Pneumococcal conjugate vaccination

Clinical impact, reduced-dose schedules and immunologic responses

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Clinical impact, reduced-dose schedules and immunologic responses

Pneumokokkenconjugaatvaccinatie
Klinische impact, gereduceerde vaccinatieschema's
en immunologische responsen

(met een samenvatting in het Nederlands)

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If I know all mysteries and all knowledge – but don't have love, I am nothing.

1 Corinthians 13:2 (World English Bible)

Contents

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Introduction

General Introduction

In 1880, both George M. Sternberg in the United States and Louis Pasteur in France isolated an organism from saliva, which proved to be the bacterium '*Streptococcus pneumoniae*' as we know it now.[1] This gram-positive, polysaccharide encapsulated bacterium has been identified as a common but transient colonizer of the human nasopharynx. Typically, *S. pneumoniae* spreads from person to person by airborne droplets or via direct physical contact. In healthy young children asymptomatic nasopharyngeal carriage rates may be as high as 70-80% or more.[2;3]

Burden of pneumococcal disease

Besides being a commensal, pneumococci can act as an opportunistic pathogen. Colonization of the nasopharynx is the first step to develop invasive pneumococcal disease (IPD) like meningitis or septicemia, but can also lead to more common respiratory tract infections like non-invasive pneumonia and otitis media.[2;4] Incidence rates of pneumococcal disease vary greatly across the world with highest infection rates and case fatalities in poor resource countries.[4;5] Risk groups for pneumococcal disease are in particular young children and elderly. According to estimations of the WHO at least 1.6 million people die of pneumococcal disease every year worldwide, of which 700.000 to 1.000.000 are under 5 years of age.[4;6] In this age-category pneumococcal infections account for 11% of overall mortality in HIV-negative children worldwide, whereas it is also a major pathogen in HIV-infected children.[4] A major determinant of the overall virulence of this bacterium is the polysaccharide capsule that surrounds the bacterium, while unencapsulated pneumococci are considered to be non-pathogenic (Figure 1).[1;5] Currently, over 90 pneumococcal serotypes have been identified on basis of polysaccharide capsule composition, each inducing unique humoral immune responses.[5;7;8] Although distribution of serotypes causing IPD differs slightly between countries worldwide, in young children most IPD is caused by only a limited number of serotypes (Figure 2).[9]

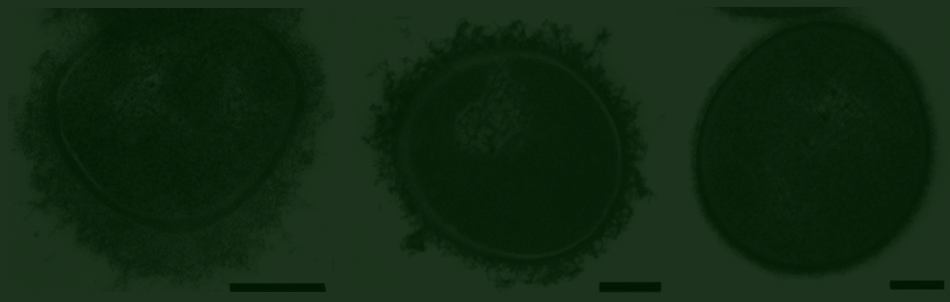

Figure 1. Pictures of polysaccharide encapsulated serotype 1 (Figure 1a), serotype 19F (Figure 1b) and unencapsulated pneumococci (Figure 1c) by field emission scanning electron microscopy after lysine-ruthenium red fixation procedure.[51]

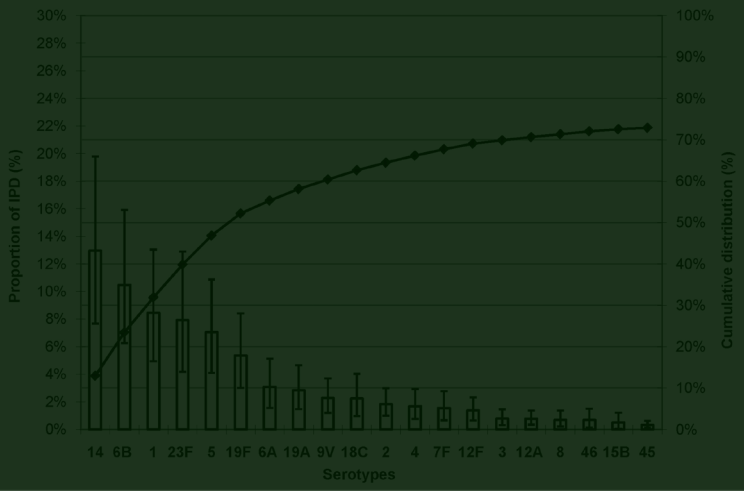

Figure 2. Proportion and cumulative distribution of IPD in young children due to the most common serotypes globally.[9]

Protein-polysaccharide conjugated vaccines

Already in 1911, around 30 years after discovery of the pneumococcus, first trials were undertaken to vaccinate humans against this pathogen and to prevent pneumonia in healthy adults.[1] Soon after the discovery that anticapsular antibodies conferred serotype-specific protection against challenge in animal models, development of multivalent pneumococcal polysaccharide vaccines started in 1931.[1] Unfortunately, in young children, with highest burden of pneumococcal disease, polysaccharides of encapsulated bacteria like *S. pneumoniae* proved to be poorly immunogenic due to immaturity of the T cell independent responses and the marginal zone B cell compartment.[10-12] The polysaccharide vaccines showed lack of efficacy against invasive diseases in most clinical trials in children.[13] Another drawback of these T cell independent polysaccharide vaccines was the lack of memory induction.[10-12] The solution was offered by the development of polysaccharide-protein-conjugated vaccines, in which polysaccharides are linked to a carrier protein, thereby enabling recruitment of CD4+ T cells. Conjugate vaccines proved to be immunogenic already in infants of a few weeks of age and to induce B cell memory.[14] In the 1980's, polysaccharide-protein vaccines against *Haemophilus influenzae* type b (Hib) were the first to be introduced, followed by conjugate vaccines against *Neisseria meningitidis* serogroup C (MenC) in the 1990's. Both conjugate vaccines conferred good immunological responses in the youngest age-groups accompanied by high clinical effectiveness.[15;16] Moreover, widespread use of the conjugate vaccines was associated with reduced disease rates in non-vaccinated age-groups, which was explained by reduced colonization of vaccine strains in vaccinees and concomitant reduced transmission rates in the community (herd effects).[15;17;18] The great success of conjugate vaccines is largely dependent on this nasopharyngeal carriage eradication of the target bacteria in vaccinees and the subsequent herd effects in the population.

Multivalent pneumococcal conjugate vaccines

In 2000, the 7-valent CRM197-conjugated pneumococcal vaccine (PCV7) was licensed in the United States for children less than 5 years of age for prevention of pneumococcal disease.[19] In North America this vaccine covered the 7 serotypes (i.e. serotypes 4, 6B, 9V, 14, 18C, 19F and 23F) which accounted for approximately 80% of the invasive disease burden in children less then 5 years of age in this continent.[5] PCV7 was licensed in a 3+1-dose vaccination schedule with 3 primary doses in the first 6 months of life, followed by a booster dose in the second year of life. In the years following introduction in the United States, a high effectiveness (>90%) in the target group of children was observed in prevention of vaccine serotype IPD, and to a lesser extent in prevention of mucosal disease like pneumonia and acute otitis media (AOM).[19;20] In line with experiences from the Hib and MenC conjugate vaccines, pneumococcal conjugate vaccines reduced nasopharyngeal vaccine serotype acquisition and colonization, resulting in decreased circulation of vaccine serotype strains in the population.[21;22] Therefore, in the United States introduction of this vaccine for children was associated with reduced vaccine serotype pneumococcal invasive disease incidences in both vaccinated and unvaccinated age groups like eldely.[21;23] Inclusion of herd effects as observed in the United States contributed substantially to the cost-effectiveness estimates of PCV7 in the Netherlands in 2005.[24] PCV7 was introduced in the Dutch national immunization program in June 2006 for all newborns born after April 1th, 2006 at the age of 2, 3, 4 and 11 months without a catch-up program for other age-groups.[24]

Disappearance of vaccine serotypes and emergence of non-vaccine serotypes

After PCV7 administration, vaccine serotypes are eradicated from the nasopharynx.[2;22] The vacant niche therefore seems an opportunity for serotypes not covered by PCV7 to increase in carriage prevalence.[25] Concomitant with the decrease of vaccine serotype carriage, an increase of non-vaccine serotype pneumococcal carriage counterbalances the reduction in vaccine serotypes and results in similar overall pneumococcal carriage.[2;22] The implications of replacement colonization for invasive pneumococcal disease are however unclear. Overall IPD rates in children in the United States decreased rapidly within the first 2 years following introduction of PCV7. However, the 77% overall reduction in IPD in children under 5 years of age already leveled off in 2002 due to gradual increase of non-vaccine serotype IPD which counterbalanced the ongoing decrease of vaccine serotype IPD.[21;26] In particular non-vaccine serotype 19A accounted for approximately 40% of all childhood IPD cases in 2005 in the United States.[26]

The observed PCV7 effectiveness in the United States in children and other age categories cannot directly be extrapolated to Europe. First of all, in the United States the IPD incidence rates in children seem much higher compared to European countries like the Netherlands and the United Kingdom.[27-29] Before introduction of PCV7 in the Netherlands, overall IPD rates were 35 cases/100,000 children <2 years of age, while in the United States, IPD incidence before introduction of PCV7 peaked at 188 cases/100,000 children <2 years of age.[23] This difference is largely explained by different blood culture practices. In the United

States, at least 60% of all blood culture or IPD are obtained from children presenting with high fever in outpatient settings.[27] In the Netherlands, primary care functions as 'gatekeeper' for patients to attend secondary care, and patients with milder clinical syndromes (transient bacteremia presenting as high fever) resolve or are treated successfully at the primary care level without admission to the hospital. Blood cultures are restricted to severely ill children who are referred to hospitals. Disease rates for severe clinical syndromes like meningitis are similar in the United States and the Netherlands.[23;29] Furthermore vaccine serotype coverage by PCV7 is slightly lower in European countries (up to 60-70%) compared with 80% coverage in the United States.[5] Also other factors like environmental exposure and ethnicity may contribute to differences.[30] The time needed for high vaccine coverage and catch-up rates for children after starting a national PCV7 program may also differ and have influenced observations. These differences underline the significance of high-quality surveillance of IPD disease rates after nationwide implementation in the Netherlands.

Correlates of Protection

The protection by pneumococcal conjugate vaccines against invasive disease is provided through induction of anticapsular serum IgG antibodies which activate complement and enhance phagocytosis of pneumococci by macrophages.[31] Considering these antibodies a mechanism of protection, the WHO agreed consensus on a post-primary antibody level of 0.35 µg/ml as a 'threshold for protection' against IPD.[32] This was estimated from data of three double blinded controlled trials performed in Northern California (United States), American Indians and South Africa with multivalent PCVs, although it should be recognized that the estimated thresholds differed largely between the different geographic regions and per serotype.[33] The 0.35 µg/ml post-primary threshold level has been recommended by the WHO as applicable for assessing the efficacy against IPD and non-inferiority of future PCVs.[32] As protection is provided by opsonin-dependent phagocytosis, the WHO has also suggested that in vitro opsonophagocytic activity (OPA) of serum is of additional value in evaluation of vaccine efficacy.[32] Though, still this assay is difficult to standardize and limited data are available on the relation between IgG antibody levels, functional OPA titers and clinical efficacy for the different vaccine serotypes.[31;34] Apart from IPD, 'correlates of protection' have also been suggested for respiratory pneumococcal disease like otitis media and for nasopharyngeal pneumococcal carriage, but these levels are rough estimates and exact data are lacking.[35;36] For nasopharyngeal colonization systemic serotype-specific IgG levels are reported to be inversely related to new nasopharyngeal acquisition of the given serotype.[37;38] It seems that much higher IgG levels are associated with eradication of vaccine serotypes at the mucosal level compared with IPD. Furthermore, the contribution of antibodies in protection against pneumococcal disease at other anatomical sites, like the mucosal surface, is largely unknown and deserves further investigation. PCVs are known to induce IgG and IgA antibodies at the mucosal level, but data about the magnitude and dynamics of these antibodies are scarce.[39-43]

Long-term protection and memory development

For sustained protection against IPD, generation and maintenance of functional circulating serum antibodies in the first years of life seem mandatory.[14] However, although protein-polysaccharide conjugate vaccines induce high serotype-specific IgG responses in young children shortly after immunization, antibody levels after the primary series wane rapidly.[44] Also, the presence of high avidity memory B cell populations are thought to be relevant for long-term protection.[14;45] After immunization, conjugate vaccines initiate naïve B cells to differentiate into plasma cells and memory B cells through involvement of CD4+ T cells. Germinal centres are formed and affinity maturation takes place through somatic hypermutation.[45] The immunological B cell memory can be evaluated by measuring the avidity of circulating antibodies. Also the induction of a rapid IgG antibody response and the detection of plasma and memory B cells shortly after administration of a challenge PCV vaccination may provide insight in memory induced by previous vaccinations.[32;45] Especially after nationwide vaccine implementation induction of immunological memory by vaccinations will become highly relevant. Natural exposure to *S. pneumoniae* by colonization probably primes B cells, initiates differentiation of memory B cells into plasma B cells and may boost capsule-specific antibody levels (natural boosting). Disappearance of circulating vaccine strains in the community by herd effects after nationwide vaccine implementation, may implicate loss of this natural boosting of the immune system.[46] The impact of decreased nasopharyngeal exposure by vaccine strains was previously shown in the United Kingdom after nationwide introduction of conjugate vaccines for Hib and MenC in infancy.[47] When limited to primary doses under 6 months of age without a booster in the second year of life, antibody levels showed rapid decreases within one year after the primary series.[48;49] For this reason, a booster injection around 12 months of age for both Hib and MenC conjugate vaccines was implemented in the United Kingdom in 2006 to prevent break-through cases under 5 years of age.[47] For pneumococcal disease, limited data are available regarding the effect of additional boosting by vaccines or natural exposure on pneumococcal antibody persistence for the different pneumococcal serotypes as well as on the markers of memory development.[14] Also whether waning anticapsular antibody levels result in waning protection against pneumococcal disease is currently unknown.

Reduced-dose schedules

Although implemented in the United States in a 3+1-dose schedule, high levels of clinical protection against IPD were already observed after less than 4 doses in the licensure study for PCV7, the Northern California Kaiser Permanente study.[19] In this study, clinical efficacy against vaccine serotype IPD was high (93.9%) despite the fact that only 58% of the 19.000 children had received the full 3+1-dose PCV7 schedule. Furthermore, the effectiveness of reduced-dose schedules in preventing vaccine serotype IPD in vaccinated children was also observed in a large case-control study from the United States showing high reductions with a 2+1-dose and even a 2-dose schedule during a period of vaccine shortage.[50] Increasingly crowded immunization programs as well as costs have prompted exploration of PCV7 schedules with fewer doses. However, immunogenicity studies comparing 2 and 3 primary

dose schedules in infants are scarce, as are data of reduced-dose PCV7 schedules regarding long-term immunity like serotype-specific memory induction, antibody persistence and functionality.

Studies of the present thesis

Invasive Pneumococcal Disease Surveillance

In the present thesis, we evaluated the impact of PCV7 introduction in the national immunization program (NIP) in the Netherlands on incidences of invasive pneumococcal disease. We assessed the epidemiology of IPD for different invasive clinical syndromes (meningitis, bacteremia, invasive pneumonia) in all age groups before and 2 years after nationwide PCV7 implementation in 2006. Isolates of all patients with culture confirmed IPD were submitted by nine sentinel laboratories to the Netherlands Reference Laboratory for Bacterial Meningitis (NRLBM), covering approximately 25% of the Dutch population and 27 hospitals well distributed over the country (Figure 3). The NRLBM is a laboratory-based surveillance system that collects nationwide bacterial isolates from blood, cerebrospinal fluid (CSF) and/or other normally sterile bodily fluids. Information about patient demographics, underlying conditions and disease course were retrospectively abstracted from hospital records by trained medical students using a standard data collection form. Pre-implementation data of IPD patients <18 years were collected of June 1, 2001 to June 1, 2006. Of patients ≥18 years of age data were collected between June 1, 2004 and June 1, 2006. Post-implementation data were collected for all ages between June 1, 2006 and June 1, 2008. Besides IPD disease incidences before and after nationwide PCV7 implementation, the relation of serotypes and disease severity was explored in adults and children based on these large surveillance studies.

Figure 3. Distribution of participating hospitals in surveillance study after invasive pneumococcal disease in the Netherlands.

We studied the immune responses in infants and toddlers after reduced–dose PCV7 schedules and a full 3+1-dose PCV7 schedule. The immunological data from reduced-dose PCV7 schedules were obtained from a randomized controlled trial in the Netherlands (MINOES trial, Figure 4) that started well before the national implementation of the vaccine in the NIP. Groups of infants received various vaccination schedules, (a) two primary doses of PCV7 at 2 and 4 months of age (2-dose group); (b) two primary doses at 2 and 4 months followed by a booster dose at 11 months of age (2+1-dose group); (c) no PCV7 vaccination (control group) (Table for study design). All parents living in the western region of the Netherlands were informed about the study by written information in their newborn's first weeks of life and asked to participate. Infants younger than 12 weeks, not yet having received any infant vaccination and living in the study region, were eligible for inclusion. Exclusion criteria were known immunodeficiency, craniofacial or chromosomal abnormalities, language barrier, or expected relocation within the follow-up period. Enrollment started on July 7, 2005, and was completed on February 9, 2006. Follow-up ended February 14, 2008. Participants did not receive any financial compensation.

Table. Study design of randomized controlled trial after reduced-dose PCV7 schedules.

	Age								
Study Group	2 months	4 months	6 months	11 months	12 months	18 months	24 months	+7-9 days	+28-42 days
2-dose schedule	**PCV7**	**PCV7**					**PCV7** *(optional)*		
2+1-dose schedule	**PCV7**	**PCV7**		**PCV7**			**PCV7** *(optional)*		
Controls							**PCV7** *(optional)*		
Nasopharyngeal swab (all)	NP Swab*		NP Swab		NP Swab	NP Swab	NP Swab		
Blood Sample (subgroups)					IgG levels		IgG levels B Cells	IgG levels B Cells	IgG levels
Saliva Sample (subgroups)					IgG / IgA levels		IgG / IgA levels		

*taken at 6 weeks

Immune responses after PCV7 schedules

After written informed consent had been obtained from either parents or guardians, infants were randomly allocated by simple randomization via a computer randomization interface during the first home-visit. PCV7 was administered intramuscularly during regular well-baby clinic visits, together with routine immunizations according to the national immunization program. Parents were aware of the child's vaccine schedule. Five home visits were conducted in which nasopharyngeal swabs were taken at the age of 6 weeks, and at 6, 12, 18 and 24 months of age. From subgroups of infants also blood samples were taken at 12 and/or 24 months. Furthermore, salivary samples were obtained from other subgroups next to nasopharyngeal swabs. After completing the study at 24 months all children were

offered a PCV7 vaccination. For subgroups who received this vaccination, an additional home-visit was planned at 7-9 days or 28-42 days after this vaccination and blood samples were collected (Table). Laboratory personnel were unaware of treatment allocation and the randomization key was not disclosed until after the study was completed.

Data from our randomized controlled study on reduced-dose PCV7 schedules were compared with immunological data from two independent population-based immune-surveillance studies from children that had received a full 3+1-dose schedule according to the NIP. The first study concerned a serological immune-surveillance study on pertussis vaccination, performed in March-June 2007. Pre- and post-booster blood samples were obtained from healthy infants aged 11 and 12 months that had started immunizations within the first 3 months of national PCV7 introduction in 2006. Thus all children had received 3 primary doses of PCV7 at 2, 3 and 4 months of age. In the other surveillance study, we collected blood samples from children 11 and 24 months of age between February and July 2009, 3 years after nationwide PCV7 implementation in the Netherlands (Kokki study, Figure 4). All children were vaccinated with PCV7 according to the NIP. Blood was drawn from: healthy 11-month-old children before or 7-9 days after the 11-month-booster and healthy 24-month-old children before or 7-9 days after an additional PCV7 challenge dose. The participants were born between April-August 2008 (11 months old) and March-June 2007 (24 months old).

Figure 4. Logo's of studies MINOES and Kokki.

Objectives & outline thesis

In this thesis we describe 3 different aspects of pneumococcal conjugate vaccination.

Part 1. Invasive pneumococcal disease in the Netherlands before and after implementation of the pneumococcal conjugate vaccine

In chapter 2 we evaluated the baseline epidemiology and burden of invasive pneumococcal disease in the Netherlands before nationwide PCV7 introduction per age-group, including clinical syndromes, disease course and underlying comorbidities. Next we assessed in chapter 3 the impact of PCV7 on different IPD manifestations in PCV7-vaccinated and

-unvaccinated age groups in the first 2 years after nationwide implementation in the Netherlands. We assessed the association between infecting serotypes and disease severity and outcome in adults in chapter 4 and in children younger then 18 years in chapter 5.

Part 2. Immune responses after reduced-dose schedules with the pneumococcal conjugate vaccine before nationwide implementation

In chapter 6, we evaluated the comparability of IgG antibody responses to PCV7 after a 2+1 and a 3+1-dose schedule before and after the booster dose at 11 months of age. In chapter 7 predictors of long term immunity (antibody avidity maturation, antibody responses upon challenge PCV7 at 24 months, and memory B cells) were evaluated at the age of 24 months after 2 primary doses with or without an 11-month PCV7 booster dose or no previous PCV7 vaccination (controls). In chapter 8 we compared the functionality of serum antibodies, i.e. in vitro opsonophagocytic activity, at the age of 12 and 24 months in children having received reduced-dose PCV7 schedules and in PCV7-unvaccinated controls. Also we assessed both quantitative and functional immune responses after a PCV7 at 24 months of age as additional booster dose or as a primary dose in previously unvaccinated controls. In chapter 9 we examined the effects of a 2-dose and 2+1-dose PCV7 schedule on mucosal salivary IgG and IgA responses compared with PCV7-unvaccinated controls.

Part 3. Effect of pneumococcal carriage on immune responses to pneumococcal conjugate vaccination after nationwide implementation

Eradication of vaccine serotype strains in the population may have impact on vaccine serotype-specific immune responses and vaccine serotype antibody maintenance after PCV7 administration. In chapter 10, we explored the impact of previous pneumococcal carriage on homologous and non-homologous serotype-specific IgG responses to a PCV7 challenge at 24 months of age. In chapter 11 we investigated the impact of vaccine serotype carriage reduction on anticapsular antibody levels by comparing IgG antibody levels towards vaccine serotypes in children who received PCV7 before or after routine implementation. In chapter 12 we described the serotype-specific memory B cell compartment of children vaccinated in the post implementation period and we compared memory B cell frequencies and IgG responses with post-PCV7 challenge responses of age-matched children vaccinated before nationwide implementation and herd effects.

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Part One

Invasive pneumococcal disease in the Netherlands before and after implementation of the pneumococcal conjugate vaccine

2

Invasive pneumococcal disease in the Netherlands: Syndromes, outcome and potential vaccine benefits

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Abstract

In this retrospective study of invasive pneumococcal disease (IPD), surveillance data were analyzed representative for 25% of the Dutch population (1275 hospitalized cases) over the period June 2004–June 2006 prior to the implementation of the 7-valent pneumococcal conjugate vaccine (PCV7). Aim of the study was to provide baseline data on IPD for the interpretation of changes after implementation of conjugate vaccines. The IPD incidence peaked in 3–5-mnth-olds (63 cases per 100,000 persons yearly) and increased in adulthood, particularly after the age of 60 yrs, from 26 cases in 60–64-yr-olds to 97 cases per 100,000 in persons ≥90 yrs. Beyond the age of 4 yrs, 19% of IPD patients were immunocompromised, and this considerable percentage may have implications for vaccine efficacy.

Introduction

The introduction of the 7-valent pneumococcal conjugate vaccine (PCV7) in the United States in 2000 was followed not only by a dramatic decrease in invasive pneumococcal disease (IPD) in the target group of children younger than 2 yrs of age, but also by a considerable decrease of IPD among unvaccinated persons of all ages which was attributed to a herd effect.[1-3] Apart from an overall decrease in IPD, a discrete emergence of non-vaccine serotype IPD was observed, in particular but not limited to serotype 19A.[4] With their high pneumococcal nasopharyngeal carriage rates, children represent the largest reservoir for spreading pneumococci in the community. Carriages studies have shown that after immunisation with PCV7 the overall pneumococcal nasopharyngeal carriage rates remain more or less similar, albeit vaccine serotypes decrease and are replaced by non-vaccine serotypes.[5] It was hypothesised that widespread use of conjugate vaccination might favour pneumococcal capsular switching, though actual replacement by non-vaccine serotypes filling the ecological vacant niche seems to be responsible for this shift in nasopharyngeal colonization.[4;6] Continuous and high-quality surveillance of IPD in different populations and at different geographic locations is therefore of utmost importance to warrant timely detection of potential shifts in serotype distribution in IPD induced by vaccination.[7]
Direct and indirect (herd) effects of PCV on IPD vary with age, presence of underlying conditions, and the disease manifestation (clinical syndrome). For example, immunocompromised persons like those with HIV seem to benefit less of herd effects because they appear prone to replacement disease.[8] Also, herd effects in adults seem to occur mainly for pneumococcal bacteremia; for meningitis, replacement by non-vaccine types reduced the benefit of the decline in vaccine-type disease in persons aged 50 yrs and older.[2] The current study aimed to provide a pre-vaccination baseline on age-specific IPD in terms of incidence, clinical syndrome, disease course, the prevalence of co-morbidity, and theoretical coverage of IPD by the current and newly developed multivalent PCV, which will help to interpret the impact of future use of PCV in Western European countries.

Methods

Pneumococcal surveillance data

Pneumococcal surveillance data were provided by the Netherlands Reference Laboratory for Bacterial Meningitis (NRLBM). The NRLBM is a laboratory-based surveillance system that collects nationwide bacterial isolates from blood and cerebrospinal fluid (CSF) of almost exclusively patients requiring hospitalization. Isolates from other normally sterile bodily fluids comprise less than 3% of all isolates. Nine sentinel microbiology laboratories spread across the Netherlands covering approximately 4.1 million inhabitants, a representative proportion of ~25% of the Dutch population, have sent all pneumococcal isolates from normally sterile sites to the NRLBM from2004 onwards. These laboratories submitted 90% of the pneumococcal isolates from CSF and 83% of all pneumococcal isolated from the blood (internal survey, personal communication, Spanjaard). Pneumococcal isolates received by

the NRLBM were typed by co-agglutination and serotyped by the capsular swelling method (Quellung reaction) using antisera (Statens Serum Institute, Copenhagen, Denmark). The current study describes all IPD data obtained from the period June 2004 to June 2006 prior to the introduction of PCV7 in the national immunisation program. Because bacterial resistance to antibiotics has traditionally been very low in the Netherlands this has not been further analysed in the current study; of the pneumococcal isolates received during the study period, 98.9% were susceptible to penicillin (minimal inhibitory concentration <0.06), 0.7% were intermediately susceptible (0.06–1.0), and 0.4% were resistant (>1.0).[9]

Case characteristics

Information about the clinical syndrome, underlying conditions, and disease course (length of hospital stay, admission to the Intensive Care Unit (ICU), and sequelae at discharge from the hospital) was retrospectively extracted from hospital records by trained medical students using a standard data collection form. Meningitis was defined as a CSF culture positive for *Streptococcus pneumoniae* (or a positive CSF PCR) or a clinical diagnosis of meningitis in combination with a blood culture positive for *S. pneumoniae*. Invasive pneumonia included a physician-diagnosed pneumonia together with a blood culture positive for *S. pneumoniae*. Bacteremia with other focus was defined as a positive blood culture in combination with a clinical focus other than meningitis or pneumonia. In case of bacteremia without focus, no clinical focus was identified.

Underlying conditions were subdivided in immunocompromising conditions and others. Immunocompromising conditions included primary immunodeficiency, HIV (with or without progression to AIDS), lymphoma, leukemia, myeloma, solid organ or stem cell transplantation, current immunosuppressive therapy for malignancy or autoimmune disease, asplenia/splenectomy, sickle cell disease, renal insufficiency/need for dialysis, and nephritic syndrome. Other co-morbidity consisted of current malignancies not considered to be immunocompromising, chronic obstructive pulmonary disease/asthma (among persons aged 5 yrs and older), diabetes mellitus, cardiovascular disease (myocardial infarction, coronary artery condition, cerebrovascular accident/transient ischemic attack, cardiomyopathy/heart failure, heart valve disease, presence of cerebral/abdominal/thoracic aneurysms), thyroid disease, liver disease, intravenous drug use, alcohol abuse, CSF leak and recent severe physical trauma/skull fracture. For cases in children, also premature birth (<37 weeks for 0–1-yr-olds and <32 for 0–4-yr-olds), serious perinatal complications for 0–1-yr-olds, congenital conditions/syndromes, and failure to thrive were recorded. Case-fatality was defined as in-hospital death and/or death within 30 days after the first reported blood/CSF culture positive for *S. pneumoniae*. Information about sequelae at discharge from the hospital was extracted from hospital records and coded as: mental retardation, deafness, paralysis/paresis, cranial-nerve palsy, aphasia, hydrocephalus, renal insufficiency requiring dialysis, peripheral gangrene requiring skin transplantation, epilepsy requiring antiepileptics, and the category others.

Dutch pneumococcal vaccination guidelines

In the Netherlands, PCV7 was introduced for all newborns born after March 31, 2006 without a catch-up program for older children. As the vaccine is administered at 2, 3, 4, and 11 months of age, eligible infants were vaccinated from June 2006 onwards. The 23-valent pneumococcal polysaccharide vaccination is recommended for risk groups but has not been routinely recommended for the elderly thus the uptake has been negligible in the Netherlands.[10]

Statistical aspects

Analyses were performed with SPSS 12.0.2. The 95% confidence intervals (95%CIs) of proportions were determined with Episheet (Rothman K. Episheet: Spreadsheets for the analysis of epidemiologic data, 2002). Average annual age-specific incidence rates of IPD were determined per 100,000 persons by adding the incidence of blood cultures positive for *S. pneumoniae* to the incidence of positive CSF cultures/PCR. The incidence of blood cultures positive for *S. pneumoniae* was estimated by dividing the number of isolates reported by the 9 sentinel labs during the study period by the estimated number of person years followed, i.e. the coverage of 25% of the Dutch population by the 9 labs multiplied by the study period in years (Statistics Netherlands, Voorburg/Heerlen, the Netherlands). Compared with blood cultures positive for *S. pneumoniae*, CSF cultures are more reliably reported nationwide (over 80%) and consequently nationwide figures were used to calculate incidences of CSF cultures positive for *S. pneumoniae*. Also the proportions of clinical syndromes within IPD were calculated. With respect to the current and future multivalent PCV, theoretical age-specific coverage of IPD by PCV7 (serotypes 4, 6B, 9V, 14,18C, 19F, 23F), and the experimental PCV10 (1, 4, 5, 6B, 7F, 9V, 14, 18C, 19F, 23F) and PCV13 (1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, 23F) was calculated. The mean (median) length of hospital stay with interquartile range, proportion patients admitted to ICU, case fatality rate, and sequelae at discharge were determined per clinical syndrome and age group.

Results

During the 2-yr study period 1275 cases of IPD were reported. Serogroup and/or serotype was available for 1264 (99%) and additional clinical information for 1226 (96%). In total, 98 cases were reported in children younger than 5 yrs of age, 216 in 5–49-yr-olds, 266 in 50–64-yr-olds, and 695 in persons aged 65 yrs and older.

The incidence rate of IPD peaked in early infancy with a maximum of 63 cases per 100,000 annually in 3–5-mnth-olds, had a nadir of 1 case per 100,000 in 10–14-yr-olds and increased again with age from 11 cases per 100,000 in 50–54-yr-olds to 26 per 100,000 in 60–64-yr-olds and 97 cases per 100,000 annually in persons aged 90 yrs and older (Figure 1). Underlying conditions were present in 30% (95%CI 21–42%) of the cases among 0–1-yr-olds, mostly concerning premature birth. The prevalence of co-morbidity increased to 80% (95%CI 76–82%) in those aged 65 yrs and older (Supporting information Table S1, Figure 2). After

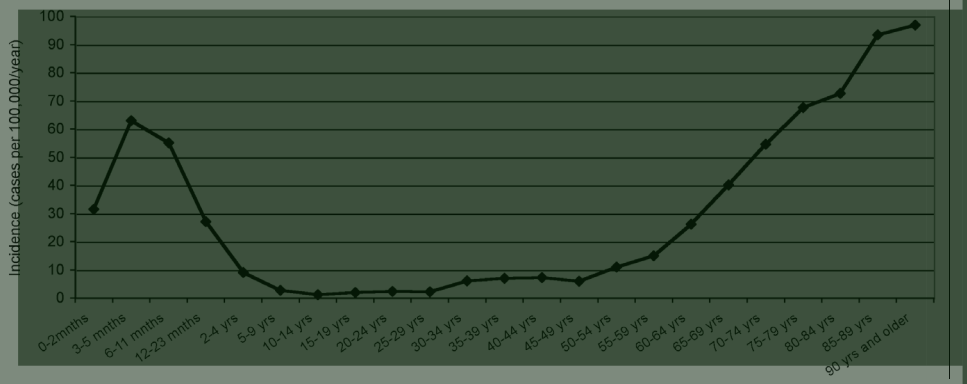

Figure 1. Incidence of invasive pneumococcal disease by age in the Netherlands over 2004–2006

the age of 4 yrs, 19% (95%CI 17–21) of the individuals with IPD had immunocompromising conditions.

In 0–1-yr-olds, the most common clinical syndrome of IPD was meningitis (44%, 95%CI 33–56%). The proportion meningitis decreased with age to 7% (95%CI 5–9%) of all IPD cases in those aged65 yrs and older (Figure 3). Invasive pneumonia accounted for 16% (95%CI 9–27%) of IPD cases in 0–1-yr-olds and 83% (95%CI 80–85%) among 65 yrs and older. Bacteremia without focus varied from20% (95%CI 13–29%) of all IPD cases in 0–4-yr-olds to 8% (95%CI 6–9%) of those in persons aged 5 yrs and older. All cases of IPD in those aged 5 yrs or younger were hospitalized, 97.6% in 5–49-yr-olds, 99.2% in 50–64-yr-olds, and 99.7% in those aged 65 yrs and older. Patients with meningitis had the longest hospital stay and the highest ICU-admission rate (Table 1). Patients aged 50 yrs and older had the longest hospital stay followed by 0–23-mnth-olds.

Overall, bacteremia without focus and meningitis were associated with the highest case-fatality rates (Table 1). Invasive pneumonia had a case-fatality of 20% in the elderly.

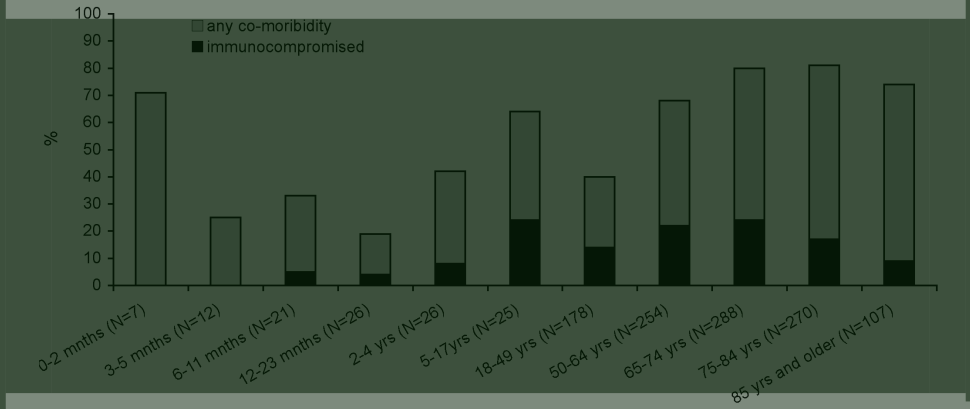

Figure 2. Prevalence of immunocompromising and any of the defined underlying conditions in invasive pneumococcal disease by age.

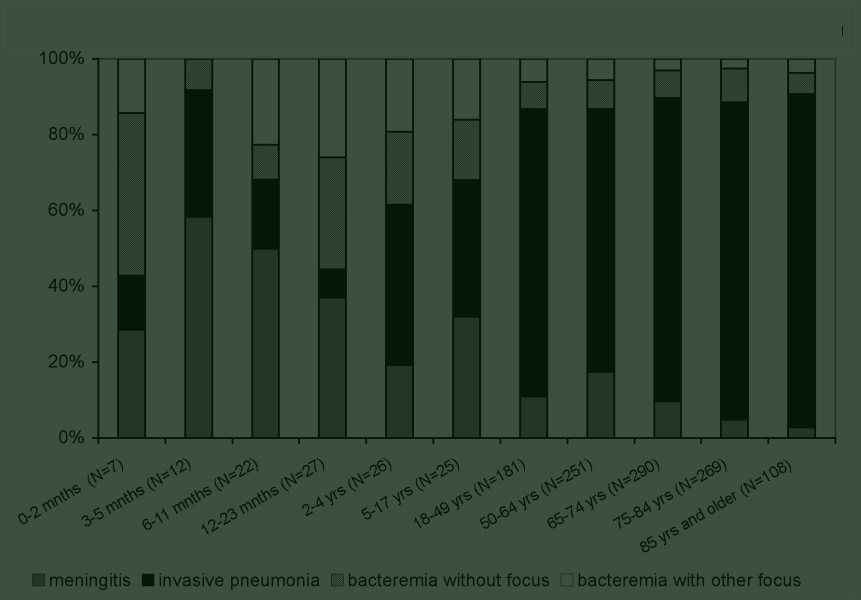

Figure 3. Distribution of clinical syndromes in invasive pneumococcal disease by age in the Netherlands.

Meningitis had the highest percentages of serious sequelae among survivors at discharge from the hospital throughout all age groups (Supporting information Table S2): 33% (95%CI 19–52%) for 0–23-mnth-olds (five cases of deafness, one case of epilepsy, three cases of mental retardation with tetraplegia, epilepsy, or deafness), 0% (95%CI 0–43) for 24–59-mnth-olds, 15% (95%CI 5–36) for 5–49-yr-olds (two cases of epilepsy, one case of peripheral paralysis/-esis), 30% (95%CI 17–47) for 50–64-yr-olds (three cases of epilepsy with or without hemiparesis or hearing loss, three cases of paralysis/-esis, two cases of deafness with or without diplopia or ataxia, one case of renal insufficiency, dialysis, and aphasia, one case of a vegetative state), and 7% (95%CI 2–23) for persons aged 65 yrs and older (one case of tetraplegia with hydrocephalus and hearing loss, one case of deafness). The percentage of serious sequelae in cases of invasive pneumonia was 0% (95%CI 0–28) for 0–23-mnth-olds, 0% (95%CI 0–26) for 24–59-mnth-olds, 1% (95%CI 0–4) for 5–49-yr-olds (one case of epilepsy), 2% (95%CI 1–6) for 50–64-yr-olds (one case of critical illness neuropathy with contractures and remains of delirium which required admission to nursing home, one case of lobectomy, one with renal dialysis requiring dialysis and critical illness neuropathy), and 0% (95%CI 0–1) for persons aged 65 yrs or older (one case of atactic hemiparalysis). Also other observed sequelae as described in (Table A2) were overall most common in meningitis, followed by bacteremia without focus.

Serotype distribution within IPD varied between children and adults (Figure 4). Compared with IPD in adulthood, serotype 14, 6B, and 18C were more prevalent in childhood IPD, whereas serotype 3, 4, and 8 accounted for a higher proportion of IPD in adults than in children. Theoretical IPD vaccine coverage by PCV7 was 29% (95%CI 8–64%) in 0–2-mnth-olds, 79% (95%CI 60–91%) in 6–11-mnth-olds, declining to 31% (95%CI 25–38%) in 18–49-yr-

Table 1. Case-fatality, length of hospital stay and intensive care unit admission rate of invasive pneumococcal disease by age and clinical syndrome.

	Age (N)	Fatality (95% CI)	Days in hospital – mean; median (IQR)	ICU-admission (95% CI)	Days on ICU mean; median (IQR)
Meningitis	0-23 mnths (30)	10% (3-26)	17; 12 (7)	27% (14-44)	1; 0 (1)
	24-59 mnths (5)	0% (0-43)	10; 11 (6)	40% (12-77)	2; 0 (5)
	5-49 yrs (27)	21% (10-40)	14; 11 (10)	46% (30-64)	2; 0 (3)
	50-64 yrs (44)	21% (11-35)	24; 17 (14)	58% (43-72)	7; 3 (9)
	≥65 yrs (44)	39% (26-53)	23; 20 (15)	58% (43-72)	6; 0 (8)
Invasive pneumonia	0-23 mnths (10)	0% (0-28)	12; 8 (13)	20% (6-51)	1; 0 (1)
	24-59 mnths (11)	0% (0-26)	5; 4 (2)	0% (0-26)	0; 0 (0)
	5-49 yrs (143)	1% (1-5)	10; 7 (6)	17% (12-24)	2; 0 (0)
	50-64 yrs (173)	9% (5-14)	16; 11 (10)	21% (15-27)	3; 0 (0)
	≥65 yrs (538)	20% (17-23)	16; 13 (11)	19% (16-23)	2; 0 (0)
Bacteremia without focus	0-23 mnths (14)	7% (1-31)	7; 6 (3)	7% (1-32)	0; 0 (0)
	24-59 mnths (5)	25% (5-70)	3; 3 (4)	0% (0-40)	0; 0 (0)
	5-49 yrs (17)	6% (1-27)	14; 9 (5)	24% (10-47)	2; 0 (1)
	50-64 yrs (19)	32% (15-54)	9; 6 (10)	16% (6-38)	1; 0 (0)
	≥65 yrs (49)	40% (28-54)	16; 10 (19)	16% (8-29)	1; 0 (0)
Bacteremia with other focus	0-23 mnths (12)	0% (0-24)	13; 8 (14)	15% (4-42)	1; 0 (0)
	24-59 mnths (5)	0% (0-43)	4; 5 (5)	0% (0-43)	0; 0 (0)
	5-49 yrs (15)	0% (0-20)	16; 13 (15)	7% (1-30)	0; 0 (0)
	50-64 yrs (14)	7% (1-31)	23; 9 (29)	21% (8-48)	4; 0 (1)
	≥65 yrs (20)	20% (8-42)	15; 14 (16)	25% (11-47)	1; 0 (1)

N, number of cases with known length of hospital stay; IQR, interquartile range; ICU, intensive care unit; 95%CI, 95% confidence interval.

olds, and increasing again to 56% (95%CI 47–64%) in elderly aged 85 yrs and older (Figure 5). The theoretical additive coverage of the experimental PCV10was on average 13% in children (0–17-yr-olds) and 18% in adults (18 yrs and older); for the experimental PCV13 this was 22 and 30%, more or less irrespective of clinical syndromes (data not shown).

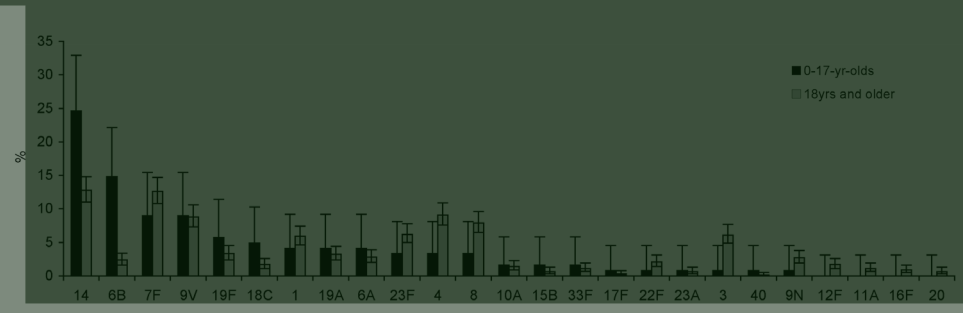

Figure 4. Distribution of serotypes in invasive pneumococcal disease.

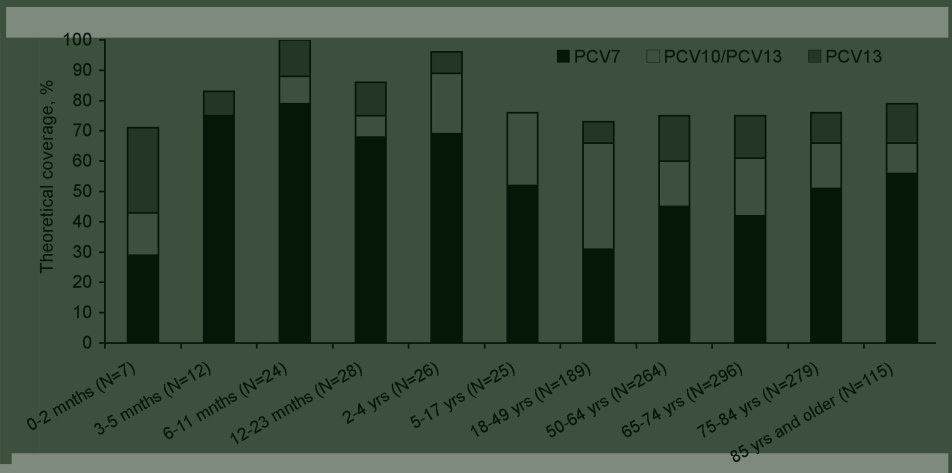

Figure 5. Theoretical coverage by pneumococcal conjugate vaccines per age group.

Discussion

This 2-yr retrospective study on IPD provides a detailed baseline of the incidence of IPD by age and clinical syndrome, prevalence of co-morbidity, disease course, and theoretical vaccine coverage in the Netherlands in the pre-vaccination era.

It has been extensively documented that the pre-vaccination incidence rates of IPD among children in Europe are lower than those reported in the United States and Canada before the implementation of PCV7 with annual rates of 160–180 cases per 100,000 in 0–1-yr-olds.[11;12] Our study confirms these lower rates in Europe as we found a peak annual incidence of 63 cases per 100,000 3–5-mnth-olds and 39 cases per 100,000 0–1-yr-olds. The differences in rates may reflect differences in ethnicity but are more likely due to variations in health care organization and blood culture sampling rates.[11-13] In the USA, blood cultures are often obtained in children presenting with high fever in the outpatient setting[11-13], while in our study virtually all reports were from children requiring hospitalization. In the Netherlands, primary care functions as 'gatekeeper' for patients to attend secondary care, and patients with milder clinical syndromes are probably treated successfully at the primary care level without admission to the hospital where blood cultures are taken. Consequently, less severe manifestations of IPD are likely to be under-diagnosed and consequently underrepresented in our study. The possibility of under-diagnosis and -representation is supported by the high proportion of the more severe clinical syndrome of meningitis among children in our study (44% of all IPD cases in 0–1-yr-olds), with even higher rates of childhood pneumococcal meningitis than reported in the USA and Canada.[14-17] Bacteremia without focus particularly accounted for a smaller proportion of IPD among children in our study compared with US studies, suggesting this manifestation is most underreported. The adult rates of IPD in our study appear higher than those reported in some other European countries but again lower than those reported in the United States.[2;16;18-20] As in

children, the rates of meningitis in adults were higher in our study than in the US, while the rates of invasive pneumonia and especially bacteremia without focus were lower. Again, underreporting of less severe cases may explain these lower rates in adults. importantly, irrespective of geographic location, both laboratory and clinically based reporting systems are prone to underascertainment of the incidence of IPD.[21]

Underlying conditions contribute to susceptibility for pneumococcal disease. Previous studies reported that 20–27% of children with IPD suffered from underlying conditions.[14;15;22;23] In our study this percentage ranged from 24 to 75%, varying with age and clinical IPD syndrome. Apart from the limited number of included childhood cases leading to imprecision of the estimates, the relatively high percentage of underlying co-morbidity in our study may be partially explained by the fact that we also considered extreme premature birth in 0–4-yr-olds and asthma in children aged 5 yrs and older as underlying conditions while most previous studies did not. Conceivably, in countries where blood cultures are not taken routinely in all children presenting with high fever (like the Netherlands), cultures may be relatively more often obtained from children with co-morbidity as they are more frequently referred to the hospital and physicians are especially attentive for complicated disease in these children. In our study, 22% of the 0–1-yr-olds with IPD other than meningitis had a premature birth compared to 8% prematurity among all live births in the Netherlands.[24] This suggests that prematurely born children are at higher risk for IPD in the first yrs of life which was also demonstrated for 0–6-mnth-olds in another recent study.[25] Immunocompromising conditions are a well-known risk factor for invasive infections.[26] We found that up to 20% of the patients with IPD older than 4 yrs had immunocompromising conditions, which compares favourably with other studies.[2;27] This considerable percentage may have important implications for both the direct effects as well as the indirect herd effects of PCV, since serotype replacement disease may be more outspoken in the immunocompromised individuals.[8] Chronic underlying conditions were present in up to 80% of the adults with IPD. In accordance with other studies, the prevalence of common chronic conditions like diabetes and COPD/asthma in IPD patients appeared to be higher than in the overall population.[24;26]

In our study, theoretical coverage of IPD by PCV7 was 69% in 0–4-yr-olds, which is comparable to other European countries but somewhat lower than the approximately 80% reported in the US.[28;29] Additional coverage by the experimental PCV10 and PCV13 was respectively 10 and 22% in this age group. In view of the high clinical efficacy of PCV7 in children, attention has also been drawn to the potential benefit of PCV in adults, particularly for the elderly.[30] Besides the traditional target group of elderly over 65 yrs of age, also 50–64-yr-olds might benefit from conjugate vaccines with broad coverage; in our study, the incidence rates of hospitalized IPD started to rise already from the age of 50 yrs, especially beyond the age of 60 yrs with a rate in 60–64-yr-olds similar to that in 12–23-mnth-olds. The theoretical coverage in our study of IPD in older adults by PCV7 was less than 50% (45% in 50–64-yr-olds and 48% in persons of 65 yrs and older) which is slightly lower than previously reported in other countries.[2;29] Theoretical coverage among 50–64-yr-olds reached 60% for the experimental PCV10 and 75% for the experimental PCV13, similar to

coverage rates in those older than 65 yrs. These vaccines therefore need to be considered for individuals of 50 yrs and older, particularly those aged 60 yrs and older. To appreciate the results of our study, some potential weaknesses should be acknowledged. Since we collected the clinical information retrospectively, we were dependent on how well patient records were kept. However, in only 4% no clinical information was available. Additionally, the number of cases in childhood was relatively small which resulted in limited precision. However, strengths of our study are that we were able to provide fairly detailed information about the epidemiology, presence of co-morbidity, clinical syndrome, and disease course of IPD representative for the Netherlands in the 2 yrs prior to introduction of PCV7 in the national infant vaccination program. Furthermore, negligible uptake of PCV7 and the 23-valent pneumococcal polysaccharide vaccine during the time period studied, together with the fact that the Netherlands is a relatively small country with a dense and relatively homogeneous population, make the Netherlands particularly suitable for providing reliable baseline information. As bacterial resistance to penicillin has traditionally been very low in the Netherlands, treatment failure will not have biased the results of our study. In conclusion, the current study provides a reliable baseline on IPD in the pre-vaccination era. Continuing monitoring of IPD is warranted after implementation of PCV, and the information provided in the current study is helpful in the interpretation of any changes accompanying future vaccination strategies in Western European countries.

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Table S1. Prevalence of underlying conditions in invasive pneumococcal disease by age.

Age (N)	Underlying conditions %, 95% confidence interval (N): specification (N)
	Meningitis
0-1 yrs (29)	24%, 12-42% (7): ●prematurity 32-36 wks (2); ●prematurity <32 wks (1); ●severe perinatal asphyxia (1); ●spina bifida/hydrocephalus (1); ●hemoglobinopathy; ●cong. adrenal hypoplasia (1)
2-4 yrs (5)	60%, 23-88% (3): ●recent trauma/skull fracture (2); ●prematurity < 32 wks (1)
5-17 yrs (8)	75%, 40-93% (6): ●skull fracture/trauma/CSF leak (4); ●suspected ID (1); ●severe perinatal asphyxia (1)
18-49 yrs (20)	45%, 26-66% (9): ●25% (5) immunocomp. *(1 organ Tx, 1 immunosuppr. for malign.; 1 AIDS, 1 renal failure, 1 asplenia)*; ●skull fracture/trauma (3); ●DM (2); ●alcohol (2); ●CVD (2); ●thyroid (1)
50-64 yrs (44)	48%, 34-62% (21): ●14% (6) immunocomp. *(1 organ transplant, 3 asplenia, 1 leukemia, 1 immunosuppr. for malign.)*; ●CVD (6); ●DM (5); ●alcohol (5); ●COPD/asthma (3); ●CSF leak/ trauma (2)
≥65 yrs (42)	52%, 38-67% (22): ●14% (6) immunocomp. *(1 organ transplant, 2 renal failure, 2 myeloma/ lymphoma, 1 immunosuppr. for AID)*; ●DM (10); ●CVD (10); ●COPD/asthma (4); ●alcohol (3); ●thyroid (3); ●AID (2)
	Other invasive pneumococcal disease
0-1 yrs (37)	35%, 22-51% (13): ●prematurity 32-36 wks (7); ●failure to thrive (2); ●prematurity <32 wks (1); ●metabolic disorder (1); ●cong. syndrome (1); ●hemoglobinopathy (1)
2-4 yrs (21)	38%, 21-59% (8): ●cong. heart disorder (2); ●organ Tx (1); ●hemoglobinopathy (1); ●Down's syndrome (1); ●achondroplasia (1); ●liver (1); ●hydrocephalus (1)
5-17 yrs (17)	59%, 36-78% (10): ●malignancy (3); ●asthma (2); ●cong. syndrome(2); ●liver disease (1); ●renal disease (1); ●hemoglobinopathy (1)
18-49 yrs (158)	40%, 33-48% (63): ●13% (20) immunocomp. *(1 primary ID, 2 organ Tx, 4 HIV, 1 AIDS, 1 nephrotic syndrome, 1 hemoglobinopathy, 1 asplenia, 4 lymphoma/leukaemia/myeloma, 5 immunosuppr. for AID)*; ●COPD/asthma (25); ●alcohol (13); ●AID (10)
50-64 yrs (206)	72%, 65-78% (148): ●24% (49) immunocomp. *(8 stem cell Tx, 1 organ Tx, 2 AIDS, 6 renal failure, 1 asplenia, 13 leukemia/lymphoma/myeloma, 14 immunosuppr. for malign. and 4 for AID)*; ●COPD/ asthma (52); ●CVD (46); ●DM (33); ●alcohol (27); ●AID (15); ●malign.** (15); ●liver (13)
≥65 yrs (622)	81%, 78-84% (506): ●19% (118) immunocomp. *(3 primary ID, 1 organ Tx, 1 HIV, 32 renal failure, 2 nephrotic syndrome, 1 hemoglobinopathy, 5 asplenia, 50 lymphoma/ myeloma/leukemia, 28 immunosuppr. for malign. and 27 for AID)*; ●CVD (225); ●COPD/asthma (207); ●DM (146); ●malign.** (61); ●AID (51); ●thyroid (51)

*The sum of percentages may exceed the total percentage with underlying conditions since multiple conditions may be present in one patient.; ** not defined below 'immunocompromised'; AID, autoimmune disease; alcohol, alcohol abuse; cong., congenital; COPD, chronic obstructive pulmonary disease; CSF, cerebrospinal fluid; CVD, cardiovascular disease; DM, diabetes mellitus; ID, immunodeficiency; immunocomp., immunocompromised; immunosuppr., immunosuppressives; liver, liver disease; malign., malignancy; thyroid, thyroid disease; Tx, transplantation.

Table S2. Sequelae of invasive pneumococcal disease by age and clinical syndrome.

Clinical syndrome (N)	Sequelae among survivors at discharge from the hospital % (95% CI)	
	Serious	Other
	0-23 mnths	
Meningitis (30)	33% (19-52%): deafness (5), epilepsy (1), mental retardation with tetraplegia, epilepsy, or deafness (3)	11% (4-28%): hearing ↓ (3)
Invasive pneumonia (10)	0% (0-28)	0% (0-28)
Bacteremia without focus (14)	0% (0-23)	8% (1-33) mild hemiparesis (1)
Bacteremia other focus (12)	0% (0-26)	0% (0-26)
	24-59 mnths	
Meningitis (5)	0% (0-43)	20% (4-62): mild ataxia/ptosis (1)
Invasive pneumonia (11)	0% (0-26)	0% (0-26)
Bacteremia without focus (4)	0% (0-56)	0% (0-56)
Bacteremia other focus (5)	0% (0-43)	20% (4-62): pre-existing condition ↓ (1)
	5-49 yrs	
Meningitis (28)	15% (5-36): epilepsy (2), peripheral paralysis/-esis (1)	25% (11-47): brain nerve damage (2), mild apraxia/aphasia (1), hearing ↓ (1), need for reconstructive bone surgery (1)
Invasive pneumonia (145)	1% (0-4)	1% (0-5): re-admission (1), pre-existing condition ↓ (1)
Bacteremia without focus (17)	7% (1-31): gangrene needing skin transplantation (1)	7% (1-31): second antibiotic course (1)
Bacteremia other focus (15)	0% (0-20)	13% (4-38): pre-existing condition ↓(1), hearing ↓ (1)
	50-64 yrs	
Meningitis (43)	30% (17-47): epilepsy w/wo hemiparesis/hearing ↓ (3), paralysis/-esis (3), deafness w/wo diplopia/ataxia (2), renal insufficiency, dialysis, and aphasia (1), vegetative state (1)	24% (13-41): hearing ↓ (4), mild hemiparesis (2), health status ↓ (1), ataxia/personality change/ cognitive problems/hearing loss (1)
Invasive pneumonia (173)	2% (1-6): critical illness neuropathy with contractures and remains of delirium which required admission to nursing home (1), lobectomy (1), renal dialysis requiring dialysis and critical illness neuropathy (1)	11% (7-17): critical illness neuropathy (2); second antibiotic course (1); re-admission (1); pre-existing condition ↓ (6); abscess drainage (1); physical therapy (1); admission to nursing home/rehabilitation clinic (4)
Bacteremia without focus (19)	0% (0-23)	15% (4-42): pre-existing condition ↓ (2)
Bacteremia other focus (14)	0% (0-23)	31% (13-58): admission rehabilitation clinic (1), critical illness neuropathy with necrosis finger tip (1), pulmonary rehabilitation (1), back brace (1)

	≥65yrs	
Meningitis (44)	7% (2-23): tetraplegia with hydrocephalus and hearing loss (1), deafness (1)	33% (19-52): secondary diabetes with or without hearing ↓ (2), hearing ↓ (3), empyema (1), admission to rehabilitation clinic (2), mild aphasia (1)
Invasive pneumonia (547)	0% (0-1): atactic hemiparalysis (1)	12% (9-15): admission to rehabilitation clinic/ nursing home (11), readmission (5), health status ↓ (1), pre-existing condition ↓ (23), decubitus (1), delirium (1), hearing ↓ (1), herpes zoster (1), physical therapy (2), help at home (1), need for oxygen (2)
Bacteremia without focus (50)	4% (0-19): renal insufficiency requiring dialysis (1)	8% (2-24): pre-existing condition ↓ (1), necrosis foot (1)
Bacteremia other focus (20)	0% (0-19)	25% (10-49): re-admission (1), arthrosis knee (1), admission to nursing home (1); help at home (1)

3

Effects of pneumococcal conjugate vaccine 2 years after its introduction, the Netherlands

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Abstract

In the Netherlands, the 7-valent pneumococcal conjugate vaccine (PCV7) was implemented in a 3+1-dose schedule in the national immunization program for infants born after April 1, 2006. To assess the vaccine's effectiveness, we compared disease incidence before and after vaccine implementation (June 2004–June 2006 and June 2006–June 2008, respectively). We serotyped 2,552 invasive pneumococcal isolates from throughout the Netherlands, covering 25% of the country's population. Clinical characteristics were extracted from hospital records. After June 2006, vaccine serotype invasive pneumococcal disease (IPD) decreased 90% (95% confidence interval [CI] 68%–97%) in children age eligible for PCV7; simultaneously, however, non-vaccine serotype IPD increased by 71% (not significant), resulting in a 44% total net IPD reduction (95% CI 7%–66%). IPD rates did not change for other age groups. In the Netherlands, PCV7 offered high protection against vaccine serotype IPD in vaccinated children, but increases of non-vaccine serotype IPD reduced net vaccine benefits.

Background

Streptococcus pneumoniae is a leading cause of invasive infections, such as meningitis, septicemia, and bacteremia, and of more common respiratory tract infections, such as pneumonia and otitis media. Young children and elderly persons are at particularly high risk for pneumococcal infection.[1] In the United States, the introduction in 2000 of the CRM197-conjugated 7-valent pneumococcal vaccine (PCV7) resulted in a 77% reduction in 2005 of invasive pneumococcal disease (IPD) in children <5 years of age from IPD rates reported in 1998–1999.[2] IPD rates in children decreased mostly within the first 2 years after introduction of PCV7; leveled off in 2002; and then stabilized, despite an ongoing decrease of vaccine serotype IPD, due to a gradual increase of non-vaccine serotype IPD, particularly serotype 19A.[2;3] In addition, use of the vaccine in children was associated with reduced IPD rates for unvaccinated age groups, which resulted from reduced nasopharyngeal colonization of vaccine serotype *S. pneumoniae* in vaccinated children and concomitant reduced transmission.[4;5] The cost effectiveness of herd immunity conferred by the conjugate vaccine in the United States prompted implementation of the vaccine in the Netherlands.[6] Data from the United States concerning both direct and indirect vaccine benefit, however, cannot be translated indiscriminately to European countries because of several major differences. Vaccine serotype coverage by PCV7 was lower in European countries (60%–70%) than in the United States (>80%)[7], which may leave more room for non-vaccine serotype replacement in European countries. Second, in the Netherlands (as in most European countries), baseline IPD incidence rates are based mainly on culture-confirmed cases in hospitalized children, resulting in markedly lower IPD incidence rates for young children in the Netherlands than for those in the United States, where blood samples are cultured for more patients. Before introduction of PCV7 in the Netherlands, overall IPD rates were 35 cases/100,000 children <2 years of age, of which 15 cases/100,000 children were meningitis.[1] In contrast, in the United States, IPD incidence before introduction of PCV7 peaked at 188 cases/100,000 children <2 years of age in 1998–1999[5], and 10 cases/100,000 children in that age group were meningitis.[8] Consequently, introduction of PCV7 may have affected IPD incidence in European countries differently than in the United States.[9]

To assess the effectiveness of PCV7 on IPD in the Netherlands, we evaluated the incidence and clinical syndromes of IPD in PCV7–vaccinated and –unvaccinated children and in other age groups during the first 2 years after implementation of PCV7.

Materials and Methods

Surveillance and Data Collection

PCV7 was introduced into the Netherlands' national immunization program (NIP) in June 2006 and was recommended for all infants born after April 1, 2006, at 2, 3, 4, and 11 months of age.[10] Our study comprised all patients with culture-confirmed IPD during June 1, 2004–June 1, 2006 (pre-implementation period) and June 1, 2006–June 1, 2008 (post-implementation period). Isolates were serotyped by the Netherlands Reference Laboratory

for Bacterial Meningitis (NRLBM), which collects nationwide bacterial isolates from blood, cerebrospinal fluid (CSF), and/or other normally sterile bodily fluids for laboratory-based surveillance. Isolates from all patients with IPD were submitted by 9 sentinel laboratories throughout the country. These laboratories covered ~4.074.412 and ~4.090.233 residents in the pre-implementation period and post-implementation period, respectively, representing ~25% of the population of the Netherlands. Laboratories were selected on the basis of their reliability for submitting pneumococcal isolates; they submitted 90%–95% of the pneumococcal isolates from CSF and ~83% of pneumococcal isolates from blood.[1] Isolates were serotyped as previously described, by using antiserum from the Statens Serum Institute (Copenhagen, Denmark).[1] Isolates with serotypes 4, 6B, 9V, 14, 18C, 19F, and 23F, the serotypes contained in PCV7, were considered vaccine serotypes. All other serotypes were considered non-vaccine serotypes.

Clinical Characteristics

Nearly all (97%–98%) IPD cases were in hospitalized patients. We retrospectively abstracted information about their clinical syndromes and underlying conditions from hospital records. Clinical syndromes were categorized as meningitis or nonmeningitis IPD (invasive pneumonia, IPD with other focus, and bacteremia without focus). Meningitis was defined as CSF culture positive for *S. pneumoniae* (or positive CSF by PCR) and/or clinical diagnosis of meningitis in combination with a blood culture positive for *S. pneumoniae*. Invasive pneumonia was physician diagnosed pneumonia and a blood culture positive for *S. pneumoniae*. IPD with other focus was a *S. pneumoniae*–positive culture of blood or other normally sterile body fluid in combination with a clinical focus other than meningitis or pneumonia. For bacteremia without focus, no clinical focus was identified. Underlying conditions were classified as immunocompromised conditions or other comorbidities, as described previously.[1] Case fatality was defined as in-hospital death and/or death within 30 days after the first reported blood/CSF culture positive for *S. pneumoniae*.

Statistical Analyses

To study the effectiveness of the vaccination program, we compared age-specific incidences during the pre-implementation and post-implementation periods. Incidence rates of IPD were calculated as number of cases per 100,000 persons per year by using 25% of the Dutch population on January 1 for each considered year, accounting for the 25% coverage of surveillance data. Changes in incidence rates from the pre-implementation to the post-implementation period were presented as incidence rate ratio with 95% confidence intervals (CI) and as percent changes. We compared the pre-implementation and post-implementation periods with regard to distribution of clinical syndromes, comorbidities, and outcomes. Theoretical coverage of IPD was based on data from the pre-implementation and post-implementation periods for future 10-valent PCV (PCV10, covering PCV7 serotypes plus serotypes 1, 5, and 7F) and 13-valent PCV (PCV13, covering PCV10 serotypes plus serotypes 3, 6A, and 19A). Proportions were tested with $\chi 2$ or Fisher exact tests, as appropriate. We considered $p < 0.05$ to be significant. Statistical analyses

were performed with SAS version 9.1.3 (SAS Institute, Cary, NC, USA), Excel 2007 (Microsoft, Redmond, WA, USA), and Episheet.[11]

Results

During the study period, the NRLBM received 2,649 *S. pneumoniae* isolates: 1,297 during the pre-implementation period and 1,352 during the post-implementation period. Medical records were assessed for 1,235 (95%) cases during the pre-implementation period and for 1,317 (97%) cases during the post-implementation period. Pneumococcal serotype was available for 1,225 and 1,304 cases (both 99%), respectively.

IPD Incidence

Overall incidence of IPD remained stable; 15.9 vs. 15.0 cases/100,000 persons during the post-implementation and pre-implementation periods, respectively. Incidence of vaccine serotype IPD did not change significantly. For non-vaccine serotype IPD, incidence increased 13% (95% CI 2%–26%; p = 0.02) (Table). In children <2 years of age, including those not vaccinated or incompletely vaccinated, the incidence of IPD decreased 35% (95% CI 4%–56%; p = 0.006), from 34.5 cases/100,000 persons in the pre-implementation period to 22.5 cases/100,000 persons in the post-implementation period (Table). Incidence of vaccine serotype IPD declined by 67% (95% CI 41%–81%; p<0.0001), from 24.3 to 8.0 cases/100,000 persons. In contrast, non-vaccine serotype IPD incidence increased, but not significantly, from 10.1 to 14.5 cases/100,000 persons (p = 0.40). Among children born after April 1 2006 (i.e., age-eligible for vaccination according to the NIP), the incidence rate of vaccine serotype IPD in the post-vaccination period (2.4 cases/100,000 persons) decreased 90% (p<0.0001) compared with that for an age-matched group in the pre-implementation period (24.2 cases/100,000) (Figure 1). Although not significant because of low numbers, the incidence of non-vaccine serotype IPD had risen by 71%, from 9.8 to 16.8 cases/100,000 persons (p = 0.12), leading to a total net reduction of 44% (95% CI 7%–66%; p = 0.02) in the birth group age-eligible for vaccination. Three vaccine serotype IPD cases occurred among children born after April 1, 2006; 2 cases after 1 vaccine dose (serotypes 9V and 23F) and 1 case within 1 week after the second dose (serotype 9V, isolated from CSF). In infants <2 years of age born before April 1, 2006 (i.e., age-ineligible for PCV7), no changes occurred in vaccine- or non-vaccine serotype IPD rates in the post-implementation period compared with those for age-matched children in the pre-implementation period.

Table. Incidence rates of invasive pneumococcal diseases before and after implementation of 7-valent pneumococcal conjugate vaccine, the Netherlands.*

Serotypes by patient age group, y	Pre-implementation period (June 2004 – June 2006)		Post-implementation period (June 2006 – June 2008)		Pre-implementation vs. post-implementation	
	No. Cases	Rate	No. Cases	Rate	IRR (95% CI)	p value†
Total						
All ages	1225	15.0	1304	15.9	1.06 (0.98-1.15)	0.14
<2 years	68	34.5	42	22.5	**0.65 (0.44-0.96)**	**0.006**
2 – 4 years	25	8.1	26	8.7	1.07 (0.62-1.86)	
5 – 49 years	206	4.1	231	4.7	1.13 (0.94-1.37)	
50 – 64 years	254	16.7	292	18.5	1.11 (0.94-1.31)	
≥65 years	672	58.8	713	60.2	1.02 (0.92-1.14)	
Vaccine serotypes ‡						
All ages	570	7.0	579	7.1	1.01 (0.90-1.14)	
<2 years	48	24.3	15	8.0	**0.33 (0.19-0.59)**	**<0.0001**
2 – 4 years	17	5.5	17	5.7	1.03 (0.53-2.02)	
5 – 49 years	69	1.4	72	1.5	1.05 (0.76-1.47)	
50 – 64 years	114	7.5	133	8.4	1.12 (0.88-1.44)	
≥65 years	322	28.2	342	28.9	1.03 (0.88-1.19)	
Non-vaccine serotypes §						
All ages	656	8.0	725	8.9	1.10 (0.99-1.23)	0.07
<2 years	20	10.1	27	14.5	1.43 (0.80-2.55)	
2 – 4 years	8	2.6	9	3.0	1.16 (0.45-3.01)	
5 – 49 years	137	2.8	159	3.2	1.17 (0.93-1.47)	
50 – 64 years	140	9.2	159	10.1	1.09 (0.87-1.37)	
≥65 years	350	30.6	371	31.3	1.02 (0.88-1.18)	

*Rate is cases/100,000 persons. IRR, incidence rate ratio; CI, confidence interval. **Boldface** indicates significant differences (p<0.05). †p values shown <0.15; incidence rates pre vs. postimplementation period. Calculated by using Fisher exact test; all p values are 2 sided.
‡*Streptococcus pneumoniae* serotypes 4, 6B, 9V, 14, 18C, 19F, and 23F.
§All other *S. pneumoniae* serotypes.

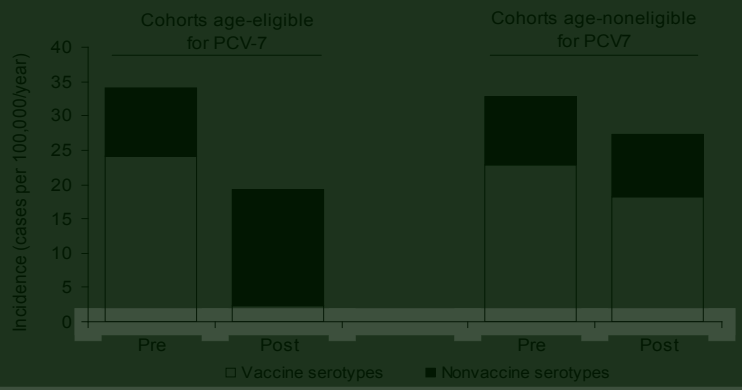

Figure 1. Incidence of invasive pneumococcal disease in children <2 years of age in the birth group born after April 1, 2006 (age eligible for 7-valent pneumococcal conjugate vaccine [PCV7]) and children born before April 1, 2006 (age noneligible for PCV7), in the post-implementation period compared with age-matched children in the pre-implementation period, the Netherlands. Incidence per 100,000 children <2 years of age per year; Pre, pre-implementation period (June 2004–June 2006); post, post-implementation period (June 2006–June 2008).

Serotype Distribution

After introduction of PCV7, for all vaccine serotypes, the number of IPD cases among the total population remained stable, except for serotype 19F (44 vs. 23 cases; p = 0.004). Also, proportions of non-vaccine serotype 1 and 22F significantly increased (Figure 2). Among children born after April 1, 2006, serotypes 1 and 7F increased in comparison with those for age-matched infants in the pre-implementation period (Figure 3). For serotype 6A, 6C and 19A, no significant changes occurred in any age group.

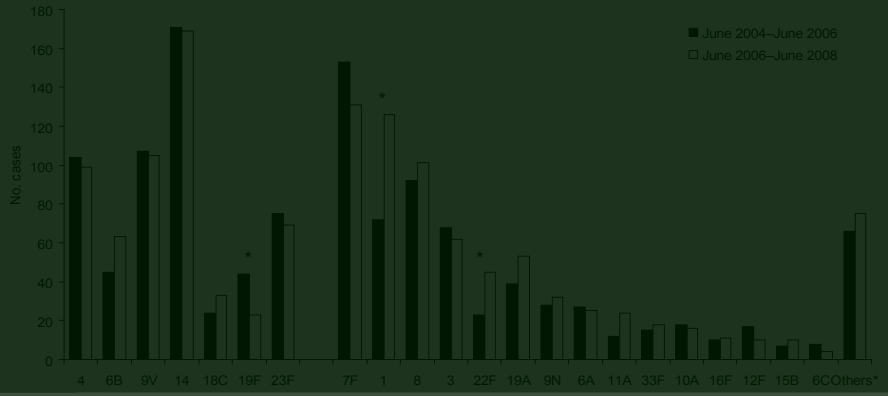

Figure 2. Serotype distribution of invasive pneumococcal disease with regard to pre-implementation and post-implementation of 7-valent pneumococcal conjugate vaccine (PCV7); among persons of all ages, the Netherlands. Pre-implementation period June 2004–June 2006; post-implementation period June 2006–June 2008; *p<0.05; proportion of serotypes pre-implementation vs.post-implementation period. Calculated using Fisher exact test; all p values are 2 sided.

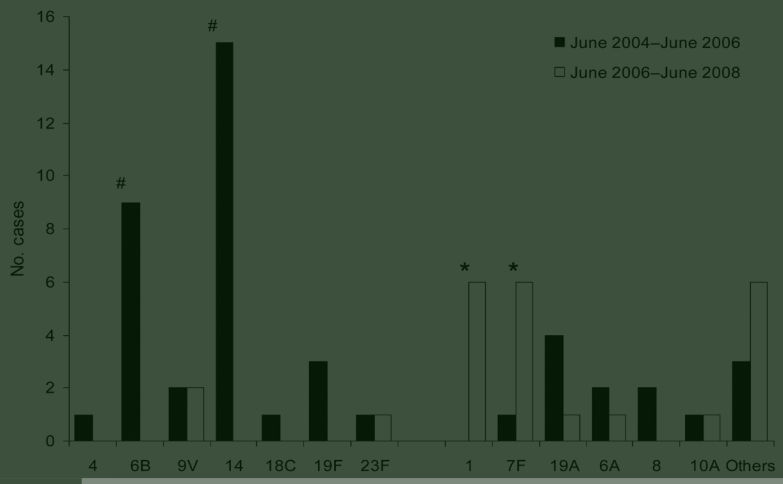

Figure 3. Serotype distribution of invasive pneumococcal disease cases among children born after April 1, 2006 (age eligible for 7-valent pneumococcal conjugate vaccine [PCV7] in the post-implementation period compared with age-matched children in the pre-implementation period, the Netherlands. Pre-implementation period, June 2004–June 2006; post-implementation period, June 2006–June 2008; other serotypes are 15A, 16F, 22F, 3, 33F, 5, and 9N. *p<0.05; pre-implementation vs. post-implementation periods. Proportions calculated using Fisher exact test; all p values are 2 sided.

Clinical Characteristics

Among children <2 years of age, incidence rates decreased for all clinical syndromes to approximately the same extent (Figure 4). Rates of meningitis declined 34% from 14.7 to 9.6 cases/100,000 children (29 vs. 18 cases) and of nonmeningitis IPD 35% from 19.8 to 12.9 cases/100,000 children in this age group (39 vs. 24 cases). Of these children, 30% (20/66) had comorbidities in the pre-implementation period, compared with 9% (4/44) in the post-implementation period (p<0.001). In all other age groups, clinical syndromes did not change from the pre-implementation to the post-implementation period, except for a 124% rise in rates of non-vaccine serotype meningitis for persons 5–49 years of age (95% CI 19%–320%; p =0.01). The proportions of adult patients with comorbidities and immunocompromising conditions were similar in the pre-implementation and post-implementation periods: 71% vs. 74% and 19 vs. 22%, respectively. For the vaccinated group of children, the case-fatality rate remained stable (9.3% vs. 8.3%) in the pre-implementation and post-implementation periods, respectively. In other age groups, casefatality rates did not differ (data not shown).

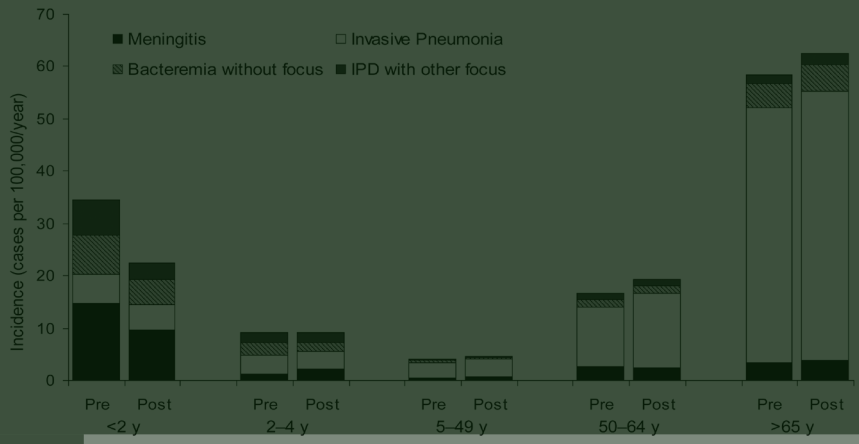

Figure 4. Age group–specific distribution of clinical invasive pneumococcal disease (IPD) syndromes in the pre-implementation and post-implementation periods of 7-valent pneumococcal conjugate vaccine (PCV7), the Netherlands. Incidence is IPD cases per 100,000 persons per year. Pre,pre-implementation period (June 2004–June 2006); post, post-implementation period (June 2006–June 2008).

Estimated Coverage by Future Vaccines

Among vaccination-eligible children, the additional coverage rates in the pre-implementation and post-implementation periods were 2.2% (1/45 cases) and 54.2% (13/24 cases) for PCV10 (p<0.0001). For PCV13, they were 19.6% (8/45 cases) and 66.7% (16/24 cases) (p<0.001) (Figure 5).

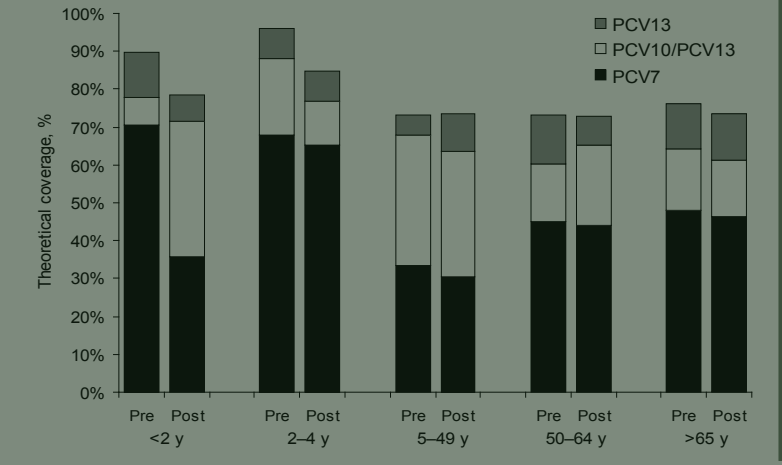

Figure 5. Age group–specific theoretical coverage of pneumococcal conjugate vaccines during the pre-implementation and post-implementation periods of 7-valent pneumococcal conjugate vaccine (PCV7), the Netherlands. IPD, invasive pneumococcal disease; PCV10/PCV13, additional coverage by PCV10 and PCV13; PCV13, additional coverage by PCV13 alone; pre, pre-implementation period (June 2004–June 2006); post, post-implementation period (June 2006–June 2008).

Discussion

The total net IPD reduction of 35% among children <2 years of age observed in the Netherlands differs from the favorable results reported in the United States, where a 69% decrease in IPD within the first 2 years after PCV7 introduction was reported despite lower vaccine uptake (i.e., estimates of national immunization coverage) rates in the United States than in the Netherlands.[2;12] United States estimates for PCV7 uptake among children born in 2001 who received >1 and >3 doses were 89% and 68%, respectively.[2] In contrast, in the Netherlands, 94.4% of all infants born in 2006 were fully vaccinated at 2 years of age.[12] Also, the decrease in meningitis incidence in the Netherlands was lower than that for United States infants <2 years of age (34% vs. 59%), whereas during the pre-implementation period, meningitis incidence was comparable in the 2 countries. In the first 2 years after implementation in the United States, IPD requiring hospitalization decreased 63% in children <2 years.[5] In contrast, all IPD in this age group decreased 35%, and almost all reported IPD cases occurred in hospitalized patients.

The difference between the impact of PCV7 in the Netherlands and the United States may be attributable to the lower proportion of vaccine serotype cases covered by PCV7 before implementation in the Netherlands and in Europe. Reports about PCV7 effectiveness by other European countries support this observation. In Germany and Norway, PCV7 effectively prevented disease in the youngest age groups during the first years after implementation, without major increase of non-vaccine serotype IPD.[13;14] In Spain, vaccine serotype IPD decreased after PCV7 implementation; however, non-vaccine serotype IPD increased.[15;16] In France, 3 years after PCV7 introduction, overall IPD cases decreased 21% among children of <2 years of age, when 44%–56% of children were vaccinated.[17] In addition, similar to findings in the Netherlands, a simultaneous increase in IPD from non-vaccine serotype pneumococci reduced the net benefit of vaccination. Differences in surveillance systems, temporal fluctuations of circulating serotypes, antimicrobial drug resistance and penicillin susceptibility of circulating pneumococcal strains, vaccination schedules, vaccine uptake, and blood sampling practices also may play a role in the differences between countries.[9;18] Like Norway, a reduced-dose PCV7 schedule has been introduced in the United Kingdom. In the first years after implementation, surveillance data from the United Kingdom have tended to show a major decline in vaccine serotype IPD in infants <2 years of age concomitant with a substantial rise in non-vaccine serotype IPD, reducing net vaccine benefits.[19;20] Unlike in the United States, where IPD in children <2 years of age stabilized within 2 years after introduction of the vaccine[2], in Europe and in our study, incidence has not yet stabilized. Our results emphasize the need for continued surveillance to monitor the long-term public health benefits of the vaccination program.

Despite the high vaccination uptake in the Netherlands, we observed no indication of herd immunity in other age groups during the first 2 years after implementation, except for a decrease in serotype 19F. This observation may be explained by the relatively short evaluation period of 2 years, a relatively small vaccinated group (2.25%) of the total population, and lack of a catch-up program for older children. In Australia and the United Kingdom, which have catch-up programs for children <2 years of age, decreases in vaccine

serotype IPD in unvaccinated children within 3 years after PCV7 implementation have been reported.[21;22]

The small increase we found in non-vaccine serotypes 1 and 7F among vaccinated children could be attributed to temporal fluctuations.[18;23] The numbers in our study were too small and the period we studied too short to enable us to draw firm conclusions about changes in serotype-specific incidence. Serotype 1 also has increased in other age groups and may cause local outbreaks, as observed in other countries before the implementation of PCV7.[24;25] In our study, we could not find evidence of outbreaks associated with serotype 1. The increase in non-vaccine serotype IPD in the vaccinated age group was not explained by more children with comorbidities or immunocompromised conditions in the years after introduction and not associated with a change in the case-fatality rate. Longer follow-up is needed to assess whether this increase and that in non-vaccine serotype IPD in the overall population are temporary or are vaccine related. Several countries have suggested that use of PCV7 might enhance the emergence of serotype 19A pneumococcal clones, often associated with penicillin resistance.[26] In the Netherlands, where use of antimicrobial drugs is restricted, few penicillin-resistant pneumococcal isolates were received during the study period; 98.8% were susceptible to penicillin (MIC <0.06 mg/L), 0.8% were intermediately susceptible (0.06–1.0 mg/L), and 0.4% were resistant (>1.0 mg/L). We did not see a prominent increase in serotype 19A among patients; only 1 isolate was penicillin resistant (>1.0 mg/L) in the first 2 years after PCV7 implementation. However, increase in serotype 19A pneumococci was found in a randomized controlled study of nasopharyngeal carriage among vaccinated children compared with unvaccinated controls before national implementation of PVC7.[27] Also, no changes in distribution of serotype 6A or 6C were reported. Theoretical coverage of the future conjugate vaccines PCV10 and PCV13 increased in the post-implementation period in vaccination-eligible children. In all other age groups, no changes were observed. Future vaccines need to be considered to improve the net benefit of immunization against pneumococcal diseases.

Some limitations should be acknowledged. Although our study covered ~25% of the population of the Netherlands, numbers of IPD cases and serotype distribution are small and need cautious interpretation. After 2 years, final vaccine benefits cannot be established. Furthermore, distribution of serotypes among invasive pneumococci may fluctuate over time, and temporal trends in serotype may vary across geographic regions and independent of PCV7 implementation.[18;23] Although the 9 sentinel laboratories submitted 25% of all pneumococcal isolates received by the NRLBM (nationwide coverage 95%), the population under surveillance might be overestimated because the laboratories were selected on reliability of stable pneumococcal isolates submission over the years. Second, our surveillance system depended on how well the 9 sentinel laboratories were submitting their isolates, and small shifts in the proportion of submission to the NRLBM cannot be excluded.[14] Blood culture rates may have influenced IPD incidence reported in this study.[28] However, these changes are not likely to be substantial. National IPD incidence rates estimated from the number of isolates submitted by the 9 sentinel laboratories are similar to those of neighboring countries, e.g., Denmark and the United Kingdom, that have

comparable health system practices.[29] The rates of submission of blood and CSF isolates for children <5 years of age have been stable in the Netherlands for the past 10 years at ~90%. Enhanced surveillance with such high submission rates and stable overall IPD rates cannot explain the 71% increase of nonvaccine serotypes. Also, and most important, the changes in IPD serotype distribution occurred only in vaccination-eligible infants. No changes in serotype distribution or signs of herd immunity were observed in unvaccinated infants. A potential bias by enhanced awareness would cause differences also in this group. Long-term surveillance data will elucidate whether the changes in serotype distribution in vaccinated and unvaccinated persons remain. Lastly, in the Netherlands, no changes in diagnostic methods or blood culture practices have been implemented recently. Strengths of our study include the detailed information about the IPD cases and the established high degree of vaccine uptake for the NIP in the Netherlands (~95% of infants of <1 year of age are fully vaccinated).[12] Vaccination with the 23-valent pneumococcal polysaccharide vaccine has not been routinely recommended for elderly persons, and uptake has been negligible in the Netherlands; thus, any influence of this vaccine can be excluded.[30] The low proportion of penicillin-resistant pneumococcal isolates and the densely living but relatively homogeneous population make the Netherlands particularly suitable for describing vaccine effects.

Our study provides accurate data from a representative group of the Dutch population with fairly detailed information about the distribution of clinical syndromes and presence of comorbidities. Shortly after introduction of PCV7 vaccination for infants, the direct vaccine effectiveness on IPD caused by vaccine serotype pneumococci appeared high in the Netherlands. However, the net benefit is partly offset by the increased incidence of nonvaccine serotypes. For this reason, future conjugate vaccines may be valuable in further reducing IPD incidence. These results further emphasize the need for ongoing surveillance.

Acknowledgments

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4

Invasive pneumococcal disease among adults: Associations among serotypes, disease characteristics, and outcome

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Abstract

Background

The *Streptococcus pneumoniae* polysaccharide capsule may be related to invasive pneumococcal disease (IPD) course.

Methods

We performed a retrospective cohort study with nationally representative surveillance data from 1075 hospitalized patients with IPD from the Netherlands from 1 June 2004 through 31 May 2006 in the prevaccination era. Serotypes were grouped according to invasive disease potential, rate of the most serious clinical syndromes of meningitis and bacteremia without focus, and case-fatality rates. Multivariable logistic regression analysis was performed to obtain odds ratios adjusted for baseline confounders for the association of serotypes and these outcomes, using the serotypes with the lowest rates as reference.

Results

IPD caused by serogroups with low invasive disease potential concerned meningitis or bacteremia without focus in 22% of cases, and 74% of patients had an underlying comorbidity. For highly invasive serogroups these figures were 10% ($p<0.01$) and 56% ($p<0.01$). Individual serotypes varied in the relative rate by which they caused meningitis or bacteremia without focus. Compared with the reference group composed of serotypes 1, 5, 7F, 15B, 20, and 33F, the group of serotypes 3, 19F, 23A, 16F, 6B, 9N, and 18C was associated with increased case-fatality rates (group adjusted odds ratio, 2.6; 95% confidence interval, 1.5–4.7).

Conclusions

The serotype appeared to be independently associated with IPD severity in adults, which indicates that careful monitoring of IPD after implementation of conjugate vaccines is necessary.

Introduction

Streptococcus pneumoniae is a common colonizer of the human nasopharyngeal tract and a major cause of invasive bacterial infections, such as meningitis and bacteremia, also called invasive pneumococcal disease (IPD). The potential of pneumococci to become invasive has been suggested to be related to the polysaccharide capsule composition (i.e., the pneumococcal serotype or serogroup).[1] Next to invasiveness, the course of disease may also be related to the serotype. Studies of this topic are scarce because large cohorts of patients are required in view of the 91 currently acknowledged serogroups[2], and the results of the available studies vary.[3-6] Moreover, most studies were not able to assess the association between infecting serotype and disease severity in the absence of any potential disturbance by antibiotic resistance and vaccine effects because pneumococcal vaccines, particularly the 23-valent pneumococcal polysaccharide vaccine (PPV23), are widely used. In the Netherlands we had a unique opportunity to assess this relation without potential disturbance by vaccine effects and antibiotic resistance. We conducted a retrospective cohort study of adults with IPD who presented from 1 June 2004 through 31 May 2006, before the implementation of the 7-valent pneumococcal conjugate vaccine (PCV7) in the infant immunization program. Coverage rates for PPV23 have been negligible in all age groups in the Netherlands[7], and antibiotic resistance has traditionally been low.[8;9]

Methods

Identification of patients

The Netherlands Reference Laboratory for Bacterial Meningitis (Academic Medical Center/ National Institute of Public Health and the Environment, Amsterdam, the Netherlands) is a laboratory-based surveillance system that collects nationwide pneumococcal isolates from blood and cerebrospinal fluid (CSF) samples. Isolates from other normally sterile bodily fluids constitute <3% of samples. Nine sentinel microbiology regional laboratories across the Netherlands, covering a representative proportion of ~25% of the Dutch population with ~4.1 million inhabitants, reported all pneumococcal isolates from sterile sites from 2004 on. These sentinel laboratories identified 1150 mainly hospitalized patients (age, ≥18 years) with IPD during 1 June 2004 through 31 May 2006, before the implementation of PCV7 in the national infant immunization program. Pneumococcal isolates received by the Netherlands Reference Laboratory for Bacterial Meningitis were typed by coagglutination and serotyped by the capsular swelling method (Quellung reaction) using antisera (Statens Serum Institute).

Diagnosis and other covariates

Information about the disease syndrome, information on the presence of underlying conditions at the time of diagnosis, and follow-up information on admission to the intensive care unit (ICU) and case fatalities (in-hospital death or death within 30 days after the first culture of a normally sterile site positive for *S. pneumoniae*) were extracted from

hospital medical records using a standard data collection form. Meningitis was defined as a CSF culture positive for *S. pneumoniae* (or a positive CSF polymerase chain reaction result) or a clinical diagnosis of meningitis in combination with a blood culture positive for *S. pneumoniae*. Invasive pneumonia included physician-diagnosed pneumonia with a blood culture positive for *S. pneumoniae*. Bacteremia with other focus was defined as a positive blood culture in combination with a clinical focus other than meningitis or pneumonia. If no clinical focus could be identified, it was recorded as bacteremia without focus.

Immunocompromising conditions included primary immunodeficiency, human immunodeficiency virus infection (with or without progression to AIDS), current lymphoma, leukemia, myeloma, solid organ or stem cell transplantation, current immunosuppressive therapy for malignancy or autoimmune disease, asplenia, sickle cell disease, renal insufficiency or need for dialysis, and nephrotic syndrome. Other comorbidities included the presence of a malignant neoplasm not considered immunocompromising, chronic obstructive pulmonary disease or asthma, diabetes mellitus, cardiovascular disease (history of myocardial infarction, coronary artery condition, history of a cerebrovascular accident or transient ischemic attack, cardiomyopathy or heart failure, heart valve disease, or cerebral, abdominal, or thoracic aneurysms), liver disease, and (a history of) long-term alcohol abuse.

Statistical analysis

All data were analyzed with the statistical software package SPSS, version 12.0.1 (SPSS), and Episheet.[10] The potential association between serogroups or serotypes and disease severity was assessed in 3 ways: (1) patient characteristics and disease severity were determined by the invasive disease potential of the infecting serotype or serogroup, (2) the serotype-specific relative rate of the more serious clinical syndromes was determined (meningitis and bacteremia without focus proved to be the most severe clinical syndromes of IPD in our data set), and (3) the serotype-specific case-fatality rate was determined.

The invasive disease potential describes the tendency of bacteria to become invasive and cause IPD while colonizing the nasopharynx. As Sjöström *et al.*[5] previously did, pneumococcal serogroups were grouped by invasive disease potential according to the meta-analysis of Brueggemann *et al.*[1] in which nasopharyngeal carriage rates of serogroups were compared with their rates of IPD. Low invasive serogroups cause invasive disease infrequently relative to their high nasopharyngeal colonization rates, whereas high invasive serogroups frequently cause IPD relative to their low colonization rates. According to Brueggemann *et al.*[1], with serotype 14 as reference and set to 1, serogroups were grouped as having high invasive disease potential when they had an odds ratio (OR) >1 (i.e., serogroups 1, 5, and 7), intermediate invasive disease potential when they had an OR >0.5 but <1 (i.e., serogroups 4, 14, 18, and 9), and low invasive disease potential when they had an OR <0.5 (i.e., serogroups 3, 6, 8, 15, 19, 23, and 33). For these groups, the relative rate of the more serious clinical syndromes of meningitis and occult bacteremia (i.e., the share of these clinical syndromes as part of all IPD cases caused by these serotypes or serogroups),

the proportion of patients with certain characteristics (e.g., age, >79 years, and presence of underlying conditions), the proportion of patients requiring ICU admission, and case-fatality rates were determined.

The relative rate of the more serious clinical syndromes of IPD was determined separately for each serogroup or serotype with at least 5 reported isolates. Taking the serogroups or serotypes with the lowest relative rate as reference, ORs with 95% confidence intervals (CIs) for the more serious disease syndromes of IPD were determined for all serogroups or serotypes in a multivariable logistic regression model, correcting for other patient and disease characteristics using $P < .05$ as the cutoff for statistical significance. The Hosmer-Lemeshow test was applied to assess goodness of fit of the model.

The ORs with 95% CIs for case fatalities were also determined for all serogroups or serotypes with at least 5 reported isolates. For statistical reasons, serotypes were subsequently clustered in groups according to their case-fatality rates. The group of serotypes with the lowest case-fatality rates served as reference in the model. Again, ORs were assessed independent of other covariates, including sex, age, and underlying conditions, and the disease syndrome was assessed using a multivariable logistic regression model. Because not all more serious disease courses are captured by the outcome of death alone, we also assessed associations with an alternative combined outcome (i.e., case-fatality [as previously defined] and/or prolonged hospitalization [defined as a hospital stay beyond the 75th percentile—that is, >19 days]).

Results

In total 1150 patients with IPD were described, and in 1142 patients (99%), the isolate could be typed. Clinical information from hospital records was available for 1107 patients (96%). This information concerned almost exclusively hospitalized patients (99%).

Disease severity and patient characteristics by invasive disease potential

Compared with IPD caused by serogroups with high invasive disease potential (serogroups 1, 5, and 7), IPD caused by serogroups with low invasive disease potential (serogroups 3, 6, 8, 15, 19, 23, and 33) concerned more often the most serious clinical syndromes of meningitis and bacteremia without focus (21.7% vs. 9.9% for the high invasive serotypes 1, 5, and 7), which had higher rates of admission to the ICU and higher case-fatality rates (table 1). More fragile persons (i.e., those who were older and/or had underlying conditions, such as immunocompromising conditions and current malignancy) were particularly affected by the intermediate and low invasive pneumococcal serogroups; high invasive serogroups or serotypes were often associated with milder disease manifestations and affected more often individuals without comorbidities.

Association between certain serotypes and disease syndrome

Serotypes varied widely in the relative rate by which they caused the clinical syndromes of meningitis or bacteremia without focus, which allowed serotypes to be entered separately into the model without the need to cluster these serotypes into groups (table 2). Within serotypes 1 and 4 IPD, relatively small proportions of meningitis or bacteremia without focus were found, whereas serotypes 6B, 9N, 10A, 16F, 18C, 19F, 20, and 22A IPD had relatively high proportions of these clinical syndromes. These differences were found to be independent of age and the presence of asthma or chronic obstructive pulmonary disease, which were statistically the only significant confounders.

Table 1. Case characteristics and disease course by invasive disease potential of serotypes.

Characteristic	Invasive disease potential of serogroups, %[a]			
	High (Serogroups 1, 5 and 7) (N=212)	Intermediate (Serogroups 4, 9, 14, 18) (N=380)	Low (Serogroups 3, 6, 8, 15, 19, 23, 33, 38) (N=380)	p[b]
Patient characteristics				
Meningitis / bacteremia	9.9	11.5	21.7	<0.01
Age, >79 years	12.4	24.1	21.3	<0.01
Comorbidity				
Any	56.1	73.3	73.5	<0.01
Immunocompromised	9.9	18.1	19.5	<0.01
Malignancy[c]	4.2	6.0	9.5	0.04
Asthma or chronic obstructive pulmonary disease	22.2	31.6	25.3	0.03
Disease course				
Intensive Care Unit	18.8	22.1	26.1	0.15
Fatality	9.9	16.8	21.1	<0.01

a Invasive disease potential was chosen according to Brueggemann *et al.* [1].
b Univariate P value for differences among the groups.
c Malignancy not defined below immunocompromised.

Association between certain serotypes and disease course

In univariate analysis, both serotype 7F, which was also among the most prevalent serotypes in the current study among adults, and serotype 1 showed case-fatality rates in the lower range, along with serotypes 5, 20, 15B, and 33F (Figure 1). The case-fatality rates of these last 4 serotypes were, however, imprecise because of the low number of reported isolates of each. Serotypes 3, 6B, 9N, 16F, 18C, 19F, and 23A had case-fatality rates in the higher range. According to their case-fatality rates, serotypes were grouped into those with the lowest case-fatality rates (reference group composed of serotypes 1, 5, 7F, 15B, 20, and 33F), intermediate case-fatality rates (serotypes 4, 6A, 8, 9V, 10A, 11A, 12F, 14, 19A, 22A, 22F, 23F, and 24F), and the highest case-fatality rates (serotypes 3, 6B, 9N, 16F, 18C, 19F, and 23A) (Figure 1). In a multivariable model, the latter group of serotypes remained significantly associated with a higher case-fatality rate, as well as immunocompromising conditions (OR, 1.5; 95% CI, 1.0–2.4), cardiovascular disease (OR, 1.6; 95% CI, 1.1–2.3), the

Table 2. Serotype and disease syndrome.

Serotype	Number of isolates	Meningitis/ occult bacteremia	Invasive pneumonia/ bacteremia with other focus	Multivariate OR (95% CI)*
5	5	0 (0)	5	Referent
1	66	2 (3.0)	64	Referent
4	100	7 (7.0)	93	Referent
11A	12	1 (8.3)	11	1.8 (0.2-16.2)
14	140	12 (8.6)	128	2.0 (0.8-5.0)
19A	33	3 (9.1)	30	2.1 (0.5-8.4)
9V	97	9 (9.3)	88	1.8 (0.7-4.9)
7F	140	18 (12.9)	122	2.6 (1.1-6.0)
3	67	9 (13.4)	58	3.1 (1.2-8.4)
8	86	12 (14.0)	74	3.1 (1.2-7.8)
33F	13	2 (15.4)	11	3.0 (0.6-15.9)
24F	5	1 (20.0)	4	5.1 (0.5-54.9)
22F	21	5 (23.8)	16	5.6 (1.6-19.3)
6A	31	8 (25.8)	23	6.5 (2.1-19.5)
23F	68	18 (26.5)	50	6.5 (2.7-15.7)
9N	26	7 (26.9)	19	8.8 (2.8-27.3)
23A	7	2 (28.6)	5	6.0 (1.0-35.8)
12F	17	5 (29.4)	12	6.0 (1.7-21.2)
6B	27	9 (33.3)	18	9.5 (3.3-27.6)
16F	10	4 (40.0)	6	11.8 (2.8-50.7)
15B	5	2 (40.0)	3	7.8 (1.1-54.0)
20	7	3 (42.9)	4	15.3 (2.7-87.5)
19F	37	16 (43.2)	21	19.1 (7.2-50.6)
10A	15	7 (46.7)	8	25.8 (7.0-95.2)
18C	18	9 (50.0)	9	21.3 (6.5-70.0)
22A	5	3 (60.0)	2	25.9 (3.7-178.4)

Note. Serotypes with statistically significant multivariable odds ratios (ORs) are presented in boldface font. CI, confidence interval.
a Serotypes ranked according to their relative rate of meningitis or bacteremia without focus.
b Independent of age and chronic obstructive pulmonary disease or asthma (the only 2 covariatesremaining significant in the multivariable model); $P = .80$, by Hosmer-Lemeshow test.

severe clinical syndromes of meningitis or bacteremia without focus (OR, 2.9; 95% CI, 1.9–4.4), and age (ORs ranging from 4.5 [95% CI, 1.0–20.0] in 40–59-yearolds to 21.2 [95% CI, 5.1–95.7] in persons aged ≥80 years). On the contrary, underlying diabetes (OR, 0.5; 95% CI, 0.3– 0.8) was found to be associated with a lower case-fatality rate.

Ranking the serotypes according to case-fatality and/or prolonged hospitalization (>19 days) showed similarity with the case-fatality rate alone, again with serotypes 1 and 7F showing a relatively low rate of this unfavorable outcome and serotypes 3, 6B, 9N, 18C, and 23F in the highest range of case-fatality and/or prolonged hospitalization (table 3). The group of serotypes with the highest rates of this outcome (serotypes 3, 6B, 9N, 18C, 22A, 22F, 23F, and 33F) remained significantly associated with a higher case-fatality or prolonged hospitalization rate (OR, 1.9; 95% CI, 1.4–2.7) in a multivariable model. In addition, older age (ORs ranging from 3.6 [95% CI, 1.7–7.9] in 40–59-year-olds to 13.5 [95% CI, 6.2–29.3] in persons aged ≥80 years), the severe clinical syndromes of meningitis or bacteremia without

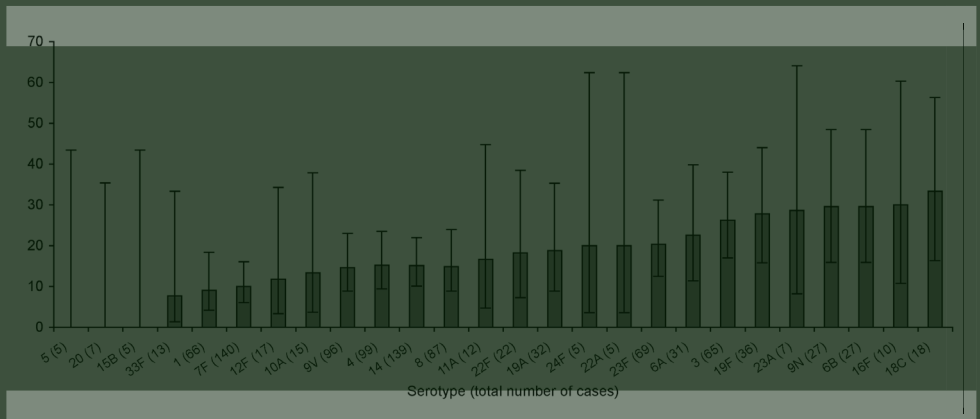

Figure 1. Serogroup- or type-specific case-fatality rates. Asterisk indicates independent of age ($p<0.01$), clinical syndrome of meningitis or occult bacteremia ($p<0.01$), diabetes mellitus ($p<0.01$), cardiovascular disease ($p =0.02$), and underlying immunocompromising conditions ($p=0.04$). $p=0.38$, by Hosmer-Lemeshow test. Error bars indicate 95% confidence intervals (CIs).

focus (OR, 3.4, 95% CI, 2.4–4.9), and (a history of) alcohol abuse (OR, 2.0; 95% CI, 1.1–3.5) were associated with higher rates of case-fatality or prolonged hospitalization, whereas underlying diabetes again was associated with a lower rate (OR, 0.7; 95% CI, 0.5–0.9).

Discussion

In this large retrospective study of hospitalized adults with IPD, we found that serogroups known to have high invasive disease potential in children[1] (serogroups 1, 5, and 7) affected relatively healthy adults, whereas those previously shown to have low or intermediate invasive disease potential (serogroups 3, 6, 8, 15, 19, 23, 33, and 38) were more likely to affect more fragile individuals at an older age and/or those with underlying conditions. Independent of patient (and disease) characteristics, a wide variation was seen among pneumococcal capsular serotypes regarding their relative rate of causing the clinically most serious syndromes of meningitis or bacteremia without focus but also regarding case-fatality rate and/or rate of prolonged hospitalization. The group of serotypes that included serotypes 3, 6B, 9N, 18C, and 19F was more often associated with an unfavorable outcome than the group that included serotypes 1 and 7F, even after correction for underlying patient and disease characteristics.

The currently licensed PCV7 is targeted against 7 serotypes common in pediatric IPD and has proven to be highly effective against vaccine-serotype invasive disease in children.[11] After implementation of PCV7 in the United States in 2000, not only was a sharp decrease in vaccine-serotype IPD in the target group of young children observed but also a considerable decrease in IPD in other unvaccinated age groups was seen, which has been attributed to a herd effect.[4] However, a gradual but progressive increase in several non-vaccine serotype

Table 3. Longer hospital stay and/or case-fatality, by serotype.

Serotype	Number of isolates	Longer hospital stay or case-fatality[a]	Unadjusted OR	Adjusted OR (95% CI)[b]
Reference serotypes				
5	5	0 (0)	Referent	Referent[c]
20	7	1 (14.3)		
15B	5	1 (20.0)		
10A	15	4 (26.7)		
1	66	18 (27.3)		
9V	96	28 (29.2%)		
12F	17	5 (29.4)		
8	85	25 (29.4)		
6A	31	10 (32.3)		
7F	137	47 (34.3)		
4	98	35 (35.7)		
Serotypes with intermediate risk of outcome				
19F	36	14 (38.9)	1.5 (1.1–2.0)	1.1 (0.8–1.6)[c]
14	137	54 (39.4)		
19A	33	13 (39.4)		
24F	5	2 (40.0)		
16F	10	4 (40.0)		
11A	12	5 (41.7)		
23A	7	3 (42.9)		
Serotypes with high risk of outcome				
33F	13	6 (46.2)	2.5 (1.8–3.4)	1.9 (1.4–2.7)[c]
23F	65	30 (46.2)		
9N	25	12 (48.0)		
22F	22	11 (50.0)		
18C	18	9 (50.0)		
6B	26	57.7% (15)		
3	64	59.4% (38)		
22A	5	80.0% (4)		

NOTE. CI, confidence interval; OR, odds ratio.

[a] Hospital stay 119 days and/or in-hospital death or death <30 days after the first blood or cerebrospinal fluid culture positive for *Streptococcus pneumoniae*.

[b] Adjusted for age (p<0.01), clinical syndrome of meningitis or occult bacteremia (p<0.01), diabetes mellitus (p=0.02), and alcohol abuse (p=0.02). p=0.68, by Hosmer-Lemeshow test.

[c] The ORs are estimated for the whole group of serotypes with the reference serotypes as a reference.

IPDs has also been observed.[12] This emergence of non-vaccine serotype IPD may be a natural temporal trend but may also be caused by widespread conjugate vaccination. Infants and toddlers represent the largest reservoir for spreading pneumococci in the community, and although after conjugate vaccination the overall pneumococcal carriage rates in children have remained more or less similar, vaccine serotypes have decreased but have been concomitantly replaced by non-vaccine serotypes in carriage studies.[13;14] In view of the results of the current study, the question is whether shifts in serotype distribution of IPD may be accompanied by a change in disease severity.

Our study was based on detailed information about the clinical syndrome and its underlying conditions, which was available for each patient, allowing assessment of the association between capsular serotype and disease severity in a multivariable model that corrected for other patient and disease characteristics. However, to appreciate the results of the current study, some potential weaknesses should be addressed. The invasive disease potential of pneumococci with certain serogroups was chosen according to Brueggemann *et al*.[1] These investigators determined the invasive disease potential of serogroups by comparing serotype distribution in asymptomatic nasopharyngeal carriage with that in IPD among children. Although we worked with the assumption that the invasive potential of serogroups in adults is similar to that in children, this is unknown and requires further study. Furthermore, despite the inclusion of 1142 patients with available pneumococcal capsular serotyping, the number of isolates per serotype was still relatively small and prohibited evaluation of case-fatality rates by individual serotype. Clustering of serotypes was necessary, and consequently no firm conclusions may be drawn about individual serotypes. In the Netherlands, as in most of Europe, referral patterns for invasive disease are considerably different from the United States for infants, but less so for adults. If proven invasive disease is present among adults, most adults will end up in the hospital for further diagnosis and treatment. We therefore do not believe that such bias has affected our results. In addition, we have estimated relative risks for different groups of serotypes that are unlikely to be affected by such referral bias.

Several studies have been performed on the relation between pneumococcal serotypes and disease. A smaller Swedish study of 494 adults with IPD showed that pneumococci with serotypes with low invasive disease potential behave as opportunistic pathogens, which means that they especially cause disease in fragile persons, whereas pneumococci with serotypes 1 and 7F, known to have high invasive disease potential, acted as primary pathogens, causing infections in previously healthy individuals.[5] Our results are also in agreement with the observation in that study that IPD caused by pneumococci with these high invasive serotypes often had a favorable outcome. In a large study of 5579 adults with IPD aged ≥50 years, serotypes 3, 11A, 19F, and 23F were found to be associated with significantly higher case-fatality rates than was serotype 14 (chosen to be the reference type because it was the most frequently occurring serotype).[4] This study was however performed during the first 4 years after introduction of PCV7 in the United States, where PPV23 is also widely used. A Danish study of 464 hospitalized adults with IPD demonstrated that infection with serotype 3 was associated with an increased risk of death, whereas serotype 1 was associated with a decreased risk of death.[3] Our findings are in agreement with the higher observed casefatality rates of serotypes 3 and 19F IPD and the lower case fatality rate of serotype 1 IPD. We also observed lower casefatality rates of serotype 7F disease. Another international study (in 10 western and nonwestern countries together) restricted to 796 hospitalized patients with bacteremia aged ≥15 years concluded that neither serotypes defined as invasive or pediatric (serotypes commonly causing IPD in children) nor PCV7 serotypes as a group were significantly associated with a higher mortality rate.[6] However, this observation does not exclude the possibility of an association

between individual serotypes and a higher or lower case-fatality rate.

In our study, diabetes appeared to be associated with a lower case-fatality rate independent of other patient and disease characteristics. This finding seems counterintuitive because diabetes is a risk factor for acquiring IPD.[15] However, there is evidence that tight glucose control during critical illness might favor the outcome.[16] It may be that diabetic patients are particularly more closely monitored for blood glucose fluctuations once hospitalized and critically ill and, consequently, may benefit from tight glucose regulation, but evidence is required to support this theory.

This and previous studies have focused on the association between capsular polysaccharide serotype and disease severity. Apart from the polysaccharide capsule, however, many other components of the pneumococcus, including its genotype, are implicated in virulence and in interaction with the immune system and may affect disease severity. This warrants further study, although the capsule is strongly linked to the bacterial genotypic clones for many serotypes circulating in a defined geographic region and for the majority of strains of a serotype causing invasive disease.[17]

The current study indicated that, in the presence of negligible coverage rates of PCV7 and the PPV23 and very low antibiotic resistance of pneumococci, disease severity of IPD, including case fatalities, varies by serogroup or serotype. Although no firm conclusions can be drawn for individual serotypes, it appears that several serotypes, including several not covered by PCV7, are associated with relatively high proportions of the more serious clinical syndromes and/or higher case-fatality rates. This information is valuable in the development and introduction of future pneumococcal conjugate vaccines. Because the introduction of PCV7 among young children has been associated with obvious shifts in serotype carriage rates and (discrete) shifts in serotype distribution in IPD, shifts in disease severity may occur. This warrants careful monitoring of nasopharyngeal carriage and IPD in the future both for serotypes and in case of disease for clinical syndromes and outcome.

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5

Outcome of childhood invasive pneumococcal infection by serotype: retrospective case series

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Abstract

Large-scale introduction of the 7-valent pneumococcal conjugate vaccine (PCV7) among infants in many countries has induced shifts in circulating pneumococcal serotypes. To predict potential consequences for the burden of invasive pneumococcal disease (IPD) among children more data on the serotype-specific prognosis are needed. A hospital chart-review of 296 IPD cases under 18 years of age was conducted in the Netherlands prior to the Netherlands implementation of PCV7 among infants. In multivariable analysis with the serotypes grouped according to their case-fatality, the group with serotype 19A en 19F, and the group with serotypes 4, 6A en 6B, had significantly higher case-fatality than the reference group consisting of serotype 1, 8, 9V, and 14.

Introduction

The introduction of the 7-valent pneumococcal conjugate vaccine (PCV7) among children under five years of age in the United States has been associated with a major decrease in the incidence of invasive pneumococcal disease (IPD) such as pneumococcal meningitis and bacteremia in the target group.[1;2] In particular IPD caused by pneumococcal serotypes covered by PCV7 (vaccine serotype IPD) was found reduced by more than 90% in children under 2 years of age.[1;2] However, a concomitant increase of non-vaccine-type IPD, although still discrete, raises concern.[3] In particular, serotype 19A now causes 40% of all childhood IPD cases under 5 years of age.[3]

The polysaccharide capsule of the pneumococcus, which defines the serotype, is an important virulence factor for invasiveness, i.e. the potential to cause IPD when colonizing the nasopharynx.[4] Also the course of IPD may differ in severity between serotypes.[5-10] More understanding of whether serotype affects disease characteristics is important to evaluate the potential consequences of shifts in serotype distribution in IPD after widespread implementation of PCV7. Available studies on this topic in children are scarce and results are inconclusive. A German study indicated that serotype 7F, but also 23F, and 3 had a high case-fatality[10], but a recent large study conducted in Denmark, could not demonstrate statistically significant serotype-mortality associations in children.[11] The current study comprising 296 childhood cases of IPD aimed to provide additional information about the relation between the infecting pneumococcal serotype and outcome in the Netherlands in the pre-vaccination era.

Methods

Cases of IPD

Cases of IPD were provided by the Netherlands Reference Laboratory for Bacterial Meningitis (NRLBM). The NRLBM is a laboratory-based surveillance system that collects nationwide bacterial isolates from blood and cerebrospinal fluid (CSF) of patients almost exclusively requiring hospitalization. Isolates from other normally sterile bodily fluids comprise less than 3% of all isolates.[12] The pneumococcal isolates were obtained from nine sentinel microbiology laboratories spread across the Netherlands that cover approximately 4.1 million inhabitants (~0.9 million 0-17-year-olds) and form a representative proportion of ~25% of the Dutch population. The sentinel laboratories submitted 90% of the pneumococcal isolates from CSF and 83% of all pneumococcal isolates from the blood (internal survey, personal communication, L. Spanjaard). We included all isolates of children aged 0 to 18 years in the period June 2001 to June 2006. In the Netherlands, PCV7 was introduced for all newborns born after March 31, 2006 without a catch-up program for older children. As the vaccine is administered at 2, 3, 4, and 11 months of age, eligible infants were vaccinated from June 2006 onwards.[13] The uptake of PCV7 in our study period was negligible since it was only recommended for children belonging to risk groups.[14] The Dutch pre-vaccination incidence rate of IPD was 21 cases per 100,000 annually in

0-4-yr-olds, 3 cases per 100,000 annually in 5-9-yr-olds, and 1 case per 100,000 annually in 10-17-yr-olds.

The NRLBM typed all pneumococcal isolates by co-agglutination and serotyped them by the capsular swelling method (Quellung reaction) using antisera (Statens Serum Institute, Copenhagen, Denmark). Bacterial resistance against antibiotics has traditionally been very low in the Netherlands.[15] Pneumococcal isolates (recovered from persons of all ages) received during the study period were for 98.9% susceptible to penicillin (minimal inhibitory concentration <0.06 µg/ml), for 0.7% intermediately susceptible (0.06-1.0 µg/ml), and for 0.4% resistant (>1.0 µg/ml). This low rate of antibiotic resistance is presumably linked to the relatively low antibiotic consumption in the Netherlands.[16;17]

Patient and disease characteristics

From all cases, information about the disease syndrome, presence of underlying conditions at the time of diagnosis and follow-up information on admission to Intensive Care Unit (ICU), sequelae at the time of discharge from the hospital, and case-fatality were extracted from hospital charts using a standard data collection form. Meningitis was defined as a CSF culture positive for *S. pneumoniae* (or a positive CSF PCR) or a clinical diagnosis of meningitis in combination with a blood culture positive for *S. pneumoniae*. Invasive pneumonia included a physician-diagnosed pneumonia together with a blood culture positive for *S. pneumoniae*. Bacteremia with other focus was defined as a positive blood culture in combination with a clinical focus other than meningitis or pneumonia. If no focus could be identified, it was recorded as bacteremia without focus.

Co-morbid conditions were considered present when the patient suffered from primary immunodeficiency, HIV (with or without progression to AIDS), current malignancy, solid organ or stem cell transplantation, current immunosuppressive therapy (for malignancy or autoimmune disease), asplenia/splenectomy, hemoglobinopathy, renal insufficiency/need for dialysis, nephrotic syndrome, asthma (in children aged 5 years or older), diabetes mellitus, cardiac disease (cardiomyopathy/heart failure, heart valve disease), severe liver disease, CSF leak and recent severe physical trauma/skull fracture, premature birth (<37 weeks for 0-1-yr-olds and <32 for 0-4-yr-olds), serious perinatal complications for 0-1-yr-olds, congenital conditions/syndromes, and serious failure to thrive.

Severe sequelae at discharge from the hospital were recorded and included mental retardation, hemi/tetraparalysis/paresis, hydrocephalus, epilepsy requiring anti-epileptics, and deafness. Case-fatality was defined as in-hospital death or death within 30 days after the first culture of a normally sterile site positive for *S. pneumoniae*).

Statistical aspects

All data were analyzed with the statistical software package SPSS 15.0 and Episheet.[18] IPD cases with an unfavourable outcome (i.e. death or non-fatal severe sequelae) were described in terms of patient characteristics (age, presence of co-morbidity), disease characteristics (disease syndrome, disease course), and infecting pneumococcal serotype.

Characteristics of IPD were also described by vaccine-type (serotype 4, 6B, 9V, 14, 18C, 19F, and 23F) and non-vaccine-type serotypes (all other serotypes), and by invasive disease potential. Like Sjöström *et al.*[8] previously did, pneumococcal serogroups were grouped by invasive disease potential according to the meta-analysis of Brueggemann *et al.*[19] in which nasopharyngeal carriage rates of serogroups were compared with their rates of IPD. Low-invasive serogroups cause invasive disease infrequently relative to their nasopharyngeal colonization rates, whereas high-invasive serogroups cause frequently IPD relative to their colonization rates. According to Brueggemann et al.[19] with serotype 14 as reference and set to 1, serogroups were grouped as having high invasive disease potential when they had an odds ratio (OR) >1, i.e. serogroups 1, 5, and 7, intermediate invasive disease potential when $0.5<OR\leq1$, i.e. serogroups 4, 14, 18, 9, and low invasive disease potential when $OR<0.5$, i.e. serogroups 3, 6, 8, 15, 19, 23, 33, and 38. P-values were determined by the chi-square test. Furthermore, the serotype-specific case-fatality with 95% confidence interval (95% CI) was determined for all serotypes with at least five reported isolates. For statistical reasons, serotypes were subsequently clustered in groups according to their case-fatality, to calculate group odds ratios (ORs) for case-fatality with the group with the lowest case-fatality as reference. ORs were assessed independently of other covariates including sex, age, and the presence of co-morbidity, and the disease syndrome in a multivariable logistic regression model using p-value< 0.05 as cut-off for statistical significance. The Hosmer and Lemeshow test was applied to assess goodness-of-fit of the model.

Results

In total, 324 childhood IPD cases were identified during the study period. For 314 cases (97%) the isolate could be serotyped and for 296 cases (91%) clinical information was available: 116 cases of meningitis, 81 cases of invasive pneumonia, 51 cases of bacteremia with other focus, and 48 cases of bacteremia without focus. Almost all cases required hospitalisation (98%). The median age was 1.5 years (interquartile range 0.6-4.2 years): 49 (17%) were younger than 6 months, 66 (22%) were 6-11 months, 61 (21%) were 12-23 months, 55 (19%) were 2-4 years, and 65 (22%) were 5-17 years of age. Overall, 60% were male.

Vaccine serotype vs. non-vaccine serotype

In 62% (177) of the IPD cases a pneumococcal serotype included in PCV7 was the infecting serotype: 22% serotype 14, 13% serotype 6B, 7% 23F, 6% 18C, 5% 19F, 5% 9V, and 3% 4. Of the non-vaccine serotypes, 7F (10%), 1 (6%), 6A (5%), 19A (3%), 8 (2%), and 3 (2%) were most common. Vaccine serotype IPD as a group did not differ significantly from non-vaccine-type IPD regarding age of the patient, presence of co-morbidity, disease manifestation, and disease course (Table 1).

Table 1. Characteristics of invasive pneumococcal disease, vaccine serotypes versus non-vaccine serotypes.

		Vaccine serotype* (N=177)	Non-vaccine serotype* (N=108)
Age median (IQR)		1.4 (0.7-3.3)	1.4 (0.6-5.8)
Manifestation	meningitis	38% (67)	37% (40)
	pneumonia	25% (44)	32% (35)
	bacteremia without focus	19% (33)	14% (15)
Co-morbidity	any	33% (57)	35% (38)
	immuno-compromised	7% (12)	6% (7)
Length of hospital stay in survivors median (IQR)		10 (5-13)	9 (6-13)
Admission to Intensive Care Unit		17% (28)	15% (15)
Death		6% (10)	6% (6)
Serious sequelae in survivors		8%	6%

* Vaccine-type serotypes include serotype 4, 6B, 9V, 14, 18C, 19F, and 23F; non-vaccine serotypes are all other serotypes including serotype 6A and 19A.

Invasiveness according to Bruegemann et al.[19]

IPD caused by serogroups with high invasive disease potential (1, 5, and 7) concerned less often the most serious clinical syndromes meningitis and bacteremia without focus (41%), than IPD caused by serogroups with intermediate (4, 9, 14, 18) and low (3, 6, 8, 15, 19, 23, 33, 38) invasive disease potential (51% and 62%, respectively) (p-value 0.04). The serogroups with high invasive disease potential appeared also associated with a lower case-fatality than the intermediate and low invasive serogroups (2%, 3%, and 9%, respectively; p-value 0.07). The percentages of IPD cases in children younger than 12 months were 31%, 37%, and 44% for these serogroups; percentages of IPD cases with any co-morbidity were 30%, 28%, and 36%; these differences were not statistically significant.

Case-fatality

The overall case-fatality rate of IPD in our study population was 5.5%: 16 children died within 30 days after the first blood/CSF culture positive for *S. pneumoniae*. All IPD cases with fatal outcome concerned bacteremia without focus (5) or meningitis (11) corresponding to a fatality rate of 11% and 10%, respectively. None of the cases of IPD with other foci, including invasive pneumonia, were fatal. Children who died had a lower median age of 0.7 (IQR 0.5-4.0) years compared with survivors who had a median age of 1.5 years (IQR 0.7-4.2). While most childhood cases with fatal outcome younger than 12 months of age had no known co-morbidity (two of the in total nine fatal cases in 0-11-mnth-olds; one with premature birth (34 weeks) and one with Down's syndrome), most fatal cases later in childhood demonstrated co-morbidity (five of seven cases). Among the fatal cases three concerned serotype 19F, three 6B, and serotype 3, 4, 7F, 10A, 14, 18C, 19A, 22F, 23F, were each responsible for one case. None of these fatal cases were vaccinated with the

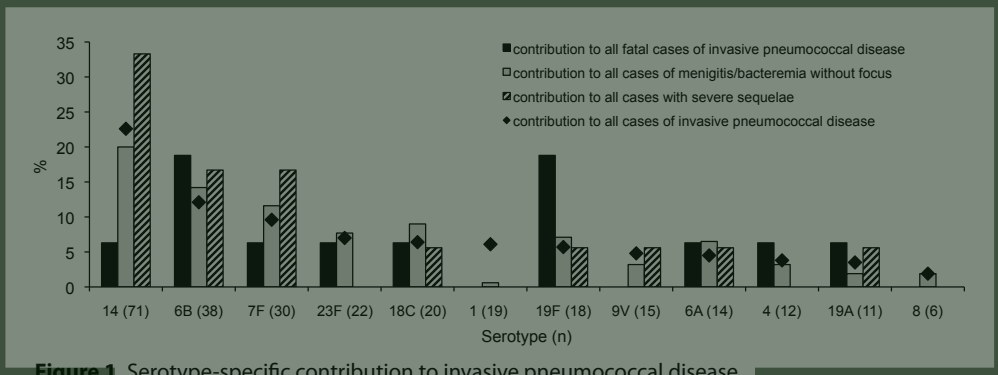

Figure 1. Serotype-specific contribution to invasive pneumococcal disease.

pneumococcal conjugate vaccine before they got the disease.

In Figure 1 the serotype-specific contributions to fatal cases are shown in comparison with their contribution to all IPD cases together, and to cases of meningitis or bacteremia without focus. The serotype-specific contribution to cases of meningitis or bacteremia without focus roughly resembles the serotype-specific contribution to all IPD cases together. The serotype-specific contribution to fatal IPD-cases shows that whereas serotype 19F causes 6% of all IPD in children, it is responsible for 19% of all fatal cases of IPD. Oppositely, serotype 14 accounts for 23% of all IPD, but only 6% of all fatal cases.

In univariate analysis, serotype 14, the most prevalent serotype in the current study in

Table 2. Serotype-specific case-fatality in children with invasive pneumococcal disease.

Serotype (N)	Case-fatality (95%CI)	Group OR (95% CI)
14 (63)	1.6 (0.3-8.5)	
8 (6)	0 (0-39.0)	Reference group
9V (13)	0 (0-22.8)	
1 (18)	0 (0-17.6)	
18C (18)	5.6 (1.0-25.8)	
23F (21)	4.8 (0.8-22.7)	4.5 (0.5-44.2)
7F (30)	3.3 (0.6-17.7)	
4 (10)	10.0 (1.8-40.4)	
6B (37)	8.1 (2.8-21.3)	9.0 (1.0-79.0)
6A (13)	7.7 (1.4-33.3)	
19F (15)	20.0 (7.0-45.2)	20.8 (2.2-196.9)
19A (8)	12.5 (2.2-47.1)	

* Hosmer and Lemeshow test p-value 1.00

children, had a case-fatality in the lower range, along with serotypes 1, 9V, and 8 (Table 2). Serotypes 19A, 19F, 4, 6A, and 6B had case-fatality rates in the high range. According to their case-fatality, serotypes were grouped into those with the lowest case-fatality (reference group composed of serotypes 1, 8, 9V, 14), intermediate case-fatality (serotypes 7F, 23F, 18C), higher case-fatality (serotypes 4, 6A, 6B), and the highest case-fatality (serotypes 19A,

19F). In multivariable analysis the group with serotypes 4, 6A, and 6B, and the group with serotype 19A and 19F remained significantly associated with a higher case-fatality (OR 9.0 (95% CI 1.0-79.0), OR 20.8 (95% CI 2.2-196.9), respectively); the co-variates age, sex, disease syndrome and presence of co-morbidity were not significant and were discarded from the model.

Follow-up information at the moment of discharge from the hospital was available for 261 (85%) children. The rate of serious sequelae was 7.3% (19 cases) in these survivors of IPD, and all occurred in children younger than 2 years of age. The following serotypes were involved: 14 (6), 7F (3), 6B (3), 6A(1), 9V (1), 9N (1), 18C (1), 19A (1), 19F (1) (one case could not be serotyped. Mostly, no known co-morbidity was present in these children; only three children who had IPD on the age of 5-7 months were known with prematurely born children (35 weeks) or perinatal asphyxia. All serious sequelae occurred after meningitis, with the exception of one case with otitis media solely as focus for IPD. Three children had a combined focus, i.e. meningitis combined with endocarditis, encephalitis, or pneumonia and mastoiditis. Most frequent sequela was deafness (14 cases); nine children got a cochlear implant. Epilepsy was the second most frequent sequela (7), followed by mental retardation (3), tetraplegia (2), hemiparesis (2), hydrocephalus (1), and aortic insufficiency (1).

Discussion

The current study comprising 296 childhood IPD cases suggested that the case-fatality of IPD differed by infecting serotype. In childhood IPD, especially serotype 19F, but also serotype 19A, 6A, 6B, and 4, appeared to be associated with a high case-fatality.

A single-site study conducted in the United States concerning 86 childhood cases of pneumococcal meningitis collected over the years 1993 to 2004, studied differences in IPD caused by vaccine serogroups and non-vaccine serogroups.[5] Their rationale behind dividing IPD by serogroup instead of serotype was a presumed cross-reactivity of the vaccine against serotypes with the same serogroup, i.e. against 6A, 9N, 19A, and 23A, although recent evidence does not actually support this for serotype 19A.[3] Nevertheless, their inclusion number was too small to allow for analysis by individual serotypes. They found no statistically significant differences between vaccine serogroup disease and non-vaccine serogroup disease in respect to the frequency of adverse outcomes although case-fatality estimates were insignificantly lower in cases caused by non-vaccine serogroups. No information was given on the influence of co-morbidity, bacterial resistance against antibiotics and vaccine-uptake in the community. In accordance with this study we also found no significant differences between vaccine-type and non-vaccine-type disease as groups. However, this does not exclude differences in individual serotypes.

A German study on 494 childhood cases of hospitalized IPD over 1997 to 2003 indicated that after correcting for potential confounders, especially serotype 7F, but also 23F, and 3 had a high case-fatality.[10] They recorded that four of the total of 27 children with serotype 7F IPD died, whereas in our study only one child died of the in total 30 children with

serotype 7F IPD. Also, serotype 23F disease was not associated with a poorer outcome in our study. The small number of cases per serotype in both studies and the consequently large statistical imprecision of the estimate may explain this discrepancy. Even a recent very large population-based cohort study conducted in Denmark over the years 1977 to 2007 with 1581 childhood IPD cases, could not demonstrate statistically significant single serotype-mortality associations, because of the very low overall childhood IPD-related mortality (less than 3%).[11]

For adults, several studies attempted to provide information on serotype-specific prognosis of IPD. In view of the immatureness of the immune system in children, in particular under 2 years of age, serotype-specific disease characteristics may be different from that in adults. Adult studies indicated a higher case-fatality for serotypes 3 and 19F, and a lower case-fatality for serotypes 1 and 14.[6-9] In a previous study of ours on the relation between pneumococcal serotype and case-fatality of IPD in adults we demonstrated that as a group, the serotypes 3, 6B, 9N, 18C, and 19F were more often associated with an unfavourable outcome than the group including serotypes 1 and 7F, even after correction for underlying patient and disease characteristics.[20] The current study may suggest that this information gathered in studies in adults may be generalized to children, which indicates that the capsular polysaccharide rather than the host immunity defines the outcome. When analyzed in a multivariable model with serotypes clustered according to unadjusted case-fatality, serotypes 19A and 19F as a group, and serotypes 4, 6A, and 6B as a group were associated with a higher case-fatality than the reference group composed of serotypes 1, 8, 9V, and 14 (OR 9.0 (95% CI 1.0-79.0) and OR 20.8 (95% CI 2.2-196.9), respectively), independent of other co-variates. In our adult study serotype 3 IPD also had a high case-fatality.[20] Like in other childhood IPD studies serotype 3 IPD was not very common in our study.[21] However, four cases of childhood serotype 3 IPD were included and one of them was fatal. Additionally, IPD caused by serotypes known to be highly invasive, i.e. serotype 1, 5, and 7F, was demonstrated to be associated with a milder disease course and also affected less often more fragile persons.[8;20] This tendency appears to be confirmed in the current study.

In our study the variables age, disease syndrome, and comorbidity were not significantly associated with mortality. However, inclusion numbers were small and consequently more subtle associations may not come forward. Furthermore, the selection of cases in our study may also have a role, since in the Netherlands, primary care functions as 'gatekeeper' for patients to attend secondary care. This means that patients with milder clinical syndromes are probably treated successfully at the primary care level without admission to the hospital where blood cultures are taken. Consequently, less severe manifestations of IPD are likely to be under-diagnosed and consequently underrepresented in our study. Presumably our cases represent the more severe manifestations of IPD requiring hospitalization. This might have lead to the fact that the variables such as disease syndrome, age and co-morbidity did not remain significant in the model and were discarded.

To appreciate the results of the current study, potential weaknesses and strengths should be acknowledged. Despite the relatively large number of cases included in our study, the numbers of cases per individual serotype are still quite small for drawing firm conclusions

about serotype-specific disease. For statistical reasons we therefore clustered serotypes. In attempts to assess the relation between serotype and disease, group analyses should however be interpreted with caution. Regardless of the basis on which serotypes are clustered, e.g. on the basis whether they are included in a vaccine or according to crude case-fatality, actual case-fatality of the individual serotype may differ from that of the group. Therefore, serotype-specific analysis is preferable. However, up to date 91 pneumococcal serotypes have been acknowledged and regarding the relative rareness of IPD in industrialized countries, very large databases would be required to provide serotype-specific analysis and allow for firm conclusion about disease characteristics of individual serotypes. Although initiatives should be encouraged to acquire such large databases, as yet these are very scarce. Therefore trend analysis with imprecise estimates of serotype-specific parameters and parameters based on group analyses may as yet be crucial for policy making. Because the polysaccharide composition of the pneumococcal capsule is considered to be crucial for virulence and is the target of for the serotype-specific protection against disease induced by the conjugate pneumococcal vaccines, we focussed on the serotype, with no consideration of the individual strains (clones) within a serotype. Further research should also address the question whether genotype or capsular type defines pneumococcal virulence. Recently, it was demonstrated using an in vitro assay that serotypes that are resistant tot neutrophil-mediated killing tended to be more heavily encapsulated.[22] Strengths of the current study are that the Netherlands is particularly suited to address the study topic, because of the very low resistance against antibiotics and the negligible uptake of pneumococcal vaccines in the study period, and consequently these aspects could not have biased the results.

In conclusion, the current study does support serotype-specific difference in virulence of IPD. Serotype 19A, 19F as well as 6A, 6B, and 4 appear associated with a higher case-fatality. However, as numbers per serotype were low, no firm conclusions may be drawn solely from this study and more studies are needed. Nevertheless the possibility of serotype-specific virulence does once again stress the importance of careful monitoring of IPD after introduction of PCV7, in particular regarding serotype replacement disease. Protection against serotypes associated with poorer prognosis is relevant for future vaccine coverage, and may substantially change the total disease burden.

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Part Two

Immune responses after reduced-dose schedules with the pneumococcal conjugate vaccine before nationwide implementation

6

Comparability of antibody response to a booster dose of 7-valent pneumococcal conjugate vaccine in infants primed with either 2 or 3 dose

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Abstract

In this cohort study we compared IgG antibody levels between infants immunized with 7-valent CRM197-conjugated pneumococcal vaccine (PCV7) at 2, 4 and 11 months and at 2, 3, 4 and 11 months of age, as measured by double adsorption ELISA. Pre- and post-booster levels following the 2+1- and 3+1-dose schedule were comparable for 5 out of 7 serotypes except for serotypes 6B and 19F. The proportion of children reaching post-booster antibody thresholds were comparable except for 6B (≥1.0 µg/ml and ≥5.0 µg/ml) and 19F (≥5.0 µg/ml). Surveillance studies are warranted for vaccine impact on 6B and 19F disease cases after reduced-dose PCV7 schedules.

Introduction

Streptococcus pneumonia is a leading cause of bacterial infections in children in the first years of life with clinical syndromes varying from non-invasive respiratory disease (pneumonia, otitis media) to invasive pneumococcal disease (IPD; sepsis, bacteremia and meningitis).[1] In 2000, the CRM197-conjugated 7-valent pneumococcal vaccine (PCV7) was licensed in the USA for infants for prevention of IPD and recommended in a 3+1 vaccination schedule with 3 primary doses in the first 6 months of life, followed by a booster dose in the second year of life. Clinically, protection against IPD after less than 4 doses was already observed in the licensure study for CRM197-conjugated PCV7, the Northern California Kaiser Permanente study.[2] In this study, clinical efficacy against vaccine serotype IPD in the intention to treat analysis was high (93.9%) despite the fact that only 58% of the children had received the full PCV7 schedule. Furthermore, protection by reduced-dose schedules in preventing vaccine serotype IPD in vaccinees was observed in a large case–control study from the USA showing high effectiveness with a 2+1-dose (98%, 95% confidence interval: 75–100%) and even a 2-dose schedule (96%, 95% confidence interval: 88–99%) during a period of vaccine shortage.[3] Increasingly crowded immunization programs have prompted exploration of PCV7 schedules with fewer doses and at present over half of the European countries have already implemented a 2+1-dose schedule, also to allow for programmatic differences and to reduce costs.[4-6]

For non-inferiority comparison between pneumococcal conjugate vaccines, an individual anticapsular serum IgG antibody concentration of 0.35 µg/ml 1 month after the primary series in infants was estimated to be associated with clinical efficacy against IPD, at least in industrialized countries like the USA.[7] In non-western countries and high-risk populations this threshold may be higher and more around 1.0 µg/ml.[8] However, threshold protective antibody levels are not well understood and seem to differ per serotype. The levels needed to prevent carriage are higher and were suggested to be around 5.0 µg/ml which is considerably higher than what is thought to be required for prevention of invasive disease.[2;8;9] Higher levels may also be required for pneumonia and otitis media compared with IPD.[10;11] A non-randomized immunogenicity study in the United Kingdom comparing a 2+1- and 3+1-dose schedule showed no consistent distinct differences between both vaccine schedules as measured by geo-metric mean concentrations (GMC) per vaccine serotype.[12] In a recent immunogenicity study in Iceland, post-primary differences in IgG GMCs were found for several serotypes following 2 or 3 primary vaccinations, yet after the booster dose the only difference observed was for serotype 18C.[13] However, since both the United Kingdom and Iceland study were performed with experimental 9-valent CRM197-conjugated pneumococcal vaccines that may have different immunogenic capacity compared with the currently licensed PCV7, potential differences between a 2+1- and 3+1-dose schedule may have been masked.[12;13] In 2005, in a single cohort of infants receiving a 2+1 PCV7 schedule, Kaythy *et al.* demonstrated low pre-booster levels for serotypes 6B and 23F.[14] Following the booster vaccination however, no differences were seen. Since immunogenicity studies comparing 2- and 3-dose primary schedules with the licensed 7-valent CRM197 pneumococcal conjugate vaccine in infants are scarce, we

evaluated individual serotype responses with the currently used PCV7 in a 2+1- and 3+1-dose schedule in infants with primary vaccination at 2 and 4 months or 2, 3 and 4 months of age and a booster dose at 11 months of age.

Subjects and methods

Study design

For this study we derived data from two separate cohorts in the Netherlands. The first study was a randomized controlled trial investigating the effects of reduced-dose PCV7 schedules on pneumococcal carriage in the first 2 years of life (ISRCTN25571720).[15] The participants were born between June and December 2005, 1 year before nationwide implementation of PCV7 in June 2006. Infants younger than 12 weeks, not yet immunized and living in the study region were eligible for inclusion. Exclusion criteria were known immunodeficiency, craniofacial or chromosomal abnormalities, language barrier or expected relocation within the follow-up period.[15] Infants were randomized to receive 2 primary doses of PCV7 at 2 and 4 months of age, followed by a booster dose at 11 months of age or no PCV7 vaccinations (controls). Infants were included in the immunogenicity arm of the study on voluntary basis with blood sampling immediately before nasopharyngeal swabs were taken. Blood samples from infants receiving 2 primary doses without a booster dose of PCV7 were collected at 12 months of age and included in the current analysis as pre-booster samples. Blood samples from infants receiving 2+1 doses were also collected at 12 months of age, 1 month after the booster dose at 11 months and included in the analysis as post-booster samples. No baseline differences were found in children who participated in the immunogenicity subset and infants participating in the main carriage trial.

The second group of infants participated in a serological immune-surveillance study on pertussis vaccination (ISRCTN97785537). The infants received a 3+1-dose PCV7 schedule at the age of 2, 3, 4 and 11 months, according the Dutch national immunization program (NIP) which was implemented for all newborns from April 2006, without a catch-up program for older children[16]. Infants in good general health eligible for the fourth DTP-IPV-Hib vaccination were qualified for inclusion. Exclusion criteria were known immunodeficiency, a history of any neurologic disorder (including epilepsy) or previous vaccination with any other vaccine than those used in the NIP. We obtained blood samples at 11 months (included as pre-booster samples in the current analysis) and 1 month after the booster dose at age 12 months (included as post-booster samples in the current analysis) from infants born from April to July 2006. Inclusion was restricted to infants born within the first 3 months after PCV7 introduction in the NIP. From both studies blood samples from high-risk infants for hepatitis B that had concomitantly received Hepatitis B immunizations were excluded from analysis. Post-booster blood samples obtained outside the estimated range of 21–42 days after receiving the booster dose were excluded. For both schedules comparable percentages of blood samples were eligible for analyses.

Study vaccines

In both studies the licensed 7-valent CRM197-conjugated pneumococcal vaccine (Wyeth Pharmaceuticals) was administered, concomitantly with DTP-IPV-Hib immunizations. Since the vaccines of the 3+1-dose schedule were administered as part of the Dutch NIP, different lot numbers were use in the two studies. Of note is that in January 2006, the DTaP-IPV-Hib vaccine (Infanrix-IPV-Hib™, GlaxoSmithKline) in the Dutch NIP was replaced by a comparable DTaP-IPV-Hib vaccine containing additional *B. pertussis* proteins (Pediacel™, Sanofi Pasteur MSD).[17] Therefore, priming DTaP-IPV-Hib vaccinations differed between both study cohorts. Both cohorts received PediacelTM as a booster dose. Informed consent was obtained from the parents or guardians of all study participants. Studies were approved by a national ethics committee.

Laboratory measurements

After collection blood was stored at 4 ◦C. Serum was separated within 24 h and stored at −20 °C until assayed. Serum IgG antibody levels were measured to the 7 vaccine pneumococcal polysaccharides 4, 6B, 9V, 14, 18C, 19F and 23F. All sera were assayed in the laboratory for infectious diseases of the National Institute for Public Health and the Environment in Bilthoven with ELISA using double adsorption with cell wall polysaccharide and 22F polysaccharide.[18]

Statistical analysis

Results of IgG antibody levels are expressed in Geometric Mean Concentration (GMC) with 95% confidence interval (95% CI). Statistical differences in IgG GMC values were assessed by log transformed unpaired t-test. Differences in percentages of subjects with antibody levels ≥0.35 µg/ml, ≥1.0 µg/ml and ≥5.0 µg/ml were calculated using Fisher's exact test. All reported p-values are 2-sided, p-values <0.05 were considered significant. The study sample sizes enabled an estimation of pneumococcal GMCs with 95% CI within 1.4-fold and detection of a 2-fold difference for comparing schedules with 80% power at a 5% significance level.[12] Analyses were performed with SPSS 15.0.

Results

Study participants

We collected 80 pre-booster and 72 post-booster serum samples from infants receiving the 2+1-dose schedule and 98 pre-booster and 90 post-booster serum samples from infants receiving the 3+1-dose schedule. For the pre-booster serum samples baseline characteristics of the participants (gender, age at time of blood collection) were comparable between the two vaccination schedules. For the post-booster samples, infants receiving the 3+1-dose schedule were up to 1 month older at the time of the booster vaccination compared to the infants receiving the 2+1-dose schedule (mean age 12.1 months vs. 11.3 months; $p < 0.001$).

Pre-booster antibody levels

No differences in pre-booster IgG GMCs between the 2 schedules for 6 out of 7 PCV7 serotypes were observed. Pre-booster GMCs ranged per serotype from 0.19 µg/ml for 18C to 1.76 µg/ml for serotype 14 (Table 1). The single exception was serotype 6B for which lower pre-booster GMCs were observed (0.23 µg/ml vs. 0.40 µg/ml, p = 0.002) after 2 and 3 primary doses, respectively. Both vaccination schedules did not differ in the proportion of infants with pre-booster antibody levels ≥0.35 µg/ml, ≥1.0 µg/ml or ≥5.0 µg/ml, with the exception of 6B. For serotype 6B, 26% of infants who received a 2+1-dose schedule showed antibody levels of ≥0.35 µg/ml compared with 52% in the 3+1-dose group (p = 0.001) (Figure 1).

Table 1. Pre- and post-booster serotype specific IgG antibody levels (GMC) in infants receiving a 2+1 and 3+1 PCV-7 schedule with early primary vaccinations at 2, 3 and 4 months or 2 and 4 months of age.

	Pre-booster samples; GMC µg/ml (95% CI)			Post-booster samples; GMC µg/ml (95% CI)		
Serotype	2 primary dose (n = 80)	3 primary dose (n = 98)	p-Value[a]	2+1-dose (n = 72)	3+1-dose (n = 90)	p-Value[b]
4	0.28 (0.23-0.34)	0.30 (0.26-0.34)	NS	2.66 (2.26-3.12)	3.05 (2.57-3.61)	NS
6B	0.23 (0.19-0.30)	0.40 (0.31-0.49)	0.002#	2.26 (1.64-3.12)	4.73 (3.62-6.17)	0.001#
9V	0.27 (0.22 0.33)	0.31 (0.27-0.36)	NS	2.21 (1.90-2.58)	2.36 (2.00-2.78)	NS
14	1.76 (1.39-2.24)	1.56 (1.23-1.81)	NS	9.43 (7.76-11.46)	10.32 (8.62-12.34)	NS
18C	0.19 (0.16-0.23)	0.22 (0.18-0.25)	NS	1.97 (1.66-2.34	1.91 (1.61-2.27)	NS
19F	0.94 (0.76-1.16)	0.96 (0.74-1.24)	NS	3.43 (2.85-4.12)	4.80 (4.01-5.76)	0.012#
23F	0.21 (0.16-0.27)	0.22 (0.18-0.26)	NS	2.61 (2.12-3.21)	3.15 (2.54-3.92)	NS

p-Value < 0.05, NS non significant
a p-Values of 2 vs. 3 primary dose. Calculated using log transformed unpaired t test
b p-Values of 2+1 vs. 3+1-dose. Calculated using log transformed unpaired t test

Post-booster antibody levels

One month after the booster dose, IgG GMCs were similar for the 2+1- and 3+1-dose schedule, except for 6B and 19F (Table 1), where the GMCs for serotype 6B were 2.26 µg/ml vs. 4.73 µg/ml (p = 0.001) and for 19F 3.43 µg/ml vs. 4.80 µg/ml (p = 0.012), respectively. Overall, the lowest GMCs were seen for serotype 18C. Comparing the proportion of children reaching threshold antibody concentrations, the percentages of infants with antibody levels ≥0.35 µg/ml were comparable between both vaccination schedules for all 7 serotypes (Figure 1). The percentages of infants reaching antibody levels ≥1.0 µg/ml differed between the two vaccination schedules for serotype 6B, with 73.6% vs. 88.9% of the infants reaching ≥1.0 µg/ml after the 2+1-dose vs. the 3+1-dose schedules, respectively (p = 0.014). Differences were also observed for the percentages of infants with antibody levels ≥5.0 µg/ml for serotypes 6B and 19F; 27.8% vs. 52.2% (p = 0.002) for 6B and 27.4% vs. 44.4% (p = 0.034) for 19F after a 2+1- or 3+1-dose schedule, respectively. For serotype 23F 19.4% vs. 33.3% of the infants reached antibody levels ≥5.0 µg/ml (p = 0.053).

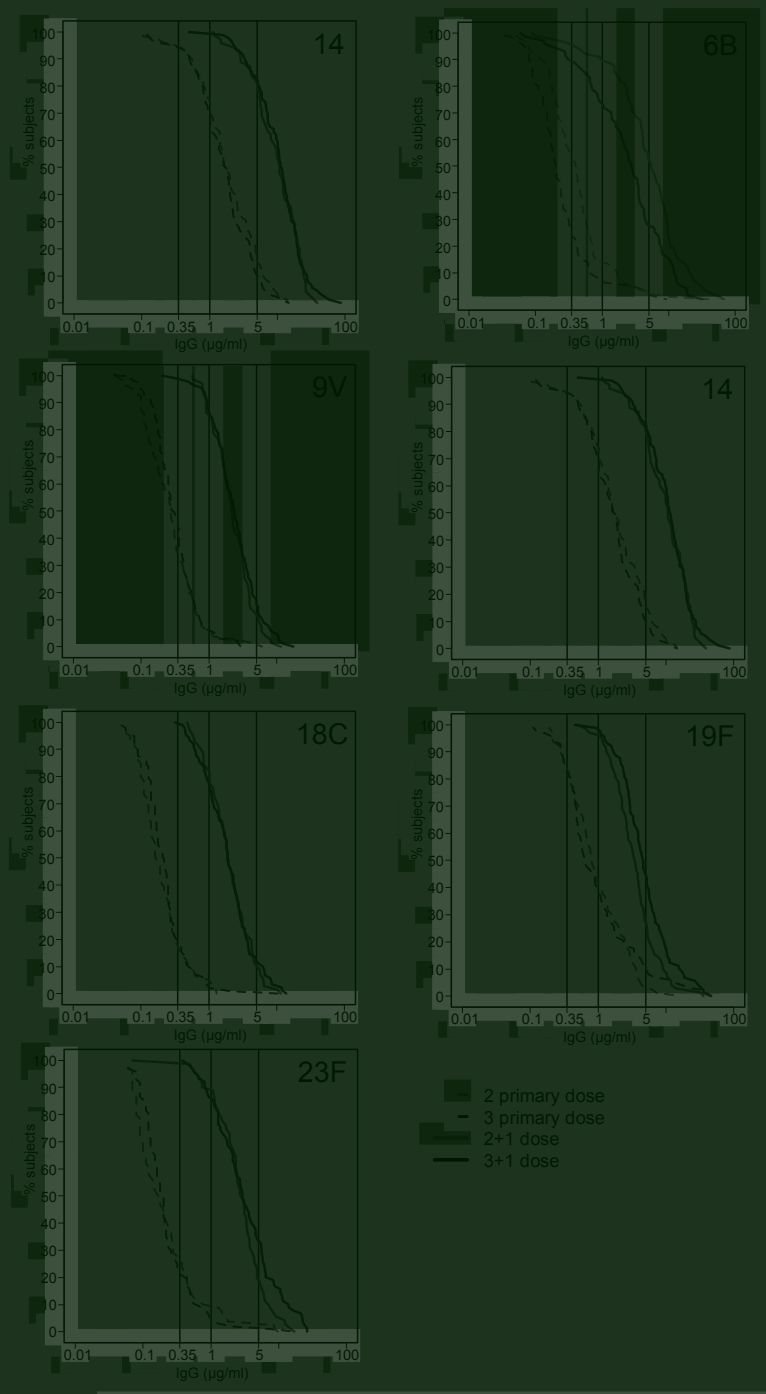

Figure 1. Reverse Cumulative Distribution Curves of infants receiving a 2+1 and 3+1 PCV-7 schedule with early primary vaccinations at 2, 3 and 4 months or 2 and 4 months of age. Vertical lines indicate levels above 0.35 µg/ml; 1.0 µg/ml and 5.0 µg/ml.

Discussion

This study compared the pre- and post-booster IgG antibody levels for the 7 vaccine serotypes following a 2+1- and a 3+1-dose schedule with the currently used 7-valent pneumococcal conjugate vaccine with early primary doses between 2 and 4 months of age. Post-primary antibody levels ≥0.35 µg/ml were estimated to be an indication for protection against IPD.[7] No pre- and post-booster threshold values have been defined around 1 year of age, but a post-booster value of 1.0 µg/ml may give some indication of protection around the age of the booster vaccination or for more vulnerable populations.[8] This study showed that pre- and post-booster antibody levels were comparable between a 2+1- and 3+1-dose PCV7 schedule, with similar proportions of infants that reached post-booster antibody levels above ≥0.35 µg/ml and ≥1.0 µg/ml for 5 of the 7 serotypes. Exceptions however were serotype 6B and 19F. Between the two vaccination schedules a difference of >10% was observed in pre- and post-booster GMCs for serotype 6B and in the post-booster GMCs for serotype 19F. These results with the licensed PCV7 differ somewhat from the results with the experimental 9-valent vaccines in the United Kingdom and Iceland, where heights of the antibody levels following the booster dose after 2- and 3-dose primary schedules were comparable for 6B and 19F.[12;13] This may be due to a different immunogenicity of the experimental 9-valent pneumococcal conjugate vaccines used in these studies. However, the age of the infants at the primary injections and timing of the booster vaccination as well as other factors like concomitant childhood vaccinations or ethnic background variability may have impact on immunogenicity of vaccines.[19;20] Difference in natural boosting of the immune system in the other countries by circulating vaccine strains may have affected the height and persistence of antibody levels and booster responses. On the other hand, the current 2+1-dose study as well as the United Kingdom immunogenicity study were both performed well before widespread PCV7 implementation in the NIP and pneumococcal carriage levels and individual circulating serotypes were comparable between the Netherlands and the United Kingdom.[15;21] Also antibody concentrations from the 3+1-dose schedule in children born during the first 3 months of implementation of PCV7 in the NIP are unlikely to be much affected by diminished natural boosting, since no catch-up was done in the Netherlands.[16] Very few children under 5 years of age had been immunized with PCV7 before national implementation of PCV7 and no herd effects were observed for IPD within the first 2 years after June 2006 (Rodenburg *et al.*, submitted).

We found no differences in pre- and post-booster GMCs for serotype 18C, unlike the Icelandic study where lower post-booster levels were observed after reduced schedules with a CRM197-conjugated 9-valent pneumococcal–meningococcal C vaccine.[13] However, identical to the Iceland study, lowest antibody levels were observed for serotype 18C. In our study the primary series were administered at 2, 3, 4 or 2 and 4 months of age. It is possible that the extra dose at 3 months of age did not add much to the overall induction of IgG antibodies because of the relatively young age and the short 1-month interval between doses. A schedule given in the United States with 3 primary doses at 2, 4 and 6 months of age is potentially more immunogenic than 2 primary doses at 2 and 4 months of

age.[19] Unfortunately, we do not have data on post-primary responses or data on comparing time intervals between schedules. For meningococcal conjugate vaccines earlier research showed higher booster responses after less primary dose, however this effect was not seen for the 7-valent pneumococcal conjugate vaccine.[22]

Successful immunological priming is important for protection in particular in the first year of life during the peak incidence of IPD between the primary series and the booster dose.[1] Looking at the results from Iceland, post-primary GMC differences were found between the 2 primary dose vs. 3 primary dose schedules, which were no longer existent in the pre-booster antibody levels at 12 months of age.[13] In contrast, these differences were not seen in post-primary antibody levels from the United Kingdom between the 2 primary dose vs. 3 primary dose schedules.[12] We found differences for 6B and 19F and possibly for 23F. Protective levels seem to vary between serotypes and lower antibody levels may suffice for clinical protection as was shown for serotype 6B IPD.[2] Furthermore, in our study serotype 6B antibody levels were in a similar range of most other vaccine serotypes and well above serotype 18C that showed the lowest pre- and post-booster antibody levels in both our vaccination groups.

The question is whether the dose-dependent antibody responses with the currently used PCV7 may have clinical consequences. Looking at other clinical data, a surveillance report from Norway on the first 2 years after national implementation of a 2+1-dose schedule (3, 5 and 12 months with catch up for infants aged 3–6 months) showed a strong decline in IPD for all vaccine serotypes including the serotypes 6B, 18C, 19F and 23F with no vaccine failures.[23] However, the later primary schedule at 3 and 5 months and the addition of a catch-up program for infants aged 3–6 months might have masked lower protection in vaccinated children after reduced primary doses since herd effects may have attributed to effectiveness due to reduction of circulating vaccine strains in the population.

Effects of reduced-dose schedules on disease in particular respiratory pneumococcal infections like pneumonia and otitis media also need to be evaluated since higher antibody levels may be required for protection. For protection against nasopharyngeal acquisition serum antibody levels above 5.0 µg/ml have been suggested.[9] We found differences in proportions of children reaching antibody above 5.0 µg/ml for serotypes 6B, 19F and possibly 23F. For serotype 23F, the difference in the proportion of children reaching the 5 µg/ml level was not significantly different ($p = 0.053$), but this is likely due to the sample size of the children. Previously Jokinen *et al*. found a GMC of 0.5 µg/ml to be more than 65% efficacious against for serotype 6B otitis media, but for serotype 19F this level had negligible protection.[10] We recently reported in our carriage study that although serotype 19F showed a decline at 18 months after 2+1 doses compared with unvaccinated controls, this was no longer present at 24 months of age. In contrast, serotypes 6B and 23F showed around 80% decline in carriage compared to unvaccinated controls at 24 months of age after a 2+1-dose schedule.[15] This highlights the serotype dependent efficacy of antibody concentrations or of other antibody characteristics like affinity.[10] From Italy and Quebec first reports show a decline in respiratory disease like otitis media and pneumonia after national PCV7 implementation in reduced-dose 2+1 schedules, but again in these countries catch-

up programmes might contribute to this observed decrease by herd effects.[24;25] Moreover, these surveillance data cannot discriminate between serotype specific protection against respiratory disease. Differences found for serotype 6B, 19F and 23F can potentially impair the effectiveness of 2+1-dose schedules for prevention of respiratory disease and also herd effects in particular before 2 years of age.[26;27] However, based on our carriage study, we predict that indirect protection via herd effects for all serotypes will eventually be obtained for both invasive and respiratory disease after a 2+1-dose schedule with primary doses also at 2 and 4 months of age followed by a booster at 11 months of age, since in the second year of life at 18 and 24 months a significant overall 60% reduction of vaccine serotypes was observed.[15] Noteworthy, our carriage study also showed that the age of carriage reduction of serotypes 6B and 19F may be dose dependent before 24 months of age with earlier reduction after more doses of PCV7.[15]

Our study has several limitations. Since the two schedules were performed in a non-randomized setting in two different cohorts some potential confounders should be assessed. Firstly, the coadministration of different DTaP-IPV-Hib vaccines in the study groups may have affected results. However, a study assessing the compatibility of concurrently administered PCV7 with acellular pertussis vaccine showed no differences in immune responses to PCV7 between groups receiving the vaccines concomitantly or separately.[28] Secondly, different lot numbers of PCV7 were used in the two studies. Thirdly, there was difference in the age of the children receiving the booster dose between study groups. Infants receiving the 3+1-dose schedule were up to 1 month older when receiving the booster compared with the infants receiving the 2+1-dose schedule. However, when comparing only infants who received their booster dose before the age of 12 months in both schedules, the difference for 6B persisted but no significant differences were observed for serotype 19F. However, the small group size does not allow firm conclusions (see also supporting information). Furthermore, all other inclusion- and exclusion criteria as well as baseline characteristics between the two vaccination schedules were comparable. Fourth, the different time periods in our study may have introduced herd effects and less natural boosting by the vaccine serotypes for the 3+1-dose group. However, our carriage study that followed children up till their second birthday during the period June 2005 till February 2008, and thus including the study period of both the 2+1- and 3+1-dose groups, no signs of herd effects were observed in unvaccinated controls.[15] Lastly, no data about post-primary IgG antibody levels were available so the period between primary vaccinations and booster vaccination cannot be compared. To summarize, we found comparable pre- and post-booster antibody responses for most vaccine serotypes between a 2+1- and 3+1-dose vaccination schedule with the currently used 7-valent pneumococcal conjugate vaccine. Between the two vaccination schedules a difference was observed in pre- and post-booster antibody levels for serotype 6B and in the post-booster antibody levels for serotype 19F. However, first surveillance reports seem to indicate clinical protection against invasive disease also with reduced-dose schedules. A recent carriage study indicated that herd effects due to diminished circulation of vaccine serotypes are to be expected after 2+1-dose schedules that will decrease also vaccine serotype respiratory disease like otitis media and pneumonia.

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Supporting information

Table. Post-booster serotype specific IgG antibody levels (GMC) in infants receiving a 2+1 and 3+1 PCV-7 schedule with early primary vaccinations at 2, 3 and 4 months or 2 and 4 months of age. Timing of the booster dose before 12 months vs. all samples.

	Post-booster samples; GMC µg/ml (95% CI) Booster dose after 12 months excluded			Post-booster samples; GMC µg/ml (95% CI) All samples		
Serotype	2+1-dose (n = 68)	3+1-dose (n = 42)	p-Value[a]	2+1-dose (n = 72)	3+1-dose (n = 90)	p-Value[a]
4	2.53 (2.15-2.98)	2.62 (2.03-3.38)	NS	2.66 (2.26-3.12)	3.05 (2.57-3.61)	NS
6B	2.24 (1.60-3.14)	3.98 (2.69-5.87)	0.035#	2.26 (1.64-3.12)	4.73 (3.62-6.17)	0.001#
9V	2.17 (1.85-2.54)	2.23 (1.74-2.87)	NS	2.21 (1.90-2.58)	2.36 (2.00-2.78)	NS
14	9.27 (7.56-11.38)	9.27 (7.41-11.60)	NS	9.43 (7.76-11.46)	10.32 (8.62-12.34)	NS
18C	1.92 (1.61-2.30)	1.81 (1.42-2.27)	NS	1.97 (1.66-2.34	1.91 (1.61-2.27)	NS
19F	3.37 (2.78-4.08)	4.16 (3.29-5.28)	0.18	3.43 (2.85-4.12)	4.80 (4.01-5.76)	0.012#
23F	2.48 (2.01-3.07)	2.55 (1.87-3.48)	NS	2.61 (2.12-3.21)	3.15 (2.54-3.92)	NS

p-Values shown <0.20; p-Value <0.05 considered significant. NS non significant
a p-Values of 2+1 vs. 3+1-dose. Calculated using log transformed unpaired t test

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7

Induction of long-term immunity after reduced-dose schedules with 7-valent pneumococcal conjugate vaccine in children; Antibody responses, avidity maturation and memory B cells

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Abstract

Background

The 7-valent CRM197-conjugated pneumococcal vaccine (PCV7) has been introduced in national immunization programmes worldwide with differences in the number and timing of doses. Data on predictors of long-term immunity induced by different PCV7 schedules are limited.

Methods

In a randomized controlled setting, infants were assigned to receive PCV7 as 2 primary doses, 2-dose and an 11-month booster (2+1-dose) or no PCV7 (controls). At 24 months of age all participants received a challenge PCV7 on voluntary basis. From different subsets of infants IgG antibody levels, avidity indices and B cell frequencies were measured.

Results

At 24 months of age, pre-vaccination parameters varied between serotypes, but were mostly comparable after 2-dose and 2+1-dose schedules and higher than controls. After the challenge PCV7 at 24 months no benefit was observed for the 11-month-booster on antibody responses, avidity indices and B cell frequencies compared to 2 primary doses only. Exception was serotype 6B where additional value of the 11-month-booster vaccination was shown by higher avidity indices and higher antibody responses 7-9 days afterPCV7 challenge. Serotype 19F showed higher antibody levels and avidity indices 1 month after the 11-month-booster, however this was no longer present at 24 months before or after the challenge vaccination.

Conclusions

These findings suggest successful induction of long-term immunity by 2 primary doses of PCV7 with a limited, serotype-dependent benefit of an early 11-month booster. The clinical impact of an early booster dose should be monitored.

Introduction

Streptococcus pneumoniae is an important cause of bacterial infections in children in the first years of life with clinical syndromes varying from respiratory disease (pneumonia, otitis media) to invasive pneumococcal disease (IPD; bacteremia and meningitis).[1;2] For IPD, the peak incidence is in the first years of life and decreases until the age of 5 years.[3] Following licensure of the CRM197-conjugated 7-valent pneumococcal vaccine (PCV7) in 2000, many countries have recommended PCV7 vaccination for infants, albeit with differences in the number of doses in the primary series and in the age at start of administrations, varying from 6 weeks to 3 months.[4] First surveillance reports confirm early direct protection against most vaccine serotype IPD after implementation of both 2 and 3 primary dose schedules.[5-7] In most countries a booster of PCV7 is scheduled, but age varies between 9 and 18 months.[4;8] The importance of a booster dose for long lasting protection was previously shown in the United Kingdom after nationwide introduction of conjugate vaccines for *Neisseria meningitidis* serogroup C (MenC) and *Haemophilus influenzae* type b (Hib) in infancy. When limited to 3-dose primary series under 6 months of age without a booster in the second year of life, antibody levels showed a rapid decline within one year after the primary series.[9;10] For this reason, a booster injection around 12 months of age for both MenC and Hib conjugate vaccines was implemented in the United Kingdom in 2006.[11] The booster vaccination at one year of age was shown to provide longer sustained levels of protective antibodies for Hib.[12]

To provide sustained protection against IPD, generation and maintenance of circulating serum antibodies and possibly the presence of high avidity memory B cell populations seem mandatory.[13] Limited data are available regarding the additional effect of the timing of the booster dose in the different PCV schedules on antibody maintenance for the different pneumococcal serotypes as well as on the markers of memory development.[14] With the loss of natural boosting as a result of carriage eradication of vaccine serotype strains as demonstrated for various PCV7 schedules, protection may become more dependent on vaccine-induced long-term immunogenicity of the various vaccine schedules.[15;16] Insight in the impact of different schedules on long-term protection is crucial for future pneumococcal vaccine policies, especially in developing countries, that have highest and most severe pneumococcal disease burden also in adulthood.[17;18]

Long-term immunity, provided by maintaining serum antibodies as well as persistence of B cell memory can be evaluated by (1) the induction of a IgG antibody increase following a challenge vaccination, (2) increased antibody avidity and (3) detection of plasma and memory B cells shortly after immunization.[19;20] In this study, we explored the induction of long-term immunity in children participating in a randomized controlled trial on PCV7 in the Netherlands, either receiving 2 primary doses at 2 and 4 months of age with and without an early booster dose at 11 months or no PCV7 until 2 years of age (controls). All infants received a challenge vaccination at 24 months of age and antibody responses were compared before and after the challenge vaccination, as well as avidity indices and B cell frequencies.

Material and Methods

Study design

Between July 2005 and February 2006, 1005 infants were included in a randomized controlled trial investigating as primary objective the effects of reduced-dose PCV7 schedules on pneumococcal carriage during the first two years of life (NCT00189020).[21] Carriage results and detailed study design are reported elsewhere. Infants younger than 12 weeks, not yet having received any infant vaccination and living in the study region, were eligible for inclusion. Exclusion criteria were known immunodeficiency, craniofacial or chromosomal abnormalities, language barrier, or expected relocation within the follow-up period. After written informed consent had been obtained from both parents or guardians, infants were randomly allocated to receive various vaccination schedules, (1) PCV7 at 2 and 4 months of age (2-dose group), (2) two primary doses at 2 and 4 months followed by a booster PCV7 at 11 months of age (2+1-dose group) or (3) no PCV7 until 24 months of age (controls). At 24 months of age a PCV7 challenge vaccination was offered to all participants on a voluntary basis. Parents were aware of the child's vaccine schedule.

In the trial, a subset of the infants were included in the immunogenicity arm of the study on voluntary basis. At 12 months and 24 months of age and 28 days after challenge vaccination 3 ml blood samples were collected for serology. In addition at 24 months of age before and 7-9 days after PCV7 challenge vaccination 8 ml blood samples were collected for serology and B cell measurements. Blood samples from high-risk infants for hepatitis B that had received Hepatitis B immunizations were excluded from analysis. Post-booster or post-challenge blood samples obtained outside the estimated range of 28 – 42 days after receiving the booster dose were excluded. No major baseline differences were found between the immunogenicity subset and the main randomised carriage trial. The study protocol was approved by an acknowledged national ethics committee from the Netherlands (Stichting Therapeutische Evaluatie Geneesmiddelen, http://www.stegmetc.org). The trial was undertaken in accordance with the European Statements for Good Clinical Practice, which includes the provisions of the Declaration of Helsinki of 1989. An external committee was appointed to review progress and advise on data eligibility for analysis. Laboratory personnel were unaware of treatment allocation during all laboratory measurements and the randomization key was not disclosed until after the study was completed.

Study Vaccines

In the first year of life the licensed 7-valent CRM197-conjugated pneumococcal vaccine (Prevenar™ Wyeth) was administered during regular well baby-clinic visits, together with routine DTaP-IPV-Hib immunizations according to the Dutch National Immunization Programme. The PCV7 challenge vaccinations at 24 months were administered during a home visit by authorized medical staff.

Measurement of serum antibodies and avidity

Serum was separated within 24 hours and stored at -20 ^{O}C until assayed. From the blood samples collected for cellular analyses plasma was stored after density gradient centrifugation. Serum and plasma IgG antibody levels were measured to all 7 vaccine pneumococcal polysaccharides. All samples were assayed by ELISA using double absorption as described before.[22] The antibody avidity to capsular polysaccharides was measured for the serotypes 4, 6B, 14 and 19F at all time-points of a random selection of blood samples with serotype-specific IgG levels of ≥0.20 µg/ml. All serum samples were adapted to an antibody concentration to the capsular polysaccharide of 0.20 µg/ml. Ammonium thiocyanate in 5 different concentrations was used to dissociate low-avidity antigen-antibody binding.[23] Antibody avidity was expressed as geometric avidity index (GMAI), corresponding to the molar concentration of ammonium thiocyanate required to produce a 50% reduction in optical density.

Measurement of plasma and memory B cell responses

Specific B cell frequencies were measured for the serotypes 6B, 14, 19F and 23F. Peripheral blood mononuclear cells (PBMCs) were isolated and serotype-specific antigen-secreting cells measured using direct (plasma B cells) and indirect ELISPOT (memory B cells) assay, see supporting information S1 for detailed description.

Statistical Analysis

The primary objective of this randomized clinical trial was the proportion of children positive for vaccine serotype pneumococcal carriage in the second year of life, and is reported elsewhere.[24] As secondary objectives antibody levels at 12 and 24 months of age were measured, as well as antibody responses to the PCV7 challenge at 24 months of age to determine memory induction in vaccinated cohort compared with controls. The study sample sizes enabled an estimation of pneumococcal IgG GMCs with 95% CI within 1.4-fold and detection of a 2-fold difference for comparing schedules with 80% power at a 5% significance level. For memory B cells and antibody avidity limited data were available at the start of the study, hampering study sample size estimates. Analyses were also conducted with all participants included and with those who had a protocol violation. Results remained consistent with the overall conclusions. Results of antibody levels are expressed in geometric mean concentration (GMC) with 95% confidence interval (95% CI). Statistical differences IgG GMC and GMAI values were assessed by log transformed unpaired t test. B cell frequencies are shown in medians and groups were compared using non-parametric Mann-Whitney test. All reported p-values are 2-sided, p-values smaller than 0.05 were considered significant. Analyses were performed with SPSS 15.0 and PRISM4.

Results

Study participants

In total 740 blood samples were collected. Overall 690 (93%) samples were included for analyses and IgG antibody levels were measured (see supporting information figure S1 for enrolment flow diagram). Baseline characteristics (gender, age at primary doses administration) of the participants were comparable in the different vaccination schedules. Antibody avidities were measured for 197 (serotype 4) to 457 (serotype 19F) samples (supporting information table S1). Plasma and memory B cells were measured in 6-10 and 16-17 samples per group pre- and post-challenge at 24 months, respectively.

IgG antibody levels at 12 and 24 months of age before challenge vaccination

At the age of 12 months, significantly higher IgG GMC values for all seven vaccine serotypes were seen in children after 2 primary doses plus a booster dose at 11 months compared with 2 primary doses only (Table 1; Figure 1). However at the age of 24 months before the challenge vaccination GMCs of all serotypes in the 2+1-dose group had decreased significantly and higher GMC values were observed only for serotype 4 and 18C compared with the 2-dose group. In the unboosted 2-dose group, between 12 and 24 months of age, a significant increase in GMC values for the frequently carried serotypes 6B, 19F and 23F was observed. In unvaccinated controls, all antibody levels were lower than in the PCV7 primed groups both at 12 and 24 months (Table 1; Figure 1).

IgG antibody responses after challenge vaccination at 24 months

After the PCV7 challenge vaccination at 24 months, GMC values of both the 2-dose and 2+1-dose group showed a significant increase for all serotypes 7-9 days and 28-42 days post-vaccination compared to pre-challenge antibody levels (Table 1; Figure 1). No differences were found in the antibody response at 7-9 days between the two PCV7 primed groups, with the exception for serotype 6B where significantly higher GMC values were found in the 2+1-dose group compared with the 2-dose group; 15.42 vs. 4.41 µg/ml (p=0.003), respectively. In the 2-dose group, significantly higher GMC values were observed 28-42 days after the challenge vaccination for serotype 4 and 9V compared with the 2+1-dose group; 5.82 vs. 3.83 µg/ml (p=0.002) for serotype 4, 4.25 vs. 3.15 µg/ml (p=0.01) for serotype 9V. In previously unvaccinated controls, the challenge vaccination gave a significant rise at day 7-9 for the serotypes 4, 9V, 18C and 23F. From day 7-9 to day 28-42 antibody levels further increased, but GMC values remained less than in the PCV7 primed groups for all serotypes, except serotype 18C (Table 1; Figure 1).

Avidity indices at 12 and 24 months of age before challenge vaccination

At 12 months of age, antibodies of serotype 4, 6B and 14 showed comparable avidity indices for the 2+1-dose group compared with the unboosted 2-dose group (Figure 2, supporting information table S1). A higher GMAI was found for the antibodies of serotype 19F after the 11-month-booster vaccination; 1.63 vs. 1.21 (p<0.001). In both PCV7 primed

Table 1. IgG GMC levels in infants after a 2-dose, 2+1-dose schedule or no vaccinations (controls). Antibody levels were measured at 12 months and 24 months of age, and 7-9 days and 28-42 days after a challenge PCV7 at 24 months.

	12 months			24 months			+7-9 days post-challenge			+28-42 days post-challenge		
	2+1-dose	2-dose	Controls	2+1-dose	2-dose	Controls	2+1-dose	2-dose	Controls	2+1-dose	2-dose	Controls
Serotype	n=72	n=80	n=28	n=74	n=80	n=77	n=17	n=16	n=16	n=71	n=79	n=80
4	2.66 [a,b] (2.26-3.12)	0.28 [c] (0.23-0.34)	0.05 (0.03-0.07)	0.26 [a,b] (0.22-0.30)	0.17 [c] (0.14-0.21)	0.10 (0.08-0.12)	5.13 [b] (3.58-7.37)	6.23 [c] (3.92-9.90)	1.08 (0.48-2.44)	3.83 [b] (3.16-4.64)	5.82 [a,c] (4.89-6.93)	2.52 (2.10-3.02)
6B	2.26 [a,b] (1.64-3.12)	0.23 [c] (0.19-0.30)	0.07 (0.06-0.10)	1.09 [b] (0.80-1.48)	0.79 [c] (0.57-1.11)	0.24 (0.20-0.28)	15.42 [a,b] (11.43-20.80)	4.41 [c] (2.11-9.18)	0.19 (0.13-0.28)	8.41 [b] (6.23-11.35)	6.48 [c] (4.75-8.85)	0.57 (0.44-0.75)
9V	2.21 [a,b] (1.90-2.58)	0.27 [c] (0.22 0.33)	0.05 (0.04-0.07)	0.30 [b] (0.24-0.37)	0.26 [c] (0.19-0.33)	0.14 (0.11-0.18)	4.60 [b] (3.37-6.28)	4.73 [c] (3.61-6.20)	0.56 (0.30-1.04)	3.15 [b] (2.64-3.75)	4.25 [a,c] (3.64-4.95)	1.80 (1.51-2.14)
14	9.43 [a,b] (7.76-11.46)	1.76 [c] (1.39-2.24)	0.07 (0.04-0.14)	1.41 [b] (1.15-1.71)	1.01 [c] (0.78-1.34)	0.41 (0.32-0.53)	12.65 [b] (8.16-19.61)	11.69 [c] (6.91-19.78)	0.61 (0.38-1.00)	11.99 [b] (9.75-14.73)	14.49 [c] (11.62-18.07)	2.58 (1.92-3.46)
18C	1.97 [a,b] (1.66-2.34)	0.19 [c] (0.16-0.23)	0.06 (0.04-0.10)	0.24 [a,b] (0.20-0.29)	0.17 [c] (0.14-0.23)	0.11 (0.09-0.14)	3.30 [b] (2.28-4.77)	2.72 [c] (1.90-3.91)	0.64 (0.30-1.34)	2.93 (2.20-3.89)	3.25 (2.80-3.76)	2.64 (2.21-3.16)
19F	3.43 [a,b] (2.85-4.12)	0.94 [c] (0.76-1.16)	0.56 (0.36-0.88)	1.83 [b] (1.42-2.36)	1.46 [c] (1.13-2.00)	0.93 (0.76-1.14)	7.29 [b] (4.79-11.08)	9.71 [c] (6.22-15.16)	0.90 (0.67-1.20)	5.90 [b] (4.89-7.12)	6.09 [c] (4.89-7.59)	1.99 (1.67-2.37)
23F	2.61 [a,b] (2.12-3.21)	0.21 [c] (0.16-0.27)	0.05 (0.04-0.06)	0.57 [b] (0.44-0.76)	0.40 [c] (0.30-0.53)	0.16 (0.13-0.19)	8.50 [b] (5.24-13.79)	3.92 [c] (1.89-8.14)	0.28 (0.15-0.51)	5.04 [b] (4.06-6.26)	4.88 [c] (3.84-6.21)	0.73 (0.54-0.99)

ap-Values <0.05; 2+1 vs. 2-dose schedule bp-Values <0.05; 2+1-dose vs. controls cp-Values <0.05; 2-dose vs. controls. Calculated using log transformed unpaired t test, p-values are 2 sided

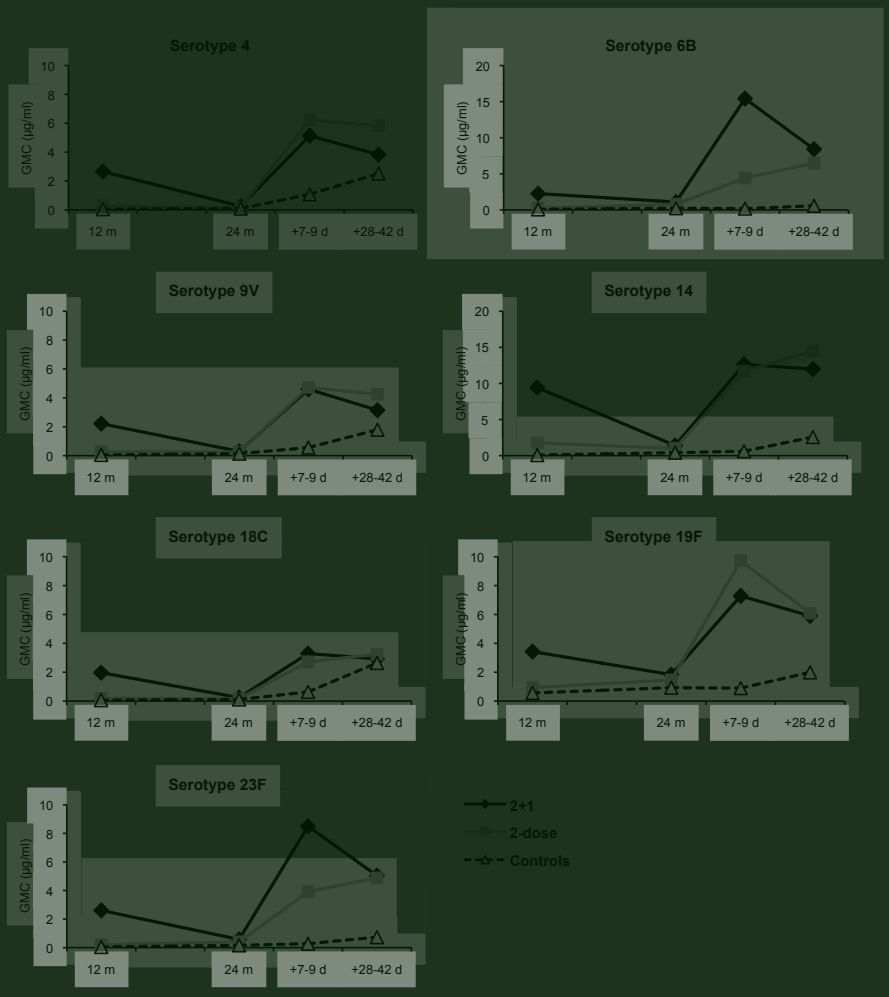

Figure 1. Pneumococcal serotype-specific IgG antibody responses after a 2-dose, 2+1-dose schedule or no vaccinations (controls). IgG responses were measured at 12 and 24 months of age and 7-9 days and 28-42 days after a challenge vaccination at 24 months of age (GMCs; µg/ml).

groups, the lowest avidity indices were seen for antibodies of serotype 6B. From 12 to 24 months of age, before the challenge vaccination was administered, a significant increase in GMAI was observed for serotype 14 (both PCV7 primed groups) and in the 2+1-dose group for serotype 6B. For this serotype a significant higher avidity index was observed compared with the 2-dose group; 1.01 vs. 1.41 (p=0.01), 2-dose vs. 2+1-dose, respectively. For serotype 4, GMAIs remained in the high range for both vaccinated groups. However, for serotype 19F, both GMAIs decreased between 12 and 24 months, leaving no residual benefit for the 11-month-boosted group. In unvaccinated controls, avidity indices were lower compared to the PCV7 primed groups for all tested serotypes, except serotype 14 (Figure 2, supporting information table S1).

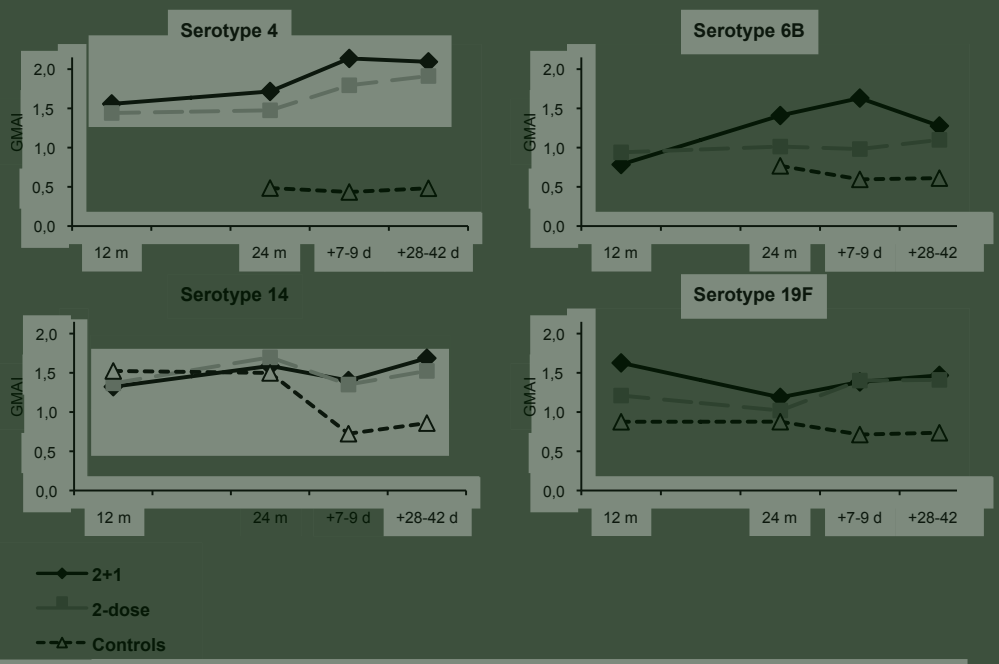

Figure 2. Geomean avidity indices of pneumococcal serotype-specific IgG antibodies after a 2-dose, 2+1-dose schedule or no vaccinations (controls). Avidity indices were measured at 12 and 24 months of age and 7-9 days and 28-42 days after a challenge vaccination at 24 months of age.

Avidity indices after challenge vaccination at 24 months

After the challenge vaccination at 24 months of age GMAIs for the serotypes 4, 6B and 14 did not increase at 7-9 days or 28-42 days after PCV7 challenge. For serotype 19F an increase in antibody avidity was observed 28-42 days after challenge: 1.02 to 1.41 (p=0.01) in the 2-dose group and 1.19 to 1.47 (p=0.07) in the 2+1-dose group (Figure 2, supporting information table S1). In controls, the post-challenge avidity indices did not change except for serotype 14 that decreased significantly already 7-9 days after vaccination; 1.50 to 0.71 ($p<0.001$). GMAIs for all analyzed serotypes were significantly lower in controls compared to the PCV7 primed groups (Figure 2, supporting information table S1)

B cell responses before and after the challenge vaccination

Before the challenge vaccination at 24 months of age, low numbers of plasma and memory B cells were detected in the 2-dose, 2+1-dose and the control-group (data not shown). After the challenge vaccination, plasma cell frequencies were comparable between the 2-dose and 2+1-dose group. Higher plasma cell frequencies could be measured only for serotype 6B in vaccinees compared with controls, medians 7.2 and 9.0 vs. 1.6 antigen-secreting cells/10^5 PBMCs (2+1-dose vs. controls, p=0.004; 2-dose vs. controls, p=0.06). For the other serotypes (14, 19F, 23F), no significant differences could be shown in circulating plasma cells. After the challenge vaccination no differences were observed in memory B cell frequencies between the two vaccination groups (Figure 3). Compared with controls

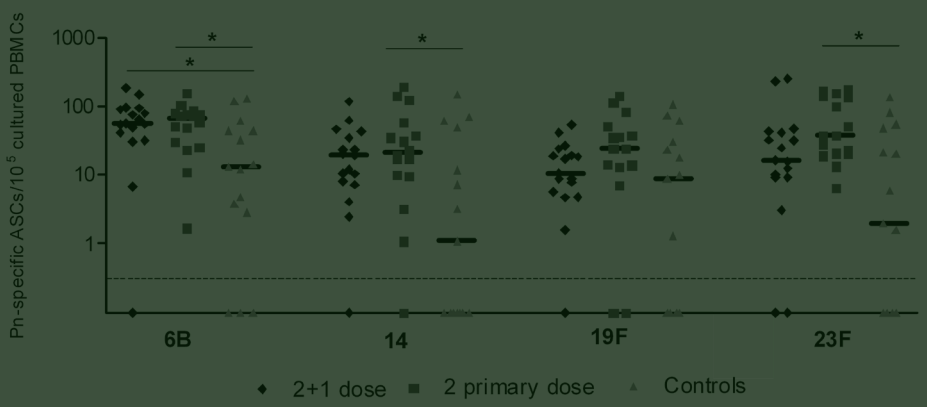

Figure 3. Pneumococcal serotype-specific memory B cell responses in infants after a 2-dose, 2+1-dose schedule or no vaccinations (controls). B cell responses were measured 7-9 days after a challenge PCV7 at 24 months (medians), *Significant difference; p<0.05.

higher memory B cell responses could be observed in the 2-dose group for serotypes 6B (p=0.02), 14 (p=0.04) and 23F (p=0.004). Although memory B cell responses in the 2+1-dose group were higher for all 4 serotypes compared to the controls, this was only significant for serotype 6B: 56.3 vs. 13.0 ASCs/10^5 cultured PBMCs (p=0.02) (Figure 3). In controls no significant increases were seen in plasma or memory B cells for any serotype after a single vaccination at 24 months.

Discussion

This study explored the impact of different reduced-dose PCV7 schedules on predictors of long-term immunity (post-challenge antibody responses, antibody avidity and B cells) at 2 years of age. In this randomized clinical trial we showed that 2 primary doses induce a memory response. No additional benefit of the 11-month-booster dose on long-term immunity could be shown at 24 months for any vaccine serotype except for the low-immunogenic serotype 6B. For this serotype additional value of a booster dose at 11 months was shown by higher avidity indices and higher antibody responses 7-9 days after the challenge vaccination at 24 months. Serotype 6B is known low-immunogenic and antibody responses to this serotype depend on the number of primary doses.[25;26] A similar trend for a booster-benefit in early antibody responses after the challenge PCV7 could also be observed for the low-immunogenic serotype 23F, but in this study the sample size was too small to reach significance.

For the serotypes 4 and 14 high avidity indices could be observed already after 2 primary doses at 12 months of age, leaving no additional value for a booster dose. Earlier studies confirm memory B cell induction shortly after PCV administration for the serotypes 4 and 14[27;28] and suggest that for high-immunogenic serotypes already after 2 primary doses a certain upper threshold is reached and the level of antigen-specific B cells cannot increase

further at that moment.[29;30] For serotype 4, as for serotype 9V, even significantly higher post-challenge antibody responses were seen without an early booster PCV7. These findings correspond with a recent study from the United Kingdom that showed decreased booster responses for the serotypes 4, 9V and 14 in infants who had received an extra third dose of PCV7 within the first year of life compared to infants only receiving 2 primary doses at 2 and 3 months of age.[31] For the MenC conjugate vaccines a reduced number of primary doses was reported to result in higher immune memory responses after revaccination.[32]

For serotype 19F a distinct pattern in booster and challenge-responses was observed. It was the only serotype where already one month after the 11-month-booster dose a rise in antibody-avidity could be observed. However, this difference was shown to be temporary, with a decrease in GMAIs following both schedules at 24 months of age. These findings suggest a different and less successful B cell priming.[33;34] Since this study confirms that long-term memory induction differs per serotype, the ability of the future 10- and 13-valent pneumococcal conjugate vaccines to induce B cell memory for the current and additional vaccine serotypes has to be investigated.

In young children, due to immaturity of the T cell independent (marginal zone) B cell compartment, polysaccharides of encapsulated bacteria like *S. pneumoniae* are poorly immunogenic and do not elicit immune memory.[35] With the use of protein-polysaccharide conjugate vaccines, naïve B cells differentiate into plasma cells and memory B cells through involvement of CD4+ T-cells. Germinal centers are formed and affinity maturation takes place through somatic hypermutation.[36] The low immune responses in controls confirm the limited capacity of encapsulated pneumococcal bacterial carriage for induction of naturally acquired B cell memory in infants at 2 years of age.[37;38] Compared with the PCV7 primed groups, limited avidity maturation was observed with no evident increase in plasma and memory B cells after a first vaccination at 24 months compared with the PCV7 vaccinated groups.

Recently, PCV7 has been implemented in the first countries with Extended Programme on Inmmunization (EPI)-schedules with no booster (The Gambia) or a very early booster at 9 months (Rwanda, South Africa) and no scheduled visit in the second year of life.[39] Long-term immunity, in particular in high-risk countries, is warranted as incidence- and mortality-rates of IPD remain high also after 5 years of age.[40;41] Considering the likely limited additional value of such an early booster dose on long-term immunity and having the disposal of three injections for the total schedule, a booster dose at a later age may probably be advantagious, when the B cell compartment has been more matured.[42] A potential drawback however, may be the lack of antibody-provided protection during the time interval between primary series and the booster dose at this young age in the period of introduction of the schedule. This might be solved several years after wide spread implementation of the vaccine and eradication of circulating vaccine serotypes. Long-term surveillance data are necessary to better assess this option.

The clinical value of each studied predictor for long-term protection against invasive pneumococcal disease still has to be elucidated. Although associated with clinical impact, the consequences of differences in avidity maturation for direct and long-term protection

against invasive pneumococcal disease in infants has yet to be determined.[43;44] Also the exact contribution of plasma and memory B cells for long-term immunity is not defined yet[45-47], although for memory B cells correlations between antibody maintenance and booster responses has been described.[48] However, in carriage of serotype 6B we earlier observed a dose-dependent decline in the second year of life, which corresponds with our immunological results.[49] For serotype 19F, we earlier showed an early loss of immunological memory and protection against 19F carriage, where high antibody levels and probably high avidity indices are needed for clinical effectiveness.[50;51] We observed only a temporary benefit of a 2+1-dose schedule at 12 and 18 months compared with unvaccinated controls but no longer at 24 months of age. In the USA, 40% of the cases of breakthrough infections in infants having received ≥3 doses were serotype 19F.[52]

Some potential limitations should be addressed. First of all, this trial was performed before nationwide introduction of PCV7 and natural boosting is likely to have occurred, as illustrated by the increase of antibody levels after 2 primary doses for the most frequently colonizing serotypes 6B, 19F and 23F.[53;54] In the coming years, the impact of decreased nasopharyngeal colonization by vaccine serotype pneumococci on vaccine responses and antibody maintenance has to be awaited. The balance between immunological protection following vaccinations on one hand and indirect protection through eradication of circulation of vaccine serotypes and subsequent herd effects on the other hand makes long-term antibody surveillance necessary. Second, the age of the infants at the primary injections, number of primary doses, as well as other factors like concomitant childhood vaccinations or ethnic background variability may have impact on immunogenicity of vaccines.[55;56] In our study population, infants were primed with 2 doses at the age of 2 and 4 months. Further study should be performed on schedules with more primary doses, with broader spreading between primary doses, or with primary doses at older ages, since this can positively influence priming and booster responses, especially for the poorly immunogenic serotypes. Designing the optimal schedule however is further complicated by the fact that pneumococcal serotypes within multivalent pneumococcal conjugate vaccines vary in functionality, kinetics as well as antibody level thresholds required for direct clinical protection against IPD in different geographic regions.[57;58] Third, no longitudinal data were available to correlate B memory cells with antibody responses and the small number of samples for B cell measurements do not allow any firm conclusions about non-differences between the two vaccination groups. Strengths of our study include the randomized controlled study design which made it possible to compare the different schedules with homogeneous groups without the influences of temporal or geographical trends in distribution of circulating pneumococcal serotypes.

In summary, after both a 2-dose and a 2+1-dose schedule successful B cell priming could be observed. However, the benefit of an early booster dose at 11 months after 2 primary doses of PCV7 on long-term immunity is limited and serotype-dependent, with no additional value for most vaccine serotypes. Only for serotype 6B and 23F a benefit could be observed at 24 months of age for an extra dose of PCV7. Insight in impact of different schedules on long-term protection is crucial for further vaccine policies also after the establishment of

herd immunity and the potential loss of natural immunity. Long-term protection data are needed to better assess the option of delaying the booster dose. The serotype-dependent manner for memory induction observed in this study also warrants further research on long-term immunity of future pneumococcal conjugate vaccines with broader serotype-coverage.

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Supporting information

S1. Measurement of plasma and memory B cell responses

Preparation of peripheral blood mononuclear cells (PBMCs)

PBMCs were isolated using 4 ml Vacutainer cell preparation tubes (CPT; Becton Dickinson) by density gradient centrifugation. Washes were done using PBS + 5% fetal calf serum (FCS; HyClone). PBMCs were resuspended in AIM-V medium containing 10% FCS and supplemented with penicillin (100 U/ml), streptomycin (100 µg/ml) and L-glutamine (200nM) (Gibco BRL).

B cell stimulation in vitro

For the indirect ELISPOT, PBMCs were resuspended and cultured at a concentration of $2x10^6$ cells/ml in AIM-V culture medium in 24-wells plates. PBMCs were stimulated polyclonally with 3 µg/ml CpG-C, PTO modified (5'-TCG TCG TCG TTC GAA CGA CGT TGA T-3') (Isogen) in the presence of 10 ng/ml IL-2 (Strathmann), 10 ng/ml IL-10 (Calbiochem) and 2 ng/ml polysaccharides 6B, 14, 19F and 23F (Netherlands Vaccin Institute) for 5 days at 37˚C and 5% CO_2. Cells were harvested by centrifugation, washed with culture medium and tested in antigen-specific ELISPOT assays.

ELISPOT assay

Multiscreen Filtration plates were pre-incubated with 35% ethanol for 1 minute, washed and coated with 100 µl PBS containing either 10 µg/ml goat-anti human IgG (SBA), 25 µg/ml polysaccharides 6B, 14, 19F or 23F or PBS only as negative control. All plates were incubated at 4˚C overnight, washed and blocked for at least 30 minutes with PBS containing 5% FCS. Afterwards 3-fold dilutions of the PBMCs suspensions (Direct ELISPOT, plasma B cells) were added to the plates at a starting concentration of $3x10^5$ cells/well, or stimulated PBMCs (Indirect ELISPOT, memory B cells) at a starting concentration of at least $0.5x10^5$ cells/well up to $2x10^5$ cells/well in AIM-V culture medium and incubated overnight at 37˚C and 5% CO_2. After washing with 0.05% Tween 20/PBS, plates were incubated with alkaline phosphatise (AP)-labelled goat-anti human IgG (1:5000) (SBA) for 2-4 hrs at 37˚C. After washes (last wash in PBS), plates were incubated with 50 µl substrate solution (1 mM 5-bromo-4-chloro-3-indolyl phosphate in H_2O; Sigma) for 30-60 minutes. Reaction was stopped by washing and dried. Plaques appearing as blue spots were measured as antigen-secreting cells by using an ELISPOT reader and software (CTL Europe).

Figure S1. Enrollment flow diagram.

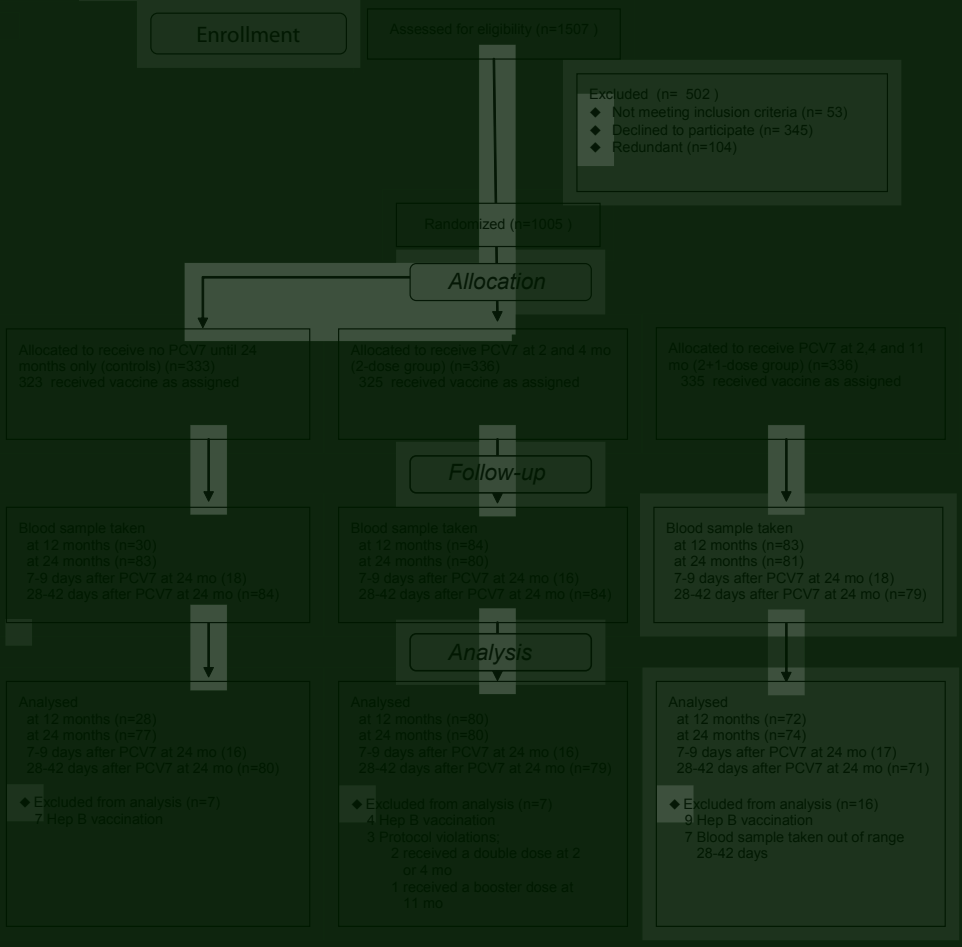

Table S1. Geomean avidity indices of pneumococcal serotype-specific antibodies in infants after a 2-dose, 2+1-dose schedule or no vaccinations (controls). Avidity indices were measured at 12 months and 24 months of age, and 7-9 days and 28-42 days after a challenge PCV7 at 24 months.

	12 months			24 months			+7-9 days post-challenge			+28-42 days post-challenge		
Serotype	2+1-dose	2-dose	Controls	2+1-dose	2-dose	Controls	2+1-dose	2-dose	Controls	2+1-dose	2-dose	Controls
4	1.56 (1.23-1.98)	1.44 (1.22-1.71)	n.a.	1.72 [b] (1.45-2.04)	1.48 [b] (1.20-1.82)	0.48 (0.38-0.63)	2.14 [b] (1.59-2.88)	1.79 [b] (1.38-2.34)	0.43 (0.29-0.67)	2.09 [b] (1.68-2.61)	1.91 [b] (1.55-2.37)	0.48 (0.38-0.61)
6B	0.78 (0.69-0.89)	0.94 (0.78-1.13)	n.a.	1.41 [a,b] (1.16-1.72)	1.01 [b] (0.86-1.19)	0.77 (0.65-0.92)	1.63 [a,b] (1.35-1.97)	0.98 [b] (0.74-1.31)	0.60 (0.43-0.82)	1.28 [b] (1.04-1.57)	1.10 [b] (0.91-1.33)	0.61 (0.51-0.74)
14	1.32 (1.18-1.49)	1.37 (1.26-1.48)	1.52 (1.24-1.88)	1.59 (1.44-1.76)	1.70 (1.51-1.92)	1.50 (1.28-1.77)	1.40 [b] (1.18-1.67)	1.35 [b] (1.13-1.61)	0.72 (0.59-0.89)	1.69 [b] (1.51-1.89)	1.52 [b] (1.35-1.73)	0.86 (0.74-1.00)
19F	1.63 [a,b] (1.47-1.81)	1.21 [b] (1.10-1.34)	0.88 (0.73-1.06)	1.19 [b] (1.03-1.38)	1.02 (0.87-1.19)	0.88 (0.75-1.03)	1.38 [b] (1.13-1.70)	1.40 [b] (1.14-1.73)	0.71 (0.62-0.83)	1.47 [b] (1.23-1.75)	1.41 [b] (1.17-1.70)	0.74 (0.64-0.85)

n.a.; not applicable because of <5 blood samples with serotype-specific IgG levels ≥0.20 µg/ml. ap-values <0.05; 2+1 vs. 2-dose schedule. bp-values <0.05; 2-dose or 2+1-dose vs. controls. Calculated using log transformed unpaired t test, p-values are 2 sided. Samples analyzed: Serotype 4: 20 samples/group (exception 24 months and +7-9 days post-challenge controls 11-12 samples), serotype 6B: 38-40 samples/group (exception 12 months 2+1-dose 68 samples; +7-9 days post-challenge 15,16 and 8 samples, 2+1-dose, 2-dose and controls, respectively), serotype 14: 36-40 samples/group (exception 12 months 72, 76 and 8 samples for 2+1-dose, 2-dose and controls, respectively; +7-9 days post-challenge 13-16 samples per group), serotype 19F: 38-40 samples/group (exception 12 months 72, 80 and 21 samples for 2+1-dose, 2-dose and controls, respectively; +7-9 days post-challenge 14-16 samples per group)

8

Antibody levels and functionality after reduced-dose schedules with the pneumococcal conjugate vaccine into the second year of life

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In Preparation

Abstract

Background

The 7-valent pneumococcal conjugate vaccine (PCV7) has been introduced in most western countries although with differences in the number and age of primary doses and timing of the booster dose. The objective of this study was to determine the quantitative and functional immune responses after a 2 primary dose schedule and the impact of PCV7 booster doses at 11 or at 24 months of age.

Methods

In a randomized controlled setting, children received PCV7 at 2 and 4 months (2-dose group); at 2, 4 and 11 months (2+1-dose group); or no PCV7 (control-group). All children received PCV7 as additional booster or primary dose (control-group) at 24 months of age. Blood samples were collected at 12, 24 and 25 months of age. Vaccine serotype-specific IgG antibodies were quantified by double adsorption ELISA. Antibody opsonophagocytic activity (OPA) was evaluated using a multiplex opsonophagocytosis assay.

Results

At 12 months, the 2-dose group showed higher vaccine serotype antibody levels compared with unvaccinated controls but OPA titers did not differ for serotypes 4, 6B, 9V and 19F. After a booster dose at 11 months, higher antibody and OPA responses were observed compared with 2 primary doses and controls for all vaccine serotypes. At 24 months, both PCV7-vaccinated groups showed higher antibody levels compared with controls, but no differences in OPA for serotypes 4, 14 and 19F were found. After an additional PCV7 vaccination at 24 months robust increases in antibody and OPA responses were observed both in the 2-dose and 2+1-dose group for all serotypes.

Conclusions

A PCV7 booster dose after 2 primary doses adds to immune responses, however after administration antibody levels and OPA titers wane rapidly. PCV7 vaccination early in life primes for robust quantitative and functional antibody responses at 24 months of age. A booster dose later in the second year of life may have positive consequences for sustained immunity.

Introduction

Streptococcus pneumonia is a main cause of serious bacterial infections in children in the first years of life with clinical syndromes varying from mucosal disease (pneumonia, otitis media) to invasive pneumococcal disease (IPD; sepsis, bacteremia and meningitis).[1;2] Pneumococcal conjugate vaccines provide protection in infants against vaccine serotype pneumococcal disease.[3;4] Following licensure of the 7-valent pneumococcal conjugate vaccine (PCV7), many developed countries have recommended PCV7 vaccination for infants. However, different schedules are currently implemented with respect to number of primary doses and timing of administration.[5] At present about half of the European countries have introduced reduced-dose schedules with 2 primary doses and a booster dose in the second year of life.[5;6] First surveillance reports confirm clinical protection against vaccine serotype IPD by these reduced-dose schedules, when accompanied with a catch-up program for other age-groups under 2 or 5 years of age.[7-9] Also herd effects will further contribute to protection after widespread implementation by disappearance of circulating vaccine strains in the community vaccination.[10;11] Reduced-dose schedules therefore may offer an attractive alternative in view of reduction of the number of injections and costs.[12] Disappearance of circulating vaccine strains in the community however may implicate the loss of natural boosting of the immune system, and render booster vaccination prerequisite for sustained seroprevalance of serotype-specific antibodies and long-term protection.[13] Currently, quantitative serotype-specific IgG antibody levels ≥0.35 µg/ml after the primary series are estimated as a threshold for protection against IPD and used for assessing non-inferiority of novel PCVs.[14] No threshold values have been defined later in infancy, but post-booster values of 1.0 µg/ml have been used as an indication of protection.[15;16] The primary mechanism for anti-capsular immunity in the host is phagocytosis, through opsonisation by anticapsular IgG antibodies and activation of complement.[17] Therefore, the WHO has suggested that functional immune responses, *e.g. in vitro* opsonophagocytic activity (OPA) of serum, are of additional value in evaluation of vaccine efficacy.[14;18] However, still limited data exist on relationships between antibody levels and OPA titers related to clinical protection for the different vaccine serotypes and after different PCV7 schedules.[17;19] The aim of the present study was to determine both quantitative and functional immune responses at 12 and 24 months of age after a 2 primary dose PCV7 schedule with or without a PCV7 booster dose at 11 months of age, compared to unvaccinated controls. Furthermore immune responses to administration of PCV7 at 24 months of age as additional booster dose or as primary dose were determined as a measure for long-term immunity.

Subjects and Methods

Study design

Between July 2005 and February 2006, before nationwide implementation of PCV7 in the Netherlands in June 2006, 1005 infants were enrolled in a randomized controlled trial investigating the effects of reduced-dose PCV7 schedules on pneumococcal carriage

during the first two years of life (NCT00189020).[10] Carriage results were described previously.[10] Healthy infants younger than 12 weeks of age, not yet having received any infant vaccination were eligible for inclusion. Randomized groups of infants received various vaccination schedules, (a) two primary doses of PCV7 at 2 and 4 months of age (2-dose group); (b) two primary doses at 2 and 4 months followed by a booster dose at 11 months of age (2+1-dose group); (c) no PCV7 vaccination (control group). At 24 months of age, an additional or primary PCV7 vaccination was offered to all study participants. From this trial, subsets of infants were included in the immunogenicity arm of the study on voluntary basis. Three ml blood samples were collected from approximately 80 children per group at 12 months and at 24 months of age and 28-42 days after PCV7 vaccination at 24 months of age. Samples from high-risk children for hepatitis B that had received Hepatitis B immunizations and post-booster samples obtained outside the estimated range of 28–42 days after vaccination were excluded from analysis. No major baseline differences were found between the immunogenicity subsets and groups in the main randomised carriage trial as reported previously.[10] Informed consent was obtained from the parents or guardians of all study participants. The study was approved by a national medical ethics committee (Stichting Therapeutische Evaluatie Geneesmiddelen, http://www.stegmetc.org) and undertaken in accordance with the European Statements for Good Clinical Practice, which includes the provisions of the Declaration of Helsinki of 1989. Laboratory personnel were unaware of treatment allocation, and the randomization key was not disclosed until the study was completed.

Study Vaccines

In the first year of life the licensed 7-valent CRM197-conjugated pneumococcal vaccine (Prevenar™ Pfizer/Wyeth) was administered during regular well baby-clinic visits, together with routine DTaP-IPV-Hib immunizations according to the Dutch National Immunization Program.[20] PCV7 vaccinations at 24 months were administered during a home visit by trained medical staff.

Laboratory measurements

After collection serum was separated and stored at -20 ^{O}C until assayed. In this report we describe the results of a subset of 40 samples in the 2-dose and 2+1-dose group, at 12 and 24 months of age, respectively, and of 20 samples in the control-group and in the 2+1-dose group at 25 months of age. Serum IgG antibody levels to the 7 vaccine pneumococcal capsular polysaccharides 4, 6B, 9V, 14, 18C, 19F and 23F were measured with ELISA using double adsorption with cell wall polysaccharide and 22F polysaccharide.[21] ELISA results of the whole immunogenicity study are described elsewhere (Chapter 7). Opsonophaygocytic activity was measured for all vaccine serotypes by a 4-fold multiplex opsonophagocytosis assay (MOPA).[22;23] In summary, 3-fold serial dilutions of heat-inactivated sera were made in 96 well microtiter plates with starting dilution 1:2. Stocks of pneumococcal strains were thawed, washed twice in opsonization buffer and 10 µl of bacterial mixture was added to each well. After incubation of 30 min at room temperature, 40 µl of differentiated HL-60 cell

suspension (ATCC) and 10 µl of rabbit complement (Pel-Freeze) were added to each well. The mixtures contained final serum dilutions starting at 1:8. After incubation of 45 min at 37°C with 5% CO_2, the mixtures were plated onto Todd-Hewitt-broth yeast extract agar plates, whereafter an overlay with selective antibiotic was added. Plates were incubated overnight at 37°C with 5% CO_2. Not phagocytosed colonies were counted and OPA titers were calculated by the software program Opsotiter 2.[23] The OPA titer was expressed as the highest serum dilution with 50% killing of the given serotype. Serum samples with a dilution <1:8 were reported as a titer of 4 for the purpose of data analysis.

Statistical Analysis

Results of IgG antibody levels are expressed in geometric mean concentration (GMC) with 95% confidence interval (95% CI). Results of OPA titers are expressed as geometric mean titers (GMT) with 95% CI. Statistical differences in IgG GMC values and OPA GMT values were assessed by log transformed unpaired t test. Of all samples with OPA titers ≥1:8 serotype-specific antibody levels required for 50% killing were calculated by dividing the IgG antibody level of a sample by the OPA titer. Proportions were tested with $\chi 2$ or Fisher exact tests, where appropriate. Correlations were assessed by Spearman correlation. All reported p-values are 2-sided, p-values smaller than 0.05 were considered significant. Analyses were performed with SPSS 15.0.

Results

IgG antibody levels at the age of 12 months

At the age of 12 months in PCV7-unvaccinated control children, IgG GMC antibody values were low (Table 1). In the 2-dose group higher IgG GMC values were observed against all vaccine serotypes compared to the control group ($p<0.001$). Proportions of subjects reaching ≥0.35 µg/ml varied in the 2-dose group between 20% (serotype 18C) and 90% (serotype 14). The proportion of children in this group reaching the higher threshold of ≥1.0 µg/ml at 12 months was ≤10% for 5 out of 7 serotypes. In the 2+1-dose group higher IgG GMCs were observed compared to the 2-dose group and control-group for all serotypes ($p<0.001$) (Table 1), as well as higher proportions of children reaching antibody levels ≥0.35 and ≥1.0 µg/ml for most serotypes. Lowest responses were observed for serotype 6B (proportions ≥0.35 and ≥1.0 µg/ml, 93% and 75%, respectively).

IgG antibody levels at the age of 24 months

At the age of 24 months in controls, IgG GMC values had increased for all serotypes compared with GMC values at 12 months (Figure 1). In the 2-dose group, IgG GMCs had increased for serotypes 6B, 19F and 23F, but had decreased for serotypes 4 and 14 at 24 months compared with values at 12 months. In the 2+1-dose group, IgG GMC values had declined for all serotypes at 24 months compared with post-booster levels at 12 months of age (Figure 1). In the 2-dose group IgG GMCs had remained higher against all serotypes at

24 months of age compared with controls. In the 2+1-dose group, no significant differences were observed anymore for 5 out of 7 serotypes compared with the 2-dose group at 24 months of age, although GMC values against all serotypes remained higher compared with levels in the control-group (Table 1). Only for serotypes 6B and 14, higher proportions of children reached the thresholds ≥0.35 and ≥1.0 µg/ml in the 2+1-dose group compared with the 2-dose group.

OPA responses at the age of 12 months

At 12 months of age, GMT OPA values in the control-group were low for all serotypes. No OPA responders (titer ≥1:8) were observed for serotypes 4, 6B and 19F (Table 1). In the 2-dose group no significant differences in OPA values and proportions of OPA responders were observed for serotypes 4, 6B, 9V and 19F compared with the control-group. For these serotypes, the percentages of OPA responders remained ≤20% in the 2-dose group (Table 1). In contrast, in the 2+1-dose group, all OPA GMT values and proportions with detectable OPA were significantly higher compared with the 2-dose and control-group. In this group >75% of the participants had reached OPA titers ≥1:8 for all serotypes (Table 1).

OPA responses at the age of 24 months

At the age of 24 months, none of the OPA GMT values had increased in the control group compared with 12 months (Figure 1). In the 2-dose group, OPA GMTs had increased for the serotypes 9V and 19F at 24 months compared with 12 months. In the 2+1-dose group, OPA GMT values for all serotypes had declined at 24 months of age compared with post-booster OPA levels at 12 months (Figure 1). In the 2-dose group, significant higher OPA GMTs and proportions of OPA responders were observed for serotypes 6B, 9V and 23F compared to the control-group (Table 1). In the 2+1-dose group, higher OPA GMT values could be observed for 5 out of 7 serotypes compared to the control-group. Compared to the 2-dose group, in the 2+1-dose group higher OPA levels were observed for the serotypes 6B, 18C and 23F. None of the participants in the 2-dose or 2+1-dose group had measurable OPA titers against serotype 4.

IgG and OPA responses after an additional PCV7 at the age of 24 months

After a first PCV7 vaccination at 24 months of age in previously unvaccinated control children, more then 95% of the participants reached the 0.35 µg/ml threshold for all vaccine serotypes except for serotypes 6B and 23F (<70%) (Table 2). For serotype 6B the proportion of OPA responders was low (40%). In the previously PCV7-vaccinated children both in the 2-dose and 2+1-dose group, higher IgG GMCs and OPA GMTs were observed following a additional 24-month booster PCV7 compared with post-PCV7 responses in previously unvaccinated controls (Table 2). A booster dose at 24 months of age resulted in higher IgG GMCs and OPA GMTs compared with the 11-month booster dose for all serotypes. After the 24-month booster dose ≥90% of the subjects reached serotype-specific antibody levels ≥1.0 µg/ml, with the only exception for serotype 18C in the 2+1-dose group (79%) (Table 2).

Relationship IgG antibody levels and OPA titers

At 12 months of age, IgG antibody levels below the threshold of 1.0 µg/ml correlated with undetectable OPA responses for the serotypes 4, 9V, 14, 19F. This counted for the 2-dose and 2+1-dose group (Figure 2). In contrast, when antibody levels did not reach the threshold ≥0.35 µg/ml for serotypes 18C and 23F, 50-53% of the children still had detectable OPA responses. A similar pattern was observed at the age of 24 months and after the additional PCV7 at 25 months (data not shown). At all time-points (12, 24 and 25 months of age) a significant correlation between IgG antibody levels and OPA titers was observed in the 2-dose and 2+1-dose group for all serotypes (Figure 2) except for serotypes 4, 9V and 18C at 12 months in the 2-dose group and serotype 9V at 12 and 25 months in the 2+1-dose group. After a primary PCV7 at 24 months in controls a significant correlation was observed between IgG antibody levels and OPA responses for the serotypes 4, 6B and 9V.

IgG antibody levels needed for 50% killing

The concentration of antibodies needed for 50% killing in the MOPA was similar between the 2-dose and 2+1-dose group for all serotypes at any time point of blood sampling (Figure 3). For serotype 23F the lowest IgG levels were needed for 50% killing (GMC 0.001 – 0.0021 µg/ml). Highest IgG levels were needed for serotypes 4 and 19F (Figure 3). Lower IgG levels against serotypes 14 ($p<0.001$) and 23F ($p=0.002$) were needed in the previously unvaccinated control-group at 25 months compared to previously vaccinated groups after an extra PCV7 at 24 months (Figure 3). At 12 and 24 months before a primary PCV7 vaccination in controls, the numbers of OPA responders were too small for comparative analyses.

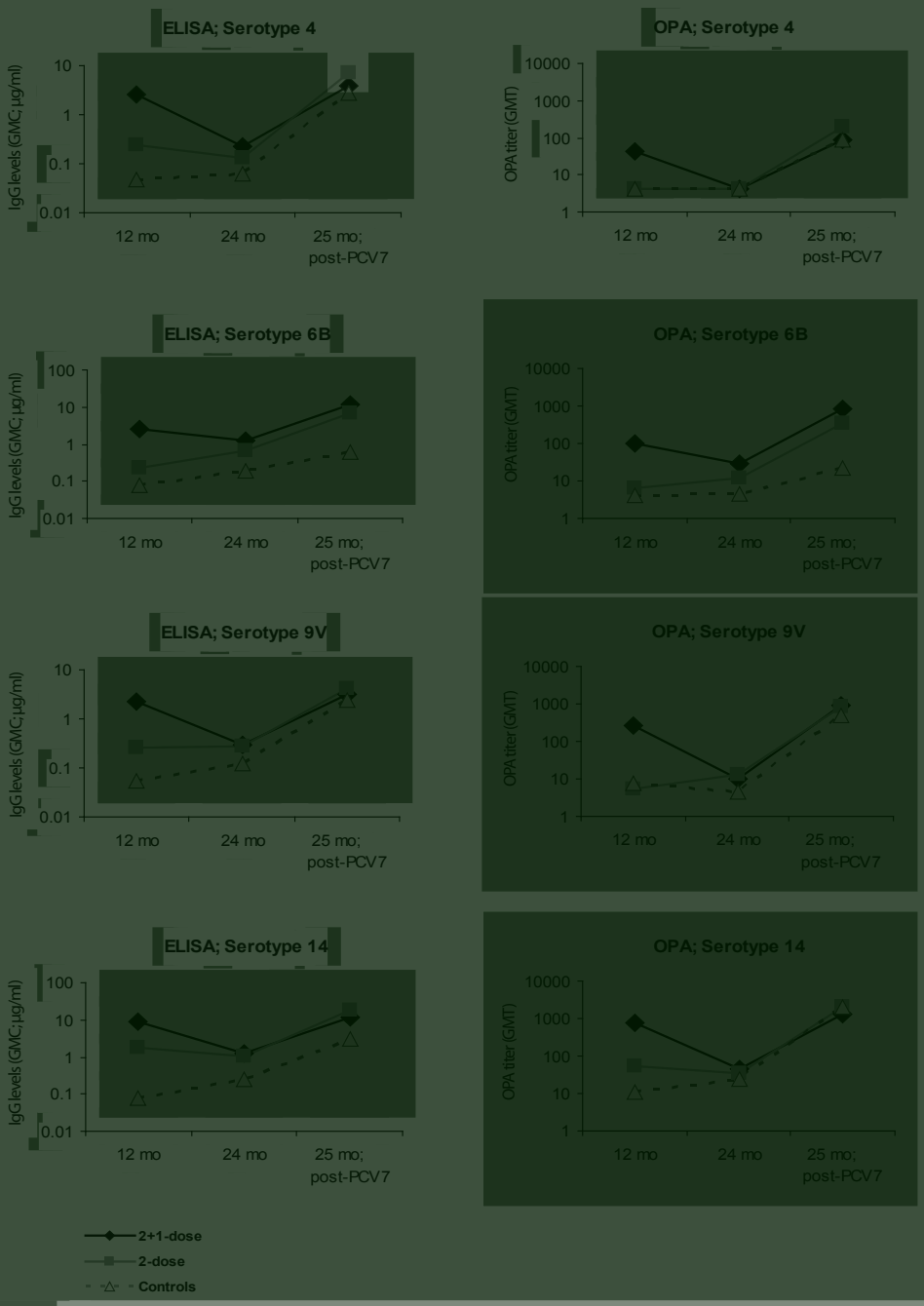
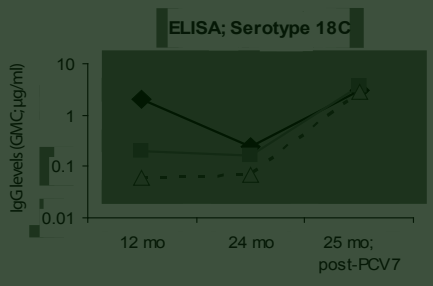
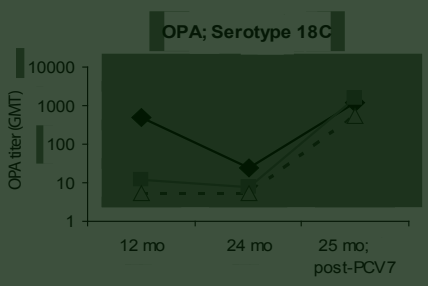
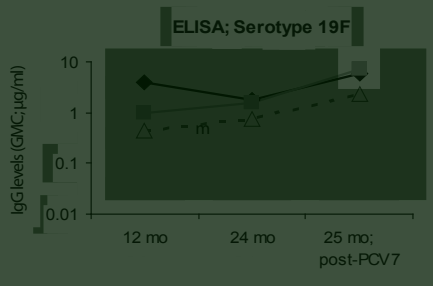
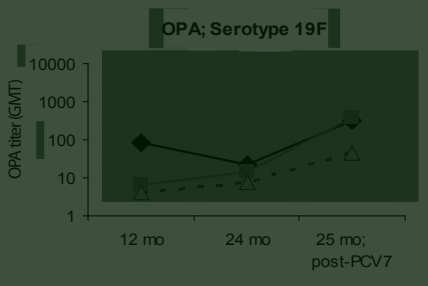


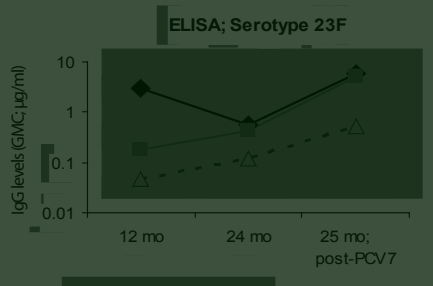
Figure 1. Serotype-specific IgG antibody levels (GMCs, µg/ml) and OPA titers (GMTs, dilution of serum able to kill 50% pneumococci) in infants after a 2-dose, 2+1-dose schedule or no vaccinations until 24 months (controls) at the age of 12 and 24 months, and at 25 months after a PCV7 at 24 months as either a booster dose (previous vaccinees) or a primary dose (previous controls).


ELISA; Serotype 18C
IgG levels (GMC; µg/ml)
10
1
0.1
0.01
12 mo
24 mo
25 mo; post-PCV7


OPA; Serotype 18C
OPA titer (GMT)
10000
1000
100
10
1
12 mo
24 mo
25 mo; post-PCV7


ELISA; Serotype 19F
IgG levels (GMC; µg/ml)
10
1
0.1
0.01
12 mo
24 mo
25 mo; post-PCV7


OPA; Serotype 19F
OPA titer (GMT)
10000
1000
100
10
1
12 mo
24 mo
25 mo; post-PCV7


ELISA; Serotype 23F
IgG levels (GMC; µg/ml)
10
1
0.1
0.01
12 mo
24 mo
25 mo; post-PCV7

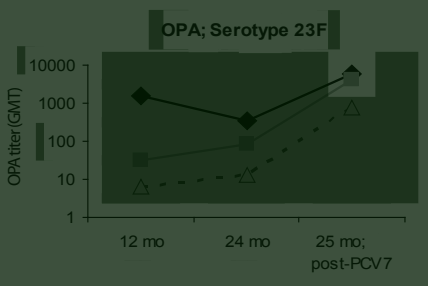

OPA; Serotype 23F
OPA titer (GMT)
10000
1000
100
10
1
12 mo
24 mo
25 mo; post-PCV7

Table 1. Serotype-specific opsonophagocytic activity (OPA) and IgG antibody responses in infants after a 2-dose, 2+1-dose schedule or no vaccinations (controls) at the age of 12 and 24 months.

	12 months				24 months			
	Controls	2-dose	2+1-dose		Controls	2-dose	2+1-dose	
	n=20	n=40	n=40	P-value	n=20	n=40	n=40	P-value
Serotype 4								
IgG GMC	0.05	0.24	2.60	α, β, γ	0.06	0.13	0.23	α, β, γ
OPA GMT	4.00	4.15	44.18	β, γ	4.00	4.00	4.00	
IgG ≥0.35 µg/ml	0%	30%	100%	β, γ	0%	13%	20%	β
IgG ≥1.0 µg/ml	0%	0%	93%	β, γ	0%	0%	3%	
OPA titer ≥1:8	0%	3%	78%	β, γ	0%	0%	0%	
Serotype 6B								
IgG GMC	0.08	0.23	2.55	α, β, γ	0.20	0.64	1.26	α, β, γ
OPA GMT	4.00	6.69	104.14	β, γ	4.44	12.34	27.85	α, β
IgG ≥0.35 µg/ml	0%	25%	93%	β, γ	25%	63%	90%	α, β, γ
IgG ≥1.0 µg/ml	0%	10%	75%	β, γ	0%	35%	50%	α, β, γ
OPA titer ≥1:8	0%	18%	90%	β, γ	5%	43%	60%	α, β
Serotype 9V								
IgG GMC	0.06	0.25	2.22	α, β, γ	0.12	0.28	0.29	α, β
OPA GMT	7.45	5.39	264.86	β, γ	4.56	12.59	10.26	α, β
IgG ≥0.35 µg/ml	0%	38%	100%	β, γ	15%	38%	35%	
IgG ≥1.0 µg/ml	0%	5%	88%	β, γ	10%	18%	8%	
OPA titer ≥1:8	10%	15%	98%	β, γ	5%	30%	30%	α, β
Serotype 14								
IgG GMC	0.08	1.71	9.06	α, β, γ	0.24	1.02	1.28	α, β
OPA GMT	11.42	54.09	768.55	α, β, γ	24.18	35.74	45.41	

IgG ≥0.35 µg/ml	25%	90%	100%	α, β	30%	85%	95%	α, β
IgG ≥1.0 µg/ml	10%	75%	100%	α, β	10%	48%	55%	α, β
OPA titer ≥1:8	20%	73%	100%	α, β, γ	35%	53%	65%	B
Serotype 18C								
IgG GMC	0.06	0.20	2.01	α, β, γ	0.07	0.17	0.24	α, β
OPA GMT	5.16	11.92	505.53	α, β, γ	5.39	7.99	24.07	β, γ
IgG ≥0.35 µg/ml	5%	20%	100%	β, γ	0%	18%	28%	β
IgG ≥1.0 µg/ml	0%	5%	83%	β, γ	0%	8%	5%	
OPA titer ≥1:8	10%	53%	100%	α, β, γ	10%	28%	63%	β, γ
Serotype 19F								
IgG GMC	0.44	0.95	3.87	α, β, γ	0.75	1.52	1.72	α,β
OPA GMT	4.00	6.20	86.56	β, γ	7.66	14.78	21.30	
IgG ≥0.35 µg/ml	60%	83%	100%	β, γ	75%	93%	98%	β
IgG ≥1.0 µg/ml	20%	45%	100%	β, γ	45%	63%	65%	β
OPA titer ≥1:8	0%	18%	85%	β, γ	20%	35%	48%	
Serotype 23F								
IgG GMC	0.05	0.17	2.96	α, β, γ	0.12	0.43	0.55	α, β
OPA GMT	6.42	30.53	1539.65	α, β, γ	13.07	81.19	341.83	α, β, γ
IgG ≥0.35 µg/ml	0%	20%	100%	β, γ	10%	48%	60%	α, β
IgG ≥1.0 µg/ml	0%	5%	93%	β, γ	5%	25%	23%	α, β
OPA titer ≥1:8	10%	60%	100%	α, β, γ	30%	70%	93%	α, β, γ

α p-Values <0.05; 2-dose vs. controls.
β p-Values <0.05; 2+1-dose vs. controls
γ p-Values <0.05; 2+1 vs. 2-dose schedule
Calculated using log transformed unpaired t test (GMCs) or chisquare/FE-test (proportions), p-values are 2 sided

Table 2. Post-vaccination IgG antibody levels and opsonophaygocytic activity titers in children who received PCV7 at 24 months as either a booster dose (previously vaccinated with 2-dose or 2+1-dose schedule) or a primary dose (previous controls). Blood samples were taken 28-42 days after vaccination.

	Post PCV7 at 24 months			
	Previous Controls n=21	Previous 2-dose n=41	Previous 2+1-dose n=19	P-value
Serotype 4				
IgG GMC	2,80	6,90	3,82	α, β, γ
OPA GMT	88,86	203,92	86,04	
IgG ≥0.35 µg/ml	100%	100%	100%	
IgG ≥1.0 µg/ml	86%	100%	95%	α
OPA titer ≥1:8	71%	93%	84%	
Serotype 6B				
IgG GMC	0,61	6,67	11,94	α, β
OPA GMT	22,84	344,76	841,79	α, β
IgG ≥0.35 µg/ml	71%	95%	100%	α, β
IgG ≥1.0 µg/ml	29%	90%	100%	α, β
OPA titer ≥1:8	48%	95%	100%	α, β
Serotype 9V				
IgG GMC	2,47	4,27	3,11	α
OPA GMT	503,83	862,47	908,90	
IgG ≥0.35 µg/ml	100%	100%	100%	
IgG ≥1.0 µg/ml	95%	95%	95%	
OPA titer ≥1:8	95%	100%	100%	
Serotype 14				
IgG GMC	3,14	18,55	12,00	α, β
OPA GMT	2035,67	1961,75	1334,50	
IgG ≥0.35 µg/ml	100%	100%	100%	
IgG ≥1.0 µg/ml	71%	98%	100%	α, β
OPA titer ≥1:8	100%	100%	100%	
Serotype 18C				
IgG GMC	2,85	3,69	2,96	
OPA GMT	547,20	1526,62	1222,49	α
IgG ≥0.35 µg/ml	100%	100%	100%	
IgG ≥1.0 µg/ml	95%	98%	79%	γ
OPA titer ≥1:8	90%	100%	100%	

Serotype 19F				
IgG GMC	2,29	6,94	6,04	α, β
OPA GMT	46,55	385,72	320,20	α, β
IgG ≥0.35 µg/ml	100%	100%	100%	
IgG ≥1.0 µg/ml	90%	100%	100%	
OPA titer ≥1:8	67%	95%	95%	α, β
Serotype 23F				
IgG GMC	0,53	5,20	5,75	α, β
OPA GMT	797,38	3953,49	5759,19	α, β
IgG ≥0.35 µg/ml	52%	100%	100%	α, β
IgG ≥1.0 µg/ml	33%	95%	100%	α, β
OPA titer ≥1:8	90%	100%	100%	

* between brackets number of samples randomly selected for OPA.
α p-Values <0.05; 2-dose vs. controls.
β p-Values <0.05; 2+1-dose vs. controls
γ p-Values <0.05; 2+1 vs. 2-dose schedule
Calculated using log transformed unpaired t test (GMCs) or chisquare/FE-test (proportions), p-values are 2 sided

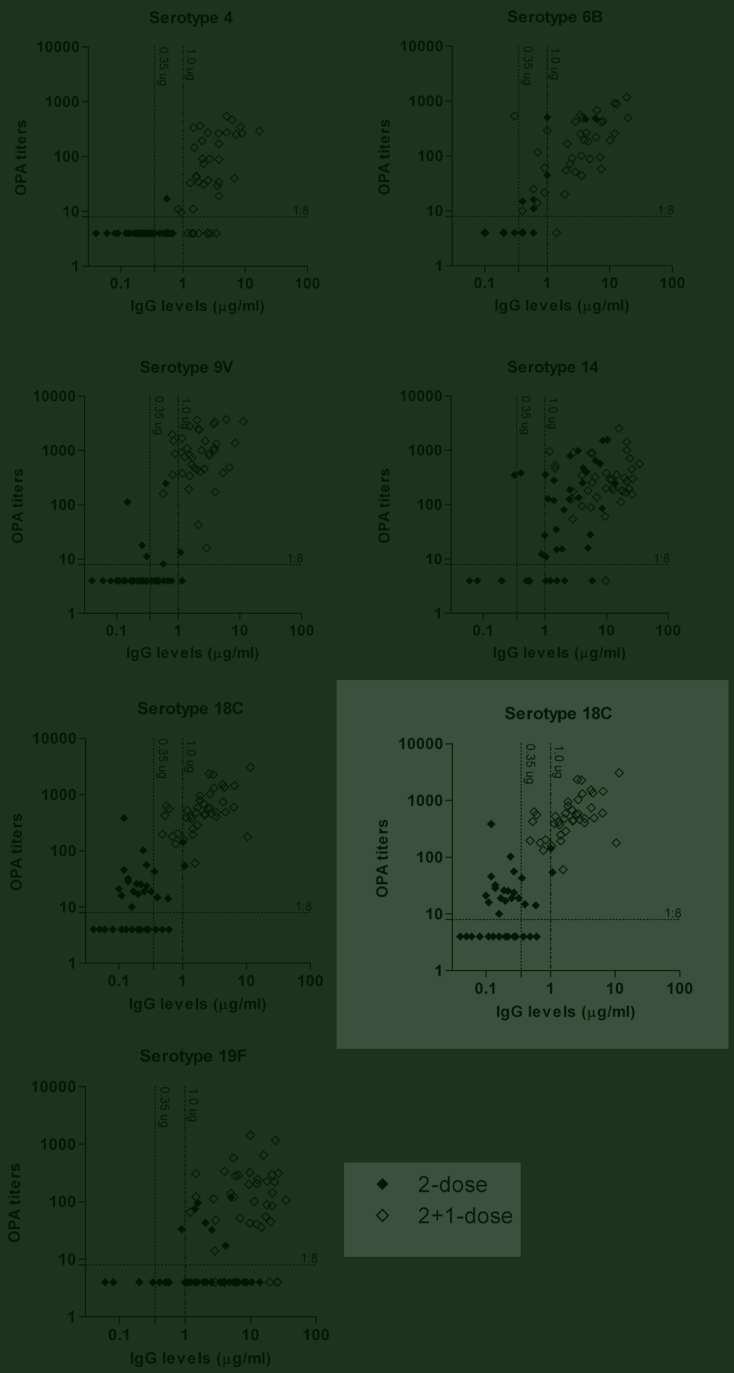


Figure 2. Relationship between participants reaching IgG antibody level thresholds (0.35 and 1.0 µg/ml) and those reaching opsonophaygocytic activity titer threshold (1:8) in children after a 2-dose (closed squares) and 2+1-dose (open squares) PCV7 schedule at 12 months of age.

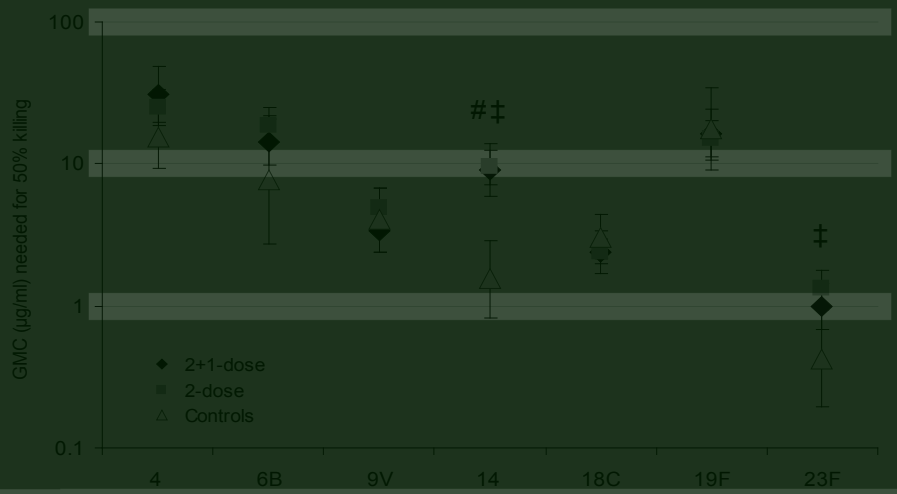


Figure 3. Post-vaccination antibody levels needed for 50% killing in the opsonophagocytic assay in children who received PCV7 at 24 months as either a booster dose (previously vaccinated with 2-dose or 2+1-dose schedule) or a primary dose (previous controls). P-values <0.05 considered significant (‡ previous controls vs. previous 2-dose group, # previous controls vs. previous 2+1-dose group).

Discussion

After repeated PCV7 administration in infancy, vaccine serotype disease is prevented by vaccine induced circulating functional antibodies and possibly the capacity to mount a proper memory B-cell response.[13] Quantitative serotype-specific IgG antibody levels are suggested by the WHO as primary comparison for non-inferiority between the various conjugate PCVs.[14] Since anticapsular immunity in the host is thought to be mediated by opsonin-dependent phagocytosis, *in vitro* opsonophagocytic assays may be a more appropriate to estimate protection.[17] However, for both quantitative antibody levels and functional OPA thresholds no definite criteria have been defined indicating vaccine-induced protection against disease.[18] In this study we showed that administration of 2 primary doses of PCV7 resulted in higher quantitative IgG antibody levels against all vaccine serotypes at both 12 and 24 months compared with unvaccinated controls. In children who had received an 11-month booster dose high post-booster antibodyresponses could be detected compared with children without a booster. However, rapid waning of antibodies was observed within 1 year after the booster dose. At 2 years of age, even in the 2+1-dose group less then half of all children had remaining IgG levels above 0.35 µg/ml for serotypes 4, 9V and 18C, which was comparable to the proportions of children who had received only 2 primary doses. In general, the kinetics of OPA titers followed similar patterns compared to IgG antibody levels for most serotypes. However, despite higher quantitative antibody levels, functional OPA responses in the 2-dose group were only marginally better for 4 serotypes compared with unvaccinated controls. On the other hand, after 2 primary doses followed by a booster dose, OPA titers were higher at 24 months than in controls for all vaccine

serotypes except for serotype 4. This rapid waning of functional immunity as measured by OPA has been earlier reported in a Finnish study after a 3+1-dose schedule (PCV7 at 2, 4, 6 and 12 months). In this study only 33% and 52% of the children had measurable OPA responses at 24 months of age against serotype 19F and 23F respectively.[24] In a study with Israelian and Finnish children receiving an 11-valent PCV, rapid waning of OPA titers was observed 6 months after 3 primary doses at 2, 4 and 6 months of age. OPA titers ≥8 were observed in only 12-37% for serotype 6B and 31-41% for serotype 14.[25]

These data suggest sustained limited protection after 2 primary doses of PCV7 and an early booster dose in the first years of life. Some studies showed lower long-term quantitative and functional antibody responses against vaccine serotypes after a 2+1-dose schedule compared with a 3+1-dose schedule.[26-28] However, after nationwide introduction of PCV7 for infants, herd effects provide protection against vaccine serotype disease in the period between primary doses and booster dose and later after the booster dose in the second year of life.[29] Indeed, after implementation of reduced-dose schedules including a catch-up strategy, a strong decline in vaccine serotype IPD is observed including not yet vaccinated newborns and infants.[7;8;30] Remarkably, in Australia after nationwide implementation of a 3 primary dose schedule without a later booster dose, a comparable ~90% effectiveness against vaccine serotype IPD was observed in children under 2 years of age, when implemented with a catch-up campaign.[31] At the moment, we do not know yet whether less clinical protection will occur at later ages due to waning immunity.[13] For better vaccine-induced immune protection, additional booster doses at later ages may be needed or delaying the booster dose may be beneficial. We observed higher immune responses after a booster vaccination at 24 months compared with a booster at 11 months of age. Priming with PCV7 under the age of 6 months clearly induced B cell memory, since children that have received two or three previous PCV7 vaccinations reached higher antibody levels after a booster vaccination at 24 months of age compared with control children receiving a first PCV7 vaccination at this age. Recently, PCV7 has been implemented in resource poor countries with a 2-dose primary schedule at 6 and 14 weeks and a very early booster at the age of 9 months (Rwanda, South Africa).[32] Especially in resource poor countries with high carriage rates with high incidence- and mortality-rates of IPD also after infancy, extra booster doses may be relevant.[33;34] In our study, we found rapidly waning immunity after the 11-month booster dose without additional value to PCV7 challenge responses at 24 months. Considering this limited long-term benefit of an early booster dose as now implemented in the EPI scheme, a later booster dose may improve long-term protection when herd effects will not have been reached yet.[13]

There is uncertainty of applicability of the quantitative and functional antibody thresholds across serotypes and of differences between different populations.[14;18] We also observed that the IgG antibody needed for 50% killing varied widely between the various vaccine serotypes.[35;36] If stated that an OPA titer ≥8 would be a more accurate measure of immunological protection against IPD than the IgG threshold ≥0.35 µg/ml, than indeed the ≥0.35 µg/ml threshold would have underestimated the level of protection reached after PCV7 vaccinations for serotypes 18C and 23F. However, this ≥0.35 µg/ml IgG treshold

would have overestimated proportion of OPA responders for the serotypes 4, 9V and 19F. These data are in correspondence with earlier studies reporting high functionality of antibodies against serotype 23F and low functionality of antibodies against serotype 19F.[19;25;35;37] A recent report furthermore suggested a role for variable susceptibility to complement deposition between serotypes and confirmed inter-serotype differences in OPA.[19] Discrepancy between IgG antibody levels and OPA responses can also be caused by other vaccine-induced circulating serotype-specific antibody isotypes, like IgM and IgA antibodies. Both have been reported to influence opsonic capacity of IgG antibodies.[38;39] Also antibodies to pneumococcal surface proteins and polyreactive antibodies may have had impact on OPA responses. These antibodies may be induced by nasopharyngeal colonization and may increase with age.[40] We found no correlations between serotype-specific IgG levels and OPA titers in PCV7-unvaccinated controls in contrast to vaccinated children for any serotype.[41] This changed after administration of a primary PCV7 at 24 months, since then reasonable correlations were observed between IgG levels and OPA responses. Naturally induced antibodies in previously unvaccinated children may explain higher OPA for serotypes 14 and 23F in children that had a first PCV7 at 24 months compared with children that were previously vaccinated already with PCV7.

In our study, no influences of herd effects on immune responses are expected.[10] However, eradication of vaccine serotypes, may result in lower serotype-specific antibody levels by loss of natural boosting. Therefore it is currently not clear whether antibody and functionality thresholds that are found now, will still prevail after establishment of herd effects. An important limitation in OPA thresholds in general and also in our study, is the *in vitro* MOPA killing assay, which is highly susceptible for inter-laboratory variation and difficult to standardize.[17] We used the standardized MOPA protocol with recommended bacterial strains and HL-60 cell line.[17;18] However, the expression of capsular polysaccharide by the used pneumococcal strain has been described as an influencing factor for serotype-specific OPA responses.[42] Also the use of the HL-60 cell line has its limitations. This cell line is homozygous for the R131 variant allele of the low-affinity FCγRII receptor, which will bind IgG1 and IgG3 but has very low affinity for IgG2, which we know is also induced by the conjugate vaccines.[43] We did not study IgG1 and IgG2 antibody subclasses in this study or IgM and IgA isotypes. Furthermore, we made a selection of blood samples for assessment in the MOPA assay. Because of the smaller groups, small differences between groups in OPA titers and proportions of responders may have been missed (type 1 error). Still, limited differences were observed between IgG GMC values of the larger groups and IgG GMC values of the smaller subset used for MOPA. Strengths of our study include the randomized controlled study design which made it possible to compare the different schedules without the influences of temporal or geographical trends in distribution of circulation pneumococcal serotypes.

To summarize, we showed that PCV7 vaccination early in life primes for robust quantitative and functional antibody responses at 24 months of age. We showed that overall quantitative IgG antibody levels are correlated to OPA titers. However IgG levels needed for 50% killing varied widely between serotypes and antibody thresholds 0.35 and 1.0 µg/ml did not necessary represent OPA titers ≥8 and vice versa. Therefore both OPA responses

and GMC antibody levels should be taken into consideration when defining correlates of protection, but more defined thresholds for different tests and serotypes are wanted. After PCV7 administration antibody levels and OPA titers waned rapidly. A PCV7 booster dose after 2 primary doses added to sustained protection as measured by OPA. Delaying or adding an extra booster dose until late in the second year of life may potentially have positive consequences for sustained vaccine responses.

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9

Mucosal immune responses to the 7-valent pneumococcal conjugate vaccine in the first 2 years of life

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Abstract

Background

The CRM197-conjugated 7-valent pneumococcal vaccine (PCV7) protects against vaccine serotype disease and nasopharyngeal carriage. Data on the role of PCV7-induced antibodies at the mucosal level are scarce.

Methods

In a randomized controlled setting, children received PCV7 at 2 and 4 months (2-dose group); at 2, 4 and 11 months (2+1-dose group); or no PCV7 (control-group). From 188 children saliva samples were collected at 12 and 24 months of age. IgG and IgA antibody levels were measured by multiplex immunoassay against the 7 vaccine serotypes and 4 non-vaccine serotypes (1, 3, 5, 7F).

Results

At 12 months of age, both vaccine groups showed higher salivary IgG-levels against vaccine serotypes compared with controls. Highest IgG-levels were observed after 2+1-doses compared with 2 primary doses only, and sustained until 24 months of age for most serotypes. Salivary IgA-levels were higher in both vaccine groups at 12 months of age compared with controls, except for serotype 19F. Differences remained for most serotypes in the 2+1-dose group until 24 months, but not in the 2-dose group where IgA-levels were similar to controls. Antibody levels against non-vaccine serotypes did not differ between randomization groups. In unvaccinated children with proven nasopharyngeal carriage of serotypes 6B, 19F and 23F higher homologous salivary antibody levels were observed compared with non-carriers.

Conclusion

PCV7 induced both salivary IgG and IgA responses with persistently higher salivary IgG-levels at 24 months of age. Differences in IgA antibody levels were less pronounced at 24 months of age and seem to respond to natural boosting. Pneumