and strains responsible for disease.[63] However, OM is a poly-microbial disease in which a complex relationship between various bacteria and viruses exists. Vaccinating against influenza also may have a significant impact on reducing the incidence of AOM in high risk cohorts during periods of influenza activity in the community.[64-66] Combining viral and bacterial vaccines will thus be of importance in the future.[63] Apart from vaccinations, a better understanding of the susceptibility of individuals toward infectious diseases will ultimately bring better vaccination and treatment strategies for individuals at increased risk to otitis media.

References

1. Veenhoven, R.; Bogaert, D.; Uiterwaal, C.; Brouwer, C.; Kiezebrink, H.; Bruin, J.; IJzerman, E.; Hermans, P.; de Groot, R.; Zegers, B.; Kuis, W.; Rijkers, G.; Schilder, A.; Sanders, E.: Effect of conjugate pneumococcal vaccine followed by polysaccharide pneumococcal vaccine on recurrent acute otitis media: a randomised study. *Lancet* **2003**; 361:2189-2195
2. van Heerbeek, N.; Straetemans, M.; Wiertsema, S.P.; Ingels, K.; Rijkers, G.T.; Schilder, A.G.; Sanders, E.A.; Zielhuis, G.A.: The effect of combined pneumococcal conjugate and polysaccharide vaccination on recurrent otitis media with effusion. *Pediatrics* **2005**;
3. Veenhoven, R.H.; Bogaert, D.; Schilder, A.G.; Rijkers, G.T.; Uiterwaal, C.S.; Kiezebrink, H.H.; van Kempen, M.J.; Dhooge, I.J.; Bruin, J.; IJzerman, E.P.; de Groot, R.; Kuis, W.; Hermans, P.W.; Sanders, E.A.: Nasopharyngeal pneumococcal carriage after combined pneumococcal conjugate and polysaccharide vaccination in children with a history of recurrent acute otitis media. *Clin.Infect.Dis.* **2004**; 39:911-919
4. McEllistrem, M.C.; Adams, J.M.; Patel, K.; Mendelsohn, A.B.; Kaplan, S.L.; Bradley, J.S.; Schutze, G.E.; Kim, K.S.; Mason, E.O.; Wald, E.R.: Acute otitis media due to penicillin-nonsusceptible Streptococcus pneumoniae before and after the introduction of the pneumococcal conjugate vaccine. *Clin.Infect.Dis.* **2005**; 40:1738-1744
5. Eskola, J.; Kilpi, T.; Palmu, A.; Jokinen, J.; Haapakoski, J.; Herva, E.; Takala, A.; Kayhty, H.; Karma, P.; Kohberger, R.; Siber, G.; Makela, P.H.: Efficacy of a pneumococcal conjugate vaccine against acute otitis media. *N.Engl.J Med.* **2001**; 344:403-409
6. Mbelle, N.; Huebner, R.E.; Wasas, A.D.; Kimura, A.; Chang, I.; Klugman, K.P.: Immunogenicity and impact on nasopharyngeal carriage of a nonavalent pneumococcal conjugate vaccine. *J.Infect.Dis.* **1999**; 180:1171-1176
7. Dagan, R.; Givon-Lavi, N.; Zamir, O.; Fraser, D.: Effect of a nonavalent conjugate vaccine on carriage of antibiotic-resistant Streptococcus pneumoniae in day-care centers. *Pediatr.Infect.Dis.J.* **2003**; 22:532-540
8. Dagan, R.; Givon-Lavi, N.; Zamir, O.; Sikuler-Cohen, M.; Guy, L.; Janco, J.; Yagupsky, P.; Fraser, D.: Reduction of nasopharyngeal carriage of Streptococcus pneumoniae after administration of a 9-valent pneumococcal conjugate vaccine to toddlers attending day care centers. *J.Infect.Dis.* **2002**; 185:927-936
9. Block, S.L.; Hedrick, J.; Harrison, C.J.; Tyler, R.; Smith, A.; Findlay, R.; Keegan, E.: Community-wide vaccination with the heptavalent pneumococcal conjugate significantly alters the microbiology of acute otitis media. *Pediatr. Infect.Dis.J.* **2004**; 23:829-833
10. Bernig, T.; Taylor, J.G.; Foster, C.B.; Staats, B.; Yeager, M.; Chanock, S.J.: Sequence analysis of the mannose-binding lectin (MBL2) gene reveals a high degree of heterozygosity with evidence of selection. *Genes Immun.* **2004**; 5:461-476
11. Cook, D.N.; Pisetsky, D.S.; Schwartz, D.A.: Toll-like receptors in the pathogenesis of human disease. *Nat.Immunol.* **2004**; 5:975-979
12. Schroder, N.W. and Schumann, R.R.: Single nucleotide polymorphisms of Toll-like receptors and susceptibility to infectious disease. *Lancet Infect.Dis.* **2005**; 5:156-164
13. Lanoue, A.; Clatworthy, M.R.; Smith, P.; Green, S.; Townsend, M.J.; Jolin, H.E.; Smith, K.G.; Fallon, P.G.; McKenzie, A.N.: SIGN-R1 contributes to protection against lethal pneumococcal infection in mice. *J.Exp.Med.* **2004**; 200:1383-1393
14. Geijtenbeek, T.B.; Torensma, R.; van Vliet, S.J.; van Duijnhoven, G.C.; Adema, G.J.; van Kooyk, Y.; Figdor, C.G.: Identification of DC-SIGN, a novel dendritic cell-specific ICAM-3 receptor that supports primary immune responses. *Cell* **2000**; 100:575-585
15. Dziarski, R.; Viriyakosol, S.; Kirkland, T.N.; Gupta, D.: Soluble CD14 enhances membrane CD14-mediated responses to peptidoglycan: structural requirements differ from those for responses to lipopolysaccharide. *Infect. Immun.* **2000**; 68:5254-5260
16. Um, S.H.; Son, E.W.; Kim, B.O.; Moon, E.Y.; Rhee, D.K.; Pyo, S.: Activation of murine peritoneal macrophages by Streptococcus pneumoniae type II capsular polysaccharide: involvement of CD14-dependent pathway. *Scand. J.Immunol.* **2000**; 52:39-45
17. da Silva, C.J.; Soldau, K.; Christen, U.; Tobias, P.S.; Ulevitch, R.J.: Lipopolysaccharide is in close proximity to each of the proteins in its membrane receptor complex. transfer from CD14 to TLR4 and MD-2. *J.Biol.Chem.* **2001**; 276:21129-21135

18. Kim, J.I.; Lee, C.J.; Jin, M.S.; Lee, C.H.; Paik, S.G.; Lee, H.; Lee, J.O.: Crystal structure of CD14 and its implications for lipopolysaccharide signaling. J.Biol.Chem. **2005**;
19. Cauwels, A.; Frei, K.; Sansano, S.; Fearns, C.; Ulevitch, R.; Zimmerli, W.; Landmann, R.: The origin and function of soluble CD14 in experimental bacterial meningitis. J.Immunol. **1999**; 162:4762-4772
20. Herrod, H.G.; Gross, S.; Insel, R.: Selective antibody deficiency to Haemophilus influenzae type B capsular polysaccharide vaccination in children with recurrent respiratory tract infection. J.Clin.Immunol. **1989**; 9:429-434
21. Ambrosino, D.M.; Siber, G.R.; Chilmonczyk, B.A.; Jernberg, J.B.; Finberg, R.W.: An immunodeficiency characterized by impaired antibody responses to polysaccharides. N.Engl.J.Med. **1987**; 316:790-793
22. Sanders, L.A.; Rijkers, G.T.; Kuis, W.; Tenbergen-Meekes, A.J.; Graeff-Meeder, B.R.; Hiemstra, I.; Zegers, B.J.: Defective antipneumococcal polysaccharide antibody response in children with recurrent respiratory tract infections. J.Allergy Clin.Immunol. **1993**; 91:110-119
23. Sanders, L.; Rijkers, G.; Tenbergen-Meekes, A.; Voorhorst-Ogink, M.; Zegers, B.: Immunoglobulin isotype-specific antibody responses to pneumococcal polysaccharide vaccine in patients with recurrent bacterial respiratory tract infections. Pediatr.Res. **1995**; 37:812-819
24. Epstein, M.M. and Gruskay, F.: Selective deficiency in pneumococcal antibody response in children with recurrent infections. Ann.Allergy Asthma Immunol. **1995**; 75:125-131
25. Prellner, K.; Harsten, G.; Lofgren, B.; Christenson, B.; Heldrup, J.: Responses to rubella, tetanus, and diphtheria vaccines in otitis-prone and non-otitis-prone children. Ann.Otol.Rhinol.Laryngol. **1990**; 99:628-632
26. Hotomi, M.; Yamanaka, N.; Saito, T.; Shimada, J.; Suzumoto, M.; Suetake, M.; Faden, H.: Antibody responses to the outer membrane protein P6 of non-typeable Haemophilus influenzae and pneumococcal capsular polysaccharides in otitis-prone children. Acta Otolaryngol. **1999**; 119:703-707
27. Breukels, M.A.; Rijkers, G.T.; Voorhorst-Ogink, M.M.; Zegers, B.J.; Sanders, L.A.: Pneumococcal conjugate vaccine primes for polysaccharide-inducible IgG2 antibody response in children with recurrent otitis media acuta. J Infect.Dis. **1999**; 179:1152-1156
28. Sorensen, R.U.; Leiva, L.E.; Giangrosso, P.A.; Butler, B.; Javier, F.C., III; Sacerdote, D.M.; Bradford, N.; Moore, C.: Response to a heptavalent conjugate Streptococcus pneumoniae vaccine in children with recurrent infections who are unresponsive to the polysaccharide vaccine. Pediatr.Infect.Dis.J **1998**; 17:685-691
29. Zielen, S.; Buhring, I.; Strnad, N.; Reichenbach, J.; Hofmann, D.: Immunogenicity and tolerance of a 7-valent pneumococcal conjugate vaccine in nonresponders to the 23-valent pneumococcal vaccine. Infect.Immun. **2000**; 68:1435-1440
30. Kilpi, T.; Ahman, H.; Jokinen, J.; Lankinen, K.S.; Palmu, A.; Savolainen, H.; Gronholm, M.; Leinonen, M.; Hovi, T.; Eskola, J.; Kayhty, H.; Bohidar, N.; Sadoff, J.C.; Makela, P.H.: Protective efficacy of a second pneumococcal conjugate vaccine against pneumococcal acute otitis media in infants and children: randomized, controlled trial of a 7-valent pneumococcal polysaccharide-meningococcal outer membrane protein complex conjugate vaccine in 1666 children. Clin.Infect.Dis. **2003**; 37:1155-1164
31. Black, S.; Shinefield, H.; Fireman, B.; Lewis, E.; Ray, P.; Hansen, J.R.; Elvin, L.; Ensor, K.M.; Hackell, J.; Siber, G.; Malinoski, F.; Madore, D.; Chang, I.; Kohberger, R.; Watson, W.; Austrian, R.; Edwards, K.: Efficacy, safety and immunogenicity of heptavalent pneumococcal conjugate vaccine in children. Northern California Kaiser Permanente Vaccine Study Center Group. Pediatr.Infect.Dis.J **2000**; 19:187-195
32. Breukels, M.A.; Jol-van der Zijde, E.; van Tol, M.J.; Rijkers, G.T.: Concentration and avidity of anti-Haemophilus influenzae type b (Hib) antibodies in serum samples obtained from patients for whom Hib vaccination failed. Clin.Infect.Dis. **2002**; 34:191-197
33. Insel, R.A.; Kittelberger, A.; Anderson, P.: Isoelectric focusing of human antibody to the Haemophilus influenzae b capsular polysaccharide: restricted and identical spectrotypes in adults. J.Immunol. **1985**; 135:2810-2816
34. Scott, M.G.; Tarrand, J.J.; Crimmins, D.L.; McCourt, D.W.; Siegel, N.R.; Smith, C.E.; Nahm, M.H.: Clonal characterization of the human IgG antibody repertoire to Haemophilus influenzae type b polysaccharide. II. IgG antibodies contain VH genes from a single VH family and VL genes from at least four VL families. J.Immunol. **1989**; 143:293-298
35. Barington, T.; Hougs, L.; Juul, L.; Madsen, H.O.; Ryder, L.P.; Heilmann, C.; Svejgaard, A.: The progeny of a single virgin B cell predominates the human recall B cell response to the capsular polysaccharide of Haemophilus influenzae type b. J.Immunol. **1996**; 157:4016-4027

36. Mosier, D.E.; Mond, J.J.; Goldings, E.A.: The ontogeny of thymic independent antibody responses in vitro in normal mice and mice with an X-linked B cell defect. J.Immunol. **1977**; 119:1874-1878
37. Fernandez, C. and Moller, G.: Immunological unresponsiveness to thymus-independent antigens: two fundamentally different genetic mechanisms of B-cell unresponsiveness to dextran. J.Exp.Med. **1977**; 146:1663-1677
38. Kirkland, T.N.; Sieckmann, D.G.; Longo, D.L.; Mosier, D.E.: Cellular requirements for antigen presentation in the induction of a thymus-independent antibody response in vitro. J.Immunol. **1980**; 124:1721-1726
39. Rosen, F.S.: T cell : B cell collaboration--the response to polysaccharide antigens. Semin.Immunol. **1989**; 1:87-91
40. Bishop, G.A. and Hostager, B.S.: Signaling by CD40 and its mimics in B cell activation. Immunol.Res. **2001**; 24:97-109
41. Sanders, L.A.; Feldman, R.G.; Voorhorst-Ogink, M.M.; De Haas, M.; Rijkers, G.T.; Capel, P.J.; Zegers, B.J.; van de Winkel, J.G.: Human immunoglobulin G (IgG) Fc receptor IIA (CD32) polymorphism and IgG2-mediated bacterial phagocytosis by neutrophils. Infect.Immun. **1995**; 63:73-81
42. Jansen, W.T.; Breukels, M.A.; Snippe, H.; Sanders, L.A.; Verheul, A.F.; Rijkers, G.T.: Fcgamma receptor polymorphisms determine the magnitude of in vitro phagocytosis of Streptococcus pneumoniae mediated by pneumococcal conjugate sera. J Infect.Dis. **1999**; 180:888-891
43. Sanders, L.A.; van de Winkel, J.G.; Rijkers, G.T.; Voorhorst-Ogink, M.M.; De Haas, M.; Capel, P.J.; Zegers, B.J.: Fc gamma receptor IIa (CD32) heterogeneity in patients with recurrent bacterial respiratory tract infections. J.Infect. Dis. **1994**; 170:854-861
44. Hoffjan, S. and Ober, C.: Present status on the genetic studies of asthma. Curr.Opin.Immunol. **2002**; 14:709-717
45. Shirakawa, I.; Deichmann, K.A.; Izuhara, I.; Mao, I.; Adra, C.N.; Hopkin, J.M.: Atopy and asthma: genetic variants of IL-4 and IL-13 signalling. Immunol.Today **2000**; 21:60-64
46. Liu, X.; Beaty, T.H.; Deindl, P.; Huang, S.K.; Lau, S.; Sommerfeld, C.; Fallin, M.D.; Kao, W.H.; Wahn, U.; Nickel, R.: Associations between total serum IgE levels and the 6 potentially functional variants within the genes IL4, IL13, and IL4RA in German children: the German Multicenter Atopy Study. J.Allergy Clin.Immunol. **2003**; 112:382-388
47. Rosenwasser, L.J.: Structure/function variants as candidate genes in asthma: linkage vs. association for relevance. Clin.Exp.Allergy **1998**; 28 Suppl 1:90-92
48. Mitsuyasu, H.; Izuhara, K.; Mao, X.Q.; Gao, P.S.; Arinobu, Y.; Enomoto, T.; Kawai, M.; Sasaki, S.; Dake, Y.; Hamasaki, N.; Shirakawa, T.; Hopkin, J.M.: Ile50Val variant of IL4R alpha upregulates IgE synthesis and associates with atopic asthma. Nat.Genet. **1998**; 19:119-120
49. Risma, K.A.; Wang, N.; Andrews, R.P.; Cunningham, C.M.; Ericksen, M.B.; Bernstein, J.A.; Chakraborty, R.; Hershey, G.K.: V75R576 IL-4 receptor alpha is associated with allergic asthma and enhanced IL-4 receptor function. J.Immunol. **2002**; 169:1604-1610
50. Strachan, D.P.: Hay fever, hygiene, and household size. BMJ **1989**; 299:1259-1260
51. Koopman, L.P.; Smit, H.A.; Heijnen, M.L.; Wijga, A.; van Strien, R.T.; Kerkhof, M.; Gerritsen, J.; Brunekreef, B.; de Jongste, J.C.; Neijens, H.J.: Respiratory infections in infants: interaction of parental allergy, child care, and siblings-- The PIAMA study. Pediatrics **2001**; 108:943-948
52. Illi, S.; von Mutius, E.; Lau, S.; Bergmann, R.; Niggemann, B.; Sommerfeld, C.; Wahn, U.: Early childhood infectious diseases and the development of asthma up to school age: a birth cohort study. BMJ **2001**; 322:390-395
53. Howard, T.D.; Koppelman, G.H.; Xu, J.; Zheng, S.L.; Postma, D.S.; Meyers, D.A.; Bleecker, E.R.: Gene-gene interaction in asthma: IL4RA and IL13 in a Dutch population with asthma. Am.J.Hum.Genet. **2002**; 70:230-236
54. Lee, J.E.; Choi, J.H.; Lee, J.H.; Lee, M.G.: Gene SNPs and mutations in clinical genetic testing: haplotype-based testing and analysis. Mutat.Res. **2005**; 573:195-204
55. Paradise, J.L.; Rockette, H.E.; Colborn, D.K.; Bernard, B.S.; Smith, C.G.; Kurs-Lasky, M.; Janosky, J.E.: Otitis media in 2253 Pittsburgh-area infants: prevalence and risk factors during the first two years of life. Pediatrics **1997**; 99:318-333
56. Rosenfeld, R.M.; Culpepper, L.; Yawn, B.; Mahoney, M.C.: Otitis media with effusion clinical practice guideline. Am.Fam.Physician **2004**; 69:2776, 2778-2776, 2779
57. Korkeila, M.; Lehtonen, H.; Ahman, H.; Leroy, O.; Eskola, J.; Kayhty, H.: Salivary anti-capsular antibodies in infants and children immunised with Streptococcus pneumoniae capsular polysaccharides conjugated to diphtheria or tetanus toxoid. Vaccine **2000**; 18:1218-1226
58. Nurkka, A.; Ahman, H.; Korkeila, M.; Jantti, V.; Kayhty, H.; Eskola, J.: Serum and salivary anti-capsular antibodies

in infants and children immunized with the heptavalent pneumococcal conjugate vaccine. Pediatr.Infect.Dis.J **2001**; 20:25-33

59. Jakobsen, H.; Saeland, E.; Gizurarson, S.; Schulz, D.; Jonsdottir, I.: Intranasal immunization with pneumococcal polysaccharide conjugate vaccines protects mice against invasive pneumococcal infections. Infect.Immun. **1999**; 67:4128-4133
60. Lynch, J.M.; Briles, D.E.; Metzger, D.W.: Increased protection against pneumococcal disease by mucosal administration of conjugate vaccine plus interleukin-12. Infect.Immun. **2003**; 71:4780-4788
61. Jakobsen, H. and Jonsdottir, I.: Mucosal vaccination against encapsulated respiratory bacteria--new potentials for conjugate vaccines? Scand.J.Immunol. **2003**; 58:119-128
62. Dagan, R.: The potential effect of widespread use of pneumococcal conjugate vaccines on the practice of pediatric otolaryngology: the case of acute otitis media. Curr.Opin.Otolaryngol.Head Neck Surg. **2004**; 12:488-494
63. Cripps, A.W.; Otczyk, D.C.; Kyd, J.M.: Bacterial otitis media: a vaccine preventable disease? Vaccine **2005**; 23:2304-2310
64. Heikkinen, T.; Ruuskanen, O.; Waris, M.; Ziegler, T.; Arola, M.; Halonen, P.: Influenza vaccination in the prevention of acute otitis media in children. Am.J.Dis.Child **1991**; 145:445-448
65. Clements, D.A.; Langdon, L.; Bland, C.; Walter, E.: Influenza A vaccine decreases the incidence of otitis media in 6- to 30-month-old children in day care. Arch.Pediatr.Adolesc.Med. **1995**; 149:1113-1117
66. Belshe, R.B.; Mendelman, P.M.; Treanor, J.; King, J.; Gruber, W.C.; Piedra, P.; Bernstein, D.I.; Hayden, F.G.; Kotloff, K.; Zangwill, K.; Iacuzio, D.; Wolff, M.: The efficacy of live attenuated, cold-adapted, trivalent, intranasal influenzavirus vaccine in children. N.Engl.J.Med. **1998**; 338:1405-1412

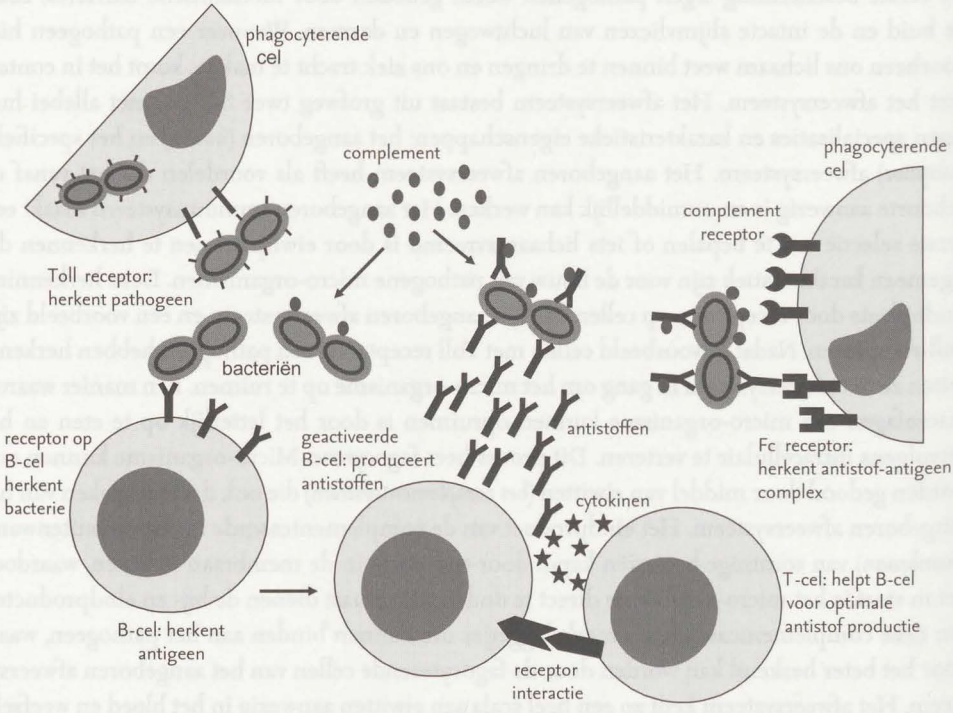
Nederlandse samenvatting

Het afweersysteem of immuunsysteem beschermt ons tegen micro-organismen die ons ziek kunnen maken zoals bacteriën, virussen, schimmels en wormen. Met een verzamelnaam heten deze ziekmakende micro-organismen ook wel *pathogenen*. Aangezien er vele verschillende soorten ziekteverwekkers bestaan, is er ook een heel scala aan afweermechanismen noodzakelijk. Het belang van een goed afweersysteem blijkt uit zeldzame syndromen waarbij kinderen geboren worden zonder goed functionerend afweersysteem of bij patiënten met het Acquired Immune Deficiency Syndrome (AIDS). Deze patiënten overlijden meestal aan het optreden van verschillende infecties – infecties die bij een persoon met een goed functionerend immuunsysteem niet of nauwelijks hinder zouden hebben veroorzaakt.

De eerste bescherming tegen pathogenen wordt geboden door mechanische barrières, zoals de huid en de intacte slijmvliezen van luchtwegen en darmen. Wanneer een pathogeen hier doorheen ons lichaam weet binnen te dringen en ons ziek tracht te maken, komt het in contact met het afweersysteem. Het afweersysteem bestaat uit grofweg twee takken, met allebei hun eigen specialisaties en karakteristieke eigenschappen: het aangeboren (*innate*) en het specifieke (*adaptive*) afweersysteem. Het aangeboren afweersysteem heeft als voordelen dat het vanaf de geboorte aanwezig is en onmiddellijk kan werken. Het aangeboren immuunsysteem maakt een eerste selectie om te bepalen of iets lichaamsvreemd is door eiwitpatronen te herkennen die algemeen karakteristiek zijn voor de bouw van pathogene micro-organismen. Deze herkenning vindt plaats door receptoren op cellen van het aangeboren afweersysteem en een voorbeeld zijn Toll-receptoren. Nadat bijvoorbeeld cellen met Toll receptoren een pathogeen hebben herkend, zetten ze het afweersysteem in gang om het micro-organisme op te ruimen. Een manier waarop macrofagen een micro-organisme kunnen opruimen is door het letterlijk op te eten en het vervolgens intracellulair te verteren. Dit proces heet fagocytose. Micro-organisme kunnen ook worden gedood door middel van eiwitten (*het complementsysteem*) die ook deel uit maken van het aangeboren afweersysteem. Het eindproduct van de complementcascade maakt de buitenwand (*membraan*) van sommige bacteriën kapot door een porie in de membraan te boren, waardoor het in staat is het micro-organisme direct te doden. Daarnaast dienen de bij- en eindproducten van deze complementcascade als merkvlaggetjes die kunnen binden aan het pathogeen, waardoor het beter herkend kan worden door de fagocyterende cellen van het aangeboren afweersysteem. Het afweersysteem kent zo een heel scala aan eiwitten aanwezig in het bloed en weefsels, waarvan sommige dienen als merkvlaggetjes om pathogenen beter zichtbaar te maken, andere leiden tot een verhoogde bloedtoevoer (locale roodheid, warmte en zwelling) en weer andere dienstdoen als boodschappermoleculen voor cellen van het immuunsysteem. Deze processen dragen allemaal bij aan het opruimen van het pathogeen.

Een tekortkoming van het aangeboren afweersysteem is dat het slechts in staat is een infectie onder controle te houden, maar dat het de infectie niet volledig kan opruimen. De tweede tak, het specifieke afweersysteem, is wel in staat een pathogeen, en dus een infectie, volledig te

elimineren. Dit specifieke afweersysteem wordt van oudsher verdeeld in twee delen: het humorale afweersysteem en het cellulaire afweersysteem. Door het humorale afweersysteem worden na herkenning van een specifieke structuur op een micro-organisme (*het antigeen*) antistoffen geproduceerd. Deze antistoffen zijn wel specifiek gericht tegen dat éne micro-organisme dat is binnengedrongen. Antistoffen worden gemaakt door cellen die oorspronkelijk uit het beenmerg komen, en daarom B-cellen worden genoemd. De antistoffen worden in grote hoeveelheden geproduceerd en binden aan het specifieke antigeen op het binnendringende micro-organisme. Vervolgens kan het antigeen-antistof complex worden herkend door receptoren voor het antistof (Fc-*receptoren*) die vóórkomen op allerlei cellen van het immuunsysteem. Na binding van het complex aan de Fc-receptor wordt het complex uit de weg geruimd door bijvoorbeeld te worden gefagocyteerd. De andere, cellulaire tak van het specifieke afweersysteem bestaat uit cellen die oorspronkelijk uit de thymus komen en daarom T-cellen worden genoemd. Cellulaire afweer is onder anderen belangrijk voor virus infecties. Zogenaamde cytotoxische T-cel-



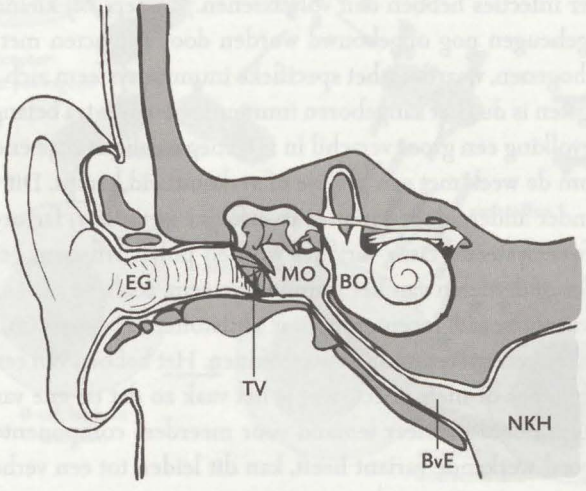
Figuur 1 Samenwerking tussen het aangeboren en het specifieke afweersysteem.
Toll-receptoren herkennen patronen op bacteriën waardoor ze deze kunnen opeten en verteren. Bacteriën worden ook herkend door receptoren op de B-cel, die vervolgens geactiveerd wordt en antistoffen gaat produceren. Geholpen door cytokinen en receptor-interacties met T-cellen, ontstaat optimale antistofproductie en immunologisch geheugen. Verder herkennen Fc- en complement-receptoren op phagocyterende cellen het antigeen-antistof complex en ruimen dit uit de weg.

len kunnen met virus geïnfecteerde cellen doden en daardoor voorkomen dat het virus zich verder in het lichaam verspreidt. Een andere categorie van T-cellen produceert groeifactoren (*cytokinen*) die het afweersysteem beïnvloeden, reguleren en coördineren. Op het oppervlak van deze T-cellen zitten specifieke receptoren die weer kunnen binden aan andere cellen van het immuunsysteem, bijvoorbeeld B-cellen. De T-cel kan met deze binding de B-cel tot productie van antistoffen aanzetten. Deze T-cellen worden daarom ook wel T-helper-cellen genoemd. De cellen van het specifieke afweersysteem herkennen dus ieder een zeer specifiek antigeen. Het grote voordeel van het specifieke afweersysteem is dat bij de tweede keer dat dit zelfde antigeen het lichaam binnendringt er nog cellen zijn die zich dit antigeen herinneren. Deze B-cellen kunnen deze tweede keer, geholpen door de T-helper-cellen, sneller en meer antistoffen produceren. Bovendien zijn deze antistoffen van een betere kwaliteit (*affiniteit*). Het immuunsysteem reageert nu dus zo snel en efficiënt dat het pathogeen geen ziekteverschijnselen meer kan veroorzaken. Dit immunologisch geheugen ontstaat na een natuurlijke infectie maar kan ook door middel van inenting (*vaccinatie*) worden opgewekt.

Het aangeboren en het specifieke afweersysteem werken nauw samen om een binnendringend pathogeen uit te roeien. Doordat het aangeboren systeem in staat is snel en aspecifiek te reageren, kan het pathogeen zich niet optimaal vermenigvuldigen en blijft de infectie dus onder controle. Het specifieke afweersysteem maakt het opruimwerk vervolgens af en zorgt voor geheugen zodat een tweede infectie nog sneller en beter te bestrijden is. Voor een optimale bescherming tegen pathogenen kan het ene systeem niet zonder het andere. Dit blijkt ook uit het feit dat kleine kinderen vaker infecties hebben dan volwassenen. Immers, bij kleine kinderen moet het immunologische geheugen nog opgebouwd worden door contacten met alle in de omgeving voorkomende pathogenen, waardoor het specifieke immuunsysteem zich geleidelijk aan verbetert. Bij de allerjongsten is dus het aangeboren immuunsysteem extra belangrijk. Toch zie je ook in de volwassen bevolking een groot verschil in infectiegevoeligheid; je ene collega is nooit ziek, terwijl de ander om de week met een griepje of verkoudheid kampt. Dit verschil in infectiegevoeligheid komt onder andere door variaties in erfelijke (*genetische*) factoren van het aangeboren en specifieke afweersysteem. Deze variaties worden polymorfismen genoemd en kunnen voorkomen in allerlei onderdelen van het immuunsysteem, zoals de bovengenoemde Toll receptoren, de complementcascade, receptoren voor antistoffen (*Fc-receptoren*), de boodschappermoleculen (*cytokinen*) en receptoren voor deze cytokinen. Het hebben van een bepaalde erfelijke variatie geeft niet een alles-of-niets effect. Wel is het vaak zo dat de ene variant beter werkt en een andere net iets minder. Wanneer iemand voor meerdere componenten van het afweersysteem een minder goed werkende variant heeft, kan dit leiden tot een verhoogde vatbaarheid voor infecties.

Een van de meest voorkomende infectieziektes bij kinderen is otitis media (OM), ofwel middenoorontsteking. Zoals in figuur 2 te zien is, staat het middenoor via de buis van Eustachius in verbinding met de neus-keelholte. In een mensenmond, en dus de neus-keelholte, zijn continu allerlei soorten bacteriën aanwezig. Het kan vóórkomen, bijvoorbeeld tijdens een verkoudheid, dat bacteriën via de buis van Eustachius het middenoor bereiken, waar ze, als ze niet bijtijds door het afweersysteem uit de weg worden geruimd, een ontsteking kunnen veroorzaken. Er zijn verschillende vormen van middenoorontsteking. Otitis media acuta (OMA) is een vorm

van acute ontsteking van het middenoor waarbij vloeistof (*effusie*) aanwezig is in het middenoor, samen met symptomen van acute ontsteking zoals koorts, roodheid van het trommelvlies en pijn. Bij otitis media met effusie (OME) is ook effusie aanwezig in het middenoor, maar nu zonder symptomen van een acute ontsteking. OME kan het gevolg zijn van een acute middenoorinfectie waarbij de effusie niet adequaat is opgeruimd en dus voor langere tijd achterblijft in het middenoor. OME lijkt echter soms ook op te treden zonder dat hier een acute infectie met symptomen aan is voorafgegaan. Naar schatting heeft ongeveer 80% van alle kinderen tenminste één keer OMA gehad als ze 3 jaar oud zijn en heeft meer dan 50% van alle kinderen al een periode met OME doorgemaakt voor hun eerste verjaardag. De huidige behandelingsmethoden voor OMA en OME, (antibiotica, het plaatsen van trommelvliesbuisjes of het knippen van de keel- en/of neusamandelen) blijken minder effectief dan gehoopt. Het zou dan ook wenselijk zijn maatregelen ter voorkóming van OM beschikbaar te hebben. Momenteel is daarom intensief onderzoek gaande naar een bruikbaar vaccin dat aan het voorkómen van OM bij zou kunnen dragen. De belangrijkste bacteriële verwekker van otitis media is *Streptococcus pneumoniae*, ofwel de pneumokok. Deze bacterie komt erg vaak voor in de neus-keelholte van jonge kinderen. Naast OM kan deze bacterie ook andere ernstige ziekten veroorzaken zoals hersenvliesontsteking, longontsteking of sepsis (bloedvergiftiging waarbij levende bacteriën aanwezig zijn in de bloedbaan). Daarom is reeds vanaf de jaren '80 veel onderzoek gedaan naar de preventie



Figuur 2 Dwarsdoorsnede van het menselijk oor.
EG: Externe gehoorkanaal;
TV: Trommelvlies;
MO: Middenoor met gehoorbeentjes;
BO: Binnenoor met slakkenhuis en evenwichtsorgaan;
BvE: Buis van Eustachius;
NKH: Neus-Keelholte.

van pneumokokkeninfecties door middel van het opwekken van een specifieke afweerrespons met pneumokokkenvaccins. De eerste vaccins bestonden uit suikers (*polysacchariden*) die aan de buitenkant van de pneumokok voorkomen en daar het kapsel vormen (gekapselde bacterie). Er kunnen op basis van verschillende polysaccharidekapsels 90 verschillende pneumokokken typen worden onderscheiden. Het op dit moment verkrijgbare pneumokokkenpolysaccharidevaccin bevat kapselsuikers van 23 verschillende typen pneumokokken (*serotypen*). Deze 23 serotypen dekken ongeveer 90% van de voorkomende pneumokokkeninfecties in de Westerse wereld. Maar pneumokokkenpolysaccharidevaccins blijken echter bij kinderen jonger dan 2 jaar een onvoldoende afweerrespons op te wekken en dus pneumokokkeninfecties niet te kunnen voorkomen. Daarom is een nieuw vaccin ontwikkeld waarin de pneumokokkensuikers gekoppeld (*geconjugeerd*) zijn aan een dragereiwit. Dit conjugaatvaccin blijkt al een antistofrespons op te kunnen wekken bij kinderen vanaf de leeftijd van 2 maanden. Het pneumokokkenconjugaatvaccin bevat echter slechts de kapselsuikers van 7 verschillende pneumokokkenserotypen. Deze top 7 dekt 75% van de pneumokokkeninfecties bij kinderen.

Kinderen met een otitis media, en vooral die kinderen die vaak een acute infectie of die vaak een langdurige OME doormaken, vormen belangrijke groepen waarvoor gezocht wordt naar betere behandeling en preventie. Daarom heeft ons onderzoek zich gericht op het in kaart brengen van het afweersysteem van kinderen met vaak terugkerende OMA en langdurige en terugkerende OME. De onderzoekspopulatie bestond uit 400 kinderen met acute middenoorontsteking en ongeveer evenzoveel kinderen met otitis media met effusie. Zij zijn onderzocht op factoren van het aangeboren en het specifieke afweersysteem.

Hoofdstuk 1 van dit proefschrift geeft een algemene inleiding over otitis media en het aangeboren en specifieke afweersysteem tegen pathogenen die otitis media kunnen veroorzaken. In de twee volgende hoofdstukken richten we ons op erfelijke (*genetische*) variaties in factoren van het aangeboren afweersysteem.

In **hoofdstuk 2** staat mannan-bindend lectine (MBL) centraal, een molecuul dat na binding aan suikers aan de buitenkant van een bacterie het complementsysteem activeert. Polymorfismen in het gen coderend voor MBL leiden tot verschillende hoeveelheden functioneel MBL in het bloed. We hebben op 12 posities in het gen erfelijke variaties onderzocht en een nieuwe variant van het MBL-gen geïdentificeerd die leidt tot lagere functionele MBL spiegels. Ook laten we zien dat kinderen die drager zijn van deze en een aantal reeds bekende andere erfelijke MBL-varianten, vaker middenoorontsteking hebben dan kinderen die een "normaal" MBL-gen en dus hogere spiegels van MBL in bloed hebben. Deze associatie werd alleen in kinderen jonger dan 2 jaar gevonden, wat nog eens benadrukt dat het aangeboren immuunsysteem vooral in de jongste kinderen van groot belang is, omdat het specifieke afweersysteem zich op die leeftijd nog verder moet ontwikkelen..

In **hoofdstuk 3** beschrijven we een andere belangrijke component van het aangeboren afweersysteem, namelijk de Toll-like receptoren (TLR) die patronen op de buitenkant van pathogenen herkennen. Een belangrijk molecuul in het Toll-like receptorcomplex is CD14, waarvan ook erferlijke varianten bekend zijn. Wij hebben aangetoond dat kinderen die drager zijn van de erfelijke variant die leidt tot lage spiegels van CD14 in het bloed minder goed antistoffen maken na pneumokokkenvaccinaties en ook vaker oorontsteking krijgen dan kinderen met de variant

waarbij meer CD14 geproduceerd wordt. De associatie tussen het polymorfisme en oorontsteking werd weer alleen gevonden in de jongste kinderen.

In de **hoofdstukken 4 en 5** beschrijven we de specifieke afweerrespons na verschillende vaccinaties in kinderen met middenoorontsteking. Er is gekeken of bij kinderen met terugkerende middenoorontstekingen eventueel sprake is van een meer algemene verzwakte specifieke afweerrespons in vergelijking tot kinderen die nooit middenoorontsteking hebben gehad. Om dit te onderzoeken is de antistofrespons na vaccinaties die alle kinderen in Nederland krijgen in het kader van het Rijksvaccinatieprogramma bepaald. Het bleek dat kinderen met OME, OMA en gezonde kinderen even goed reageerden op mazelen, *Haemophilus influenzae*-type B, tetanus en difterievaccinaties.

De kinderen die aan onze studie deelnamen reageerden over het algemeen bovendien goed op de toegediende pneumokokkeninentingen en maakten voldoende antistoffen. Vervolgens hebben we onderzocht of verschillende vaccinatieschema's, met eerst één of twee keer een pneumokokkenconjugaatvaccinatie gevolgd door een pneumokokkenpolysaccharide inenting, leidden tot verschillen in antistofkwaliteit (affiniteit). Wij hebben laten zien dat de polysaccharidevaccinatie, afhankelijk van het aantal toegediende pneumokokkenconjugaatvaccinaties, inderdaad betere antistoffen oplevert. De antistoffen opgewekt na een extra polysaccharidevaccinatie binden beter aan bacteriën dan antistoffen die ontstaan na alleen conjugaatvaccinatie. Ook werden bacteriën die in een reageerbuisje geïncubeerd werden met antistoffen opgewekt na de extra polysaccharidevaccinatie beter gefagocyteerd dan bacteriën geïncubeerd met antistoffen opgewekt na alleen een conjugaat inenting. Deze kwalitatieve karakteristieken na de polysaccharidevaccinatie zijn over het algemeen beter als daarvóór twee pneumokokkenconjugaatvaccinaties zijn toegediend.

In de **hoofdstukken 6 en 7** hebben we bestudeerd of er ook variaties in erfelijke factoren van het afweersysteem voorkomen, die de effectiviteit van vaccinaties of de hoogte van de antistofrespons beïnvloeden. In **hoofdstuk 6** werd gekeken naar erfelijke variaties die bestaan in de Fc-receptoren (zie figuur 1). Het blijkt dat kinderen die drager zijn van een Fc-receptor die minder goed antistoffen van de IgG2 klasse bindt, vaker last hebben van terugkomende middenoorontsteking in vergelijking tot kinderen die de goed IgG2 bindende Fc-receptor hebben. Het slechter binden van IgG2 aan de Fc-receptor leidt waarschijnlijk tot een minder goede opname van pneumokokken door immuuncellen (slechtere fagocytose) lokaal in het middenoor.

De sterkte en de aard van een antistofrespons wordt mede bepaald door de aanwezigheid van de immunologische boodschappermoleculen (*cytokinen*). Ook in deze cytokinen en receptoren voor cytokinen komen polymorfismen voor. Een cytokine dat productie van antistoffen door B-cellen stimuleert is interleukine-4 (IL-4). Wij hebben in **hoofdstuk 7** gekeken of polymorfismen in IL-4 en de receptor daarvoor leiden tot een verschil in de antistofrespons na pneumokokkenvaccinatie. Als maar één enkele erfelijke variant werd bekeken, waren de verschillen in antistofrespons klein. Toen echter gekeken werd naar de combinatie van de polymorfisme in IL-4 en de IL-4 receptor tegelijkertijd (*een haplotype*), werd wel een associatie gezien met een minder goede antistofrespons.

De vraag is welke componenten van het immuunsysteem die lijken bij te dragen aan het ontstaan van acute middenoorontsteking, ook bijdragen aan het ontstaan van otitis media met

effussie. In **hoofdstuk 8** werden daarom een aantal van deze immunologische factoren onderzocht in kinderen met otitis media met effusie. We hebben gekeken naar immuunglobulinen- en mannan-bindend lectine (MBL) spiegels in serum en naar de polymorfismen in Fc-receptoren. MBLspiegels en variaties in Fc-receptoren lijken mogelijk wel geassocieerd te zijn met het terugkomen van OME, immuunglobulinenspiegels daarentegen niet.
In **hoofdstuk 9** worden de bevindingen beschreven in dit proefschrift samengevat en bediscussieerd in de context van reeds bestaande literatuur en worden tevens suggesties gedaan voor mogelijk vervolgonderzoek.
Samenvattend zijn we er door middel van het door ons uitgevoerde onderzoek achter gekomen dat een aantal erfelijke variaties in het aangeboren afweersysteem bijdragen aan het ontstaan van middenoorontsteking, maar alleen in de jongste kinderen van onze studie. De productie van specifieke antistoffen lijkt in kinderen met middenoorontsteking voldoende. Vaccinatie met verschillende pneumokokkenvaccins leidt tot goede antistoffen, waarvan de kwaliteit wel verschilt afhankelijk van het type vaccin (puur bacterieel suiker of suiker gekoppeld aan een eiwit) en het aantal vaccinaties. Wederom zijn het genetische verschillen in de receptoren die deze antistoffen moeten binden voordat ze effect kunnen hebben die wel verder bijdragen tot een verhoogd risico op middenoorontsteking.
Om echter tot de ware invloed van genetische polymorfismen in het aangeboren en specifieke afweersysteem te komen en om het effect van omgevingsfactoren en andere immuun-gerelateerde eigenschappen, zoals bijvoorbeeld het hebben van allergie, verder te onderzoeken, is vervolgonderzoek noodzakelijk. Het beste zou hier een studie voor kunnen worden opgezet waarin baby's vanaf hun geboorte deelnemen en waarbij zowel de afweer, omgevingsfactoren (crèchebezoek, roken in huis, het hebben van broertjes en zusjes), het ontwikkelen van allergieën en het doormaken van infecties gedurende lange tijd zorgvuldig wordt gevolgd. Pas dan kan de bijdrage van deze verschillende factoren op waarde worden beoordeeld. Mogelijk leidt deze kennis dan ook tot een betere preventie en behandeling van middenoorontsteking en andere ontstekingen veroorzaakt door de pneumokok, waarvan veel kinderen over de gehele wereld zouden kunnen profiteren.

Dankwoord

Feeling gratitude and not expressing it is like wrapping a present and not giving it.
William Arthur Ward

Het is dan ook de hoogste tijd om aan een heel aantal mensen kenbaar te maken dat ik ze dankbaar ben... Iedereen die op wat voor manier dan ook bij heeft gedragen aan het tot stand komen van dit proefschrift en de afgelopen jaren met me heeft meegeleefd, dank daarvoor. Met het gevaar iemand te vergeten, wil ik een aantal mensen toch bij naam noemen.

Beste Lieke, graag wil ik je bedanken voor de manier waarop je de afgelopen jaren sturing hebt gegeven aan mijn promotieonderzoek. Steeds betrokken, maar mij wel de vrijheid gevend dát te doen waarvan ik dacht dat het goed was. Jouw kritische blik was onmisbaar, zeker op de momenten dat ik dacht dat ik er eigenlijk al wel was. Bedankt voor de mogelijkheden die je me hebt gegeven, onder andere om de samenwerking met het lab van Peter LeSouëf in Australië verder uit te bouwen. De twee keer dat ik in Perth geweest ben zijn voor mij zeer leerzaam geweest, zowel op werkvlak, alsook voor mij persoonlijk, en ik heb hier enorm van genoten! Bedankt voor alles tot nu toe!

Beste Ger, jij bent voor mij de afgelopen jaren de grote vraagbaak en kennisbron geweest. Je deur stond altijd open en je had altijd tijd even een vraag te beantwoorden. Aangezien je werkomgeving voor jou de afgelopen tijd niet altijd even inspirerend is geweest, waardeer ik je blijvende enthousiasme, steun en betrokkenheid des te meer. Je enorme kennis en liefde voor het vak zijn bewonderingwaardig, en ook de manier waarop je dit weet over te dragen op anderen vind ik zeer bijzonder. Daarnaast heb ik ontzettend veel plezier beleefd aan je muziek-quizzen, heb je me enthousiast gekregen voor hardlopen en waren de tour- en voetbalpoules elk jaar weer een succes. Bedankt voor deze vier onvergetelijke jaren!

Beste Vanessa, bedankt voor de enorme hoeveelheid werk die je verzet hebt en het geduld waarmee je mij van alles hebt geleerd. Jouw handigheid, zorgvuldigheid en precisie zijn moeilijk te evenaren. Daarnaast was je ook een gezellige aanwinst op het lab en organiseerde je regelmatig concert-, schaats-, en filmuitjes. Bedankt voor alles en ik ben erg blij dat je m'n paranimf bent! Nathalie, dank voor je hulp met kweken, tritium en Luminex, maar vooral voor het feit dat je altijd jezelf bent. En natuurlijk voor Cienema! Carla, bedankt voor al je praktische hulp op het lab in Utrecht en helemaal voor de berg werk die je in Amsterdam op het CLB hebt verzet. Die data staan dan wel niet in dit boekje, maar we hebben er wel weer veel van geleerd!

Een groot aantal mensen heeft zich door de jaren ingezet voor het goed lopen van het KNOOP4 project. Iedereen op de KNO-, OK-, lab en poli- afdelingen van de deelnemende ziekenhuizen, en natuurlijk alle ouders en kinderen die hebben meegedaan aan KNOOP4, ontzettend bedankt!
Beste Gerhard Zielhuis, als projectleider van KNOOP4 was jij, misschien dan wel op afstand maar zeker continu, zeer betrokken bij mijn promotieonderzoek. Jouw kennis van zaken vind ik indrukwekkend en je kritische vragen tijdens de regelmatige projectvergaderingen heb ik als zeer nuttig ervaren. Dank daarvoor!
Edith, Masja en Niels, jullie waren al flink op stoom toen ik me bij KNOOP aansloot en ik had dan ook heel wat extra uitleg nodig. Dank voor jullie geduld daarmee, voor alle vakkennis die jullie gedeeld hebben en natuurlijk voor de gezellige congressen en etentjes. Sonja, bedankt voor jouw voortdurende goede organisatie en zorg.

Na een tijdje breidde mijn werkterrein zich met de OMAVAX studie uit naar de acute middenoorontsteking. Iedereen die aan de OMAVAX studie heeft gewerkt (ik ken niet iedereen bij naam) in Haarlem, Rotterdam en Utrecht wil ik heel hartelijk bedanken voor zijn/haar inzet. Reinier, ontzettend bedankt voor de manier waarop jij dit onderzoek met hart en ziel hebt uitgevoerd. De door jou zeer nauwkeurig verzamelde onderzoeksgegevens hebben mede geleid tot een paar mooie hoofdstukken in dit boekje en zullen hopelijk binnenkort tot nog een aantal geaccepteerde gezamenlijke papers leiden.

Lieve kamergenoten en andere Aio's op en rond het lab, bedankt voor alle gezelligheid, steun en discussies. Enikö, Evelyn, Harriët, Marc, Pieter, en Stella, bedankt voor jullie goede voorbeelden! Ismé, jij was mijn meest recente directe voorbeeld. Over de manier waarop jij gestaag en rustig op je doel afging, dacht ik: "zo wil ik het ook wel doen...". Ik heb genoten van de twee jaar die we naast elkaar hebben doorgebracht, bedankt! Anne, je was er aan het begin van m'n aioschap en je bent er nu ook weer bij, leuk dat je terug bent! Cora, Wendy en Michael succes de komende jaren, volgens mij zit dat wel goed! Niels, misschien moeten we het koekjesreceptenboek toch nog een keer uitgeven, of ach, laat ook maar... Bastiaan, succes met je nieuwe toekomstplannen. Patricia, ik heb bewondering voor jouw doorzettingsvermogen; de finish is nu echt in zicht, aangezien je ook bent gaan hardlopen: succes met de eindsprint! Maike, jij bent er mijn hele vier jaar bij geweest! Altijd tijd voor een luisterend oor en een vriendelijk woord. Bedankt voor je gezelligheid en je statistiekhulp. Loes, gelukkig heeft Ger een voorliefde voor het aannemen van Brabo's want daardoor blijft het gezellig op het lab en in de aio kamer. We hebben een cluppie opgericht, voor mensen met een... mag ik er ook bij?

Alle collegae van de Pediatrische Immunologie en de Psycho Neuro Immunologie, ik noem niet iedereen bij naam, bedankt voor jullie interesse, hulp en gezelligheid op het lab en in de koffiekamer. Jullie zijn fantastische collegae geweest! Marleen, bedankt voor je hulp bij de honderden Elisa's, 't was een hele klus... Wilma en Tineke, bedankt voor de immuunglobuline- en subclassebepalingen en Vera Kamp voor alle IgE's.

Berent Prakken en iedereen van het "Prakken-lab", bedankt voor de input tijdens de werkbesprekingen en natuurlijk alle gezelligheid daaromheen. Alvin, Arash (see you in the Super De Boer!) Bas, Berber (we moeten toch echt nog een keer sámen een wedstrijdje lopen), Evelien, Huib, Joost (mocht je nog een keer terug naar Perth willen...), Lianne (met jou altijd een gesprek op gelijke hoogte, prettig!), Mark, Sarah, Sylvia (nu jij!!!), Titia (niet een echte Prakker maar wel bij de werkbesprekingen... succes met de Panda's) Wilco (Mister Luminex, dank voor je hulp!) en Yvonne bedankt en veel succes met jullie promoties, klinisch werk en verdere toekomstplannen!

Grada van Bleek, bedankt voor je scherpe blik en tong. Anne Schilder, voor je input vanuit de KNO en je kritische blik op abstracts en manuscripten.

Guy Berbers bedankt voor de mogelijkheid om op jouw lab op het RIVM de vele antistofbepalingen uit te voeren. Pieter van Gageldonk en Fiona van der Klis, bedankt voor de warme ontvangst en de perfecte begeleiding op het lab aldaar. Susan van den Hof, bedankt voor de Pienter data en alle uitleg daarover.

Kors van de Ent en Henk Ruven, bedankt voor de prettige samenwerking en wekelijkse SNP besprekingen. Martijn Slieker (ja je staat er echt in!) bedankt voor je hulp bij het ontwerpen van primers, de ritjes naar Nieuwegein en vooral voor je gezelligheid. Jan Broess, ontzettend bedankt voor de duizenden SNP-analyses die jij zo nauwkeurig hebt gedraaid op het KCL van het Antonius in Nieuwegein.

Voor alle "Dames van Montreal", laten we dat nog eens een keer herhalen! Ik heb al even gekeken, iemand zin in ICI 2007, Rio de Janeiro, Brazil??

Next to the work done in the Netherlands, I have spent five months in the molecular biotechnology/UDP lab of the Telethon Institute for Child Health Research in Perth, Western Australia. This has been an excellent adventure for me, for which I am grateful to a lot of people.

Dear Peter LeSouëf, I want to thank you for offering me this great opportunity to come to Perth, which gave me the ability to further develop myself in the field of immunology and genetics. This experience has enriched me workwise, as well as personally. Thanks for sharing your knowledge, your enthusiasm, and trust.

Dear Jack Goldblatt, the way you transfer your expertise, in a precise and indulgent way, is impressive. Thanks for your critical views and scientific input.

Dear Kim, thank you for your extraordinary technical help, patience with explaining things over and over again and support with all the statistical abracadabra. However, what I am most grateful for, are your understanding, kindness, and friendship. You made my stay in Perth unforgettable; we'll hopefully soon have another barbie, crabbing- or Penguin Island expedition!

Dear Gareth, as a real geneticist you have thought me about how to analyze haplotypes, Hardy-Weinberg, gene-environment interactions etc. etc. etc. Thanks for bearing with me. Soon the three-headed-monster will be reunited! Brad, your stats help was indispensable; we could never have done this without you. Dear Ingrid, thanks for your input on the data, project plans and papers, and especially for your and Mario's friendship. Margareth River was great, as well as the Tikka.22/250! Joelene, Pierre, Claire, Jamie, Amanda and everybody else from the institute, thanks for the barbecues, parties, movie- and gaming nights; you made my stay in Perth great fun!

En dan zijn er nog al die familie en vrienden, die misschien niet direct aan de inhoud van dit boekje hebben bijgedragen, maar zonder wie de afgelopen vier jaar toch een stuk minder aangenaam waren geweest. Dank dat jullie er altijd zijn!
Martijn en Matthijs, dankzij jullie enorme inzet en bereidheid tot meedenken, zijn de buiten- en binnenkant van dit boekje fantastisch geworden. Ontzettend bedankt voor jullie hulp en enthousiasme!
Adonut+ en iedereen die zich hieromheen beweegt, bedankt voor jullie interesse en de afleiding door middel van barbecues, pub-quizen en feestjes. Ralf bedankt voor het draaiende houden van Foebal, dat heeft deze vier jaar een stuk veiliger gemaakt!
Iels, Melis, Pien, San en Siets, lieve dames, dank voor jullie continue steun, vriendschap, gezelligheid bij etentjes en weekendjes weg, maar vooral voor het feit dat jullie allemaal zijn wie jullie zijn.
Mien en Piet, jullie immer weer welkome onthaal en de manier waarop jullie met ons meeleven is hartverwarmend, dank daarvoor!
Suus en Daan, wat hebben we heerlijke relaxte en gezellige avonden en weekendjes met z'n vieren. Dat dat nog maar lang zo mag blijven; dank voor alles!
Emo en Hanka, we komen snel weer een keer loungen in Bloemendaal! En ik ben erg blij dat mijn grote broer op de voor mij spannendste dag van dit jaar dicht naast me staat.
Pap en mam, dank voor alle mogelijkheden die jullie mij altijd gegeven hebben en het vertrouwen dat jullie in mij stellen. Jullie onophoudelijke steun heeft me gebracht waar ik nu ben, een plek waar ik het zeer naar m'n zin heb; iets dat me overigens niet tegenhoudt toch ook weer verder te kijken...

Guido, we hebben vaak grappen gemaakt over wat hier als laatste woorden in mijn dankwoord zou komen te staan. Toch wil ik je zeggen dat het mét jou gewoon zo veel fijner, leuker, spannender en makkelijker is; dank dat je er voor me bent!

Selma

Leave the beaten track occasionally and dive into the woods. Every time you do so you will be certain to find something that you have never seen before. Follow it up, explore all around it, and before you know it, you will have something worth thinking about to occupy your mind. All really big discoveries are the results of thought.

Alexander Graham Bell

Curriculum vitae

Selma Wiertsema werd op 31 maart 1977 geboren te Bunnik. Na het behalen van haar VWO- diploma (Athenaeum) in 1995, aan het Strabrecht College te Geldrop, begon zij aan de studie Medische Biologie aan de Universiteit Utrecht. Als onderdeel van deze studie verrichtte zij in 1998, onder begeleiding van Dr. ir. G.T. Rijkers, zes maanden onderzoek bij de afdeling Pediatrische Immunologie in het Wilhelmina Kinderziekenhuis te Utrecht. Vervolgens voerde zij gedurende het collegejaar 1998/1999 een stage-onderzoek van negen maanden uit bij de afdeling Pathologie van het Universitair Medisch Centrum in Utrecht. Dit onderzoek, dat onder begeleiding van Dr. M.G. Tilanus stond, richtte zich op de expressie van HLA klasse I op hoofd-hals tumorcellijnen. Na deze stage nam zij voor een jaar (1999-2000) als wedstrijdcommissaris zitting in het bestuur van de Algemene Utrechtse Studenten Roeivereniging Orca, waarvoor zij een bestuursbeurs van de Universiteit Utrecht ontving. Na het schrijven van haar scriptie volgde zij een extra-curriculair semester aan Brock University in St. Catherines, Canada. In 2001 sloot zij haar studie af met het behalen van het doctoraalexamen, waarna zij onder begeleiding van Prof. Dr. E.A.M. Sanders en Dr. ir. G.T. Rijkers als assistent in opleiding bij de afdeling Pediatrische Immunologie in het Wilhelmina Kinderziekenhuis te Utrecht werd aangenomen. Zij werd aangesteld op een NWO programmasubsidie (KNOOP4 genaamd) waarin vanuit een multidisciplinair samenwerkingsverband (KNO, Epidemiologie, Immunologie, Dierexperimenteel) van de Radboud Universiteit Nijmegen en het Universitair Medisch Centrum Utrecht, het ontstaan en mogelijk voorkómen van otitis media met effusie werd onderzocht. Ook het immunologisch aspect van acute middenoorontsteking ontwikkelde zich door de OMAVAX studie tot haar werkterrein. Gedurende haar onderzoeksperiode is zij meerdere malen te gast geweest bij het Laboratory for Vaccine preventable Diseases van het Rijksinstituut voor Volksgezondheid en Milieu in Bilthoven en bij het Telethon Institute for Child Health Research in Perth, Western Australia.

De resultaten van haar onderzoek zijn beschreven in dit proefschrift.

Tot 1 februari 2006 geniet zij een aanstelling bij de afdeling Pediatrische Immunologie. Verder is zij op het moment bezig met de voorbereidingen voor een baan als post-doc bij het Telethon Institute for Child Health Research in Perth, Western Australia.

Curriculum vitae

List of publications

Selma P. Wiertsema, Reinier H. Veenhoven, Vanessa Walraven, Cuno S.P.M. Uiterwaal, Anne G.M. Schilder, Ger T. Rijkers, Elisabeth A.M. Sanders: *Pneumococcal vaccine efficacy for mucosal pneumococcal infections depends on Fcγ receptor IIa polymorphism*
Vaccine, 2005 (in press)

Niels van Heerbeek, Masja Straetemans, **Selma P. Wiertsema**, Koen J.A.O. Ingels, Ger T. Rijkers, Anne G.M. Schilder, Elisabeth A.M. Sanders, Gerhard A. Zielhuis: *The effect of combined pneumococcal conjugate and polysaccharide vaccination on recurrent otitis media with effusion*
Pediatrics, 2005 (in press)

Selma P. Wiertsema, Reinier H. Veenhoven, Elisabeth A.M. Sanders, Ger T. Rijkers: *Immunological screening of children with recurrent otitis media*
Curr Allergy Asthma Rep. 2005 Jul; 5(4): 302-7.

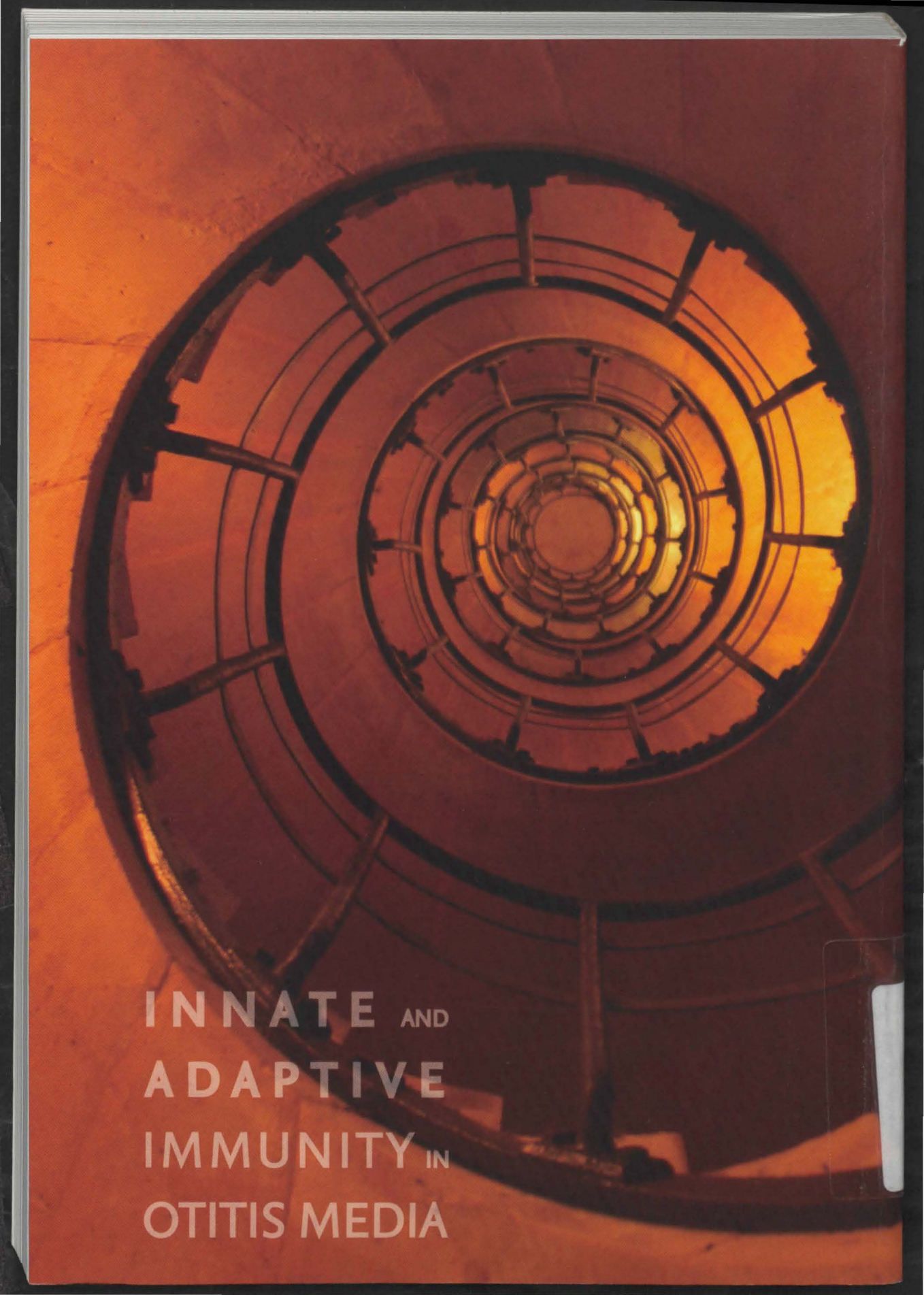
Masja Straetemans, **Selma P. Wiertsema**, Elisabeth A.M. Sanders, Ger T. Rijkers, Kees Graamans, Bert van der Baan, Gerhard A. Zielhuis: *Immunological status in the Aetiology of Recurrent Otitis media with Effusion: Serum Immunoglobulin Levels, Functional Mannose- binding Lectin and Fc Receptor Polymorphisms for IgG*
J Clin Immunol. 2005 Jan; 25(1): 78-86

Selma P. Wiertsema, Elisabeth A.M. Sanders, Reinier H. Veenhoven, Niels van Heerbeek, Susan van den Hof, Guy A.M. Berbers, Ger T. Rijkers: *Antibody levels after regular childhood vaccinations in the immunological screening of children with recurrent otitis media*
J Clin Immunol. 2004 Jul; 24(4): 354-60

M. Feenstra, M. Veltkamp, J. van Kuik, **S. Wiertsema**, P. Slootweg, J. van den Tweel, R. de Weger, M. Tilanus: *HLA class I expression and chromosomal deletions at 6p and 15q in head and neck squamous cell carcinomas*
Tissue Antigens, 1999 Sep; 54(3):235-45

List of abbreviations

AOM	acute otitis media
DGGE	denaturing gradient gel electrophoresis
ELISA	enzyme linked immuno sorbent assay
FcγR	Fc gamma receptor
IL	interleukin
LPS	lipo-polysaccharide
MASP	MBL-associated serine protease
MBL	mannan-binding lectin
NTHi	non-typeable *Haemophilus influenzae*
OM	otitis media
OME	otitis media with effusion
PAMP	pathogen-associated molecular pattern
PCR	polymerase chain reaction
PCV7	7-valent pneumococcal conjugate vaccine
PPV23	23-valent pneumococcal polysaccharide vaccine
PRR	pattern-recognition receptor
PS	polysaccharide
RSV	respiratory syncytial virus
SNP	single nucleotide polymorphism
TLR	Toll-like receptor



INNATE AND ADAPTIVE IMMUNITY IN OTITIS MEDIA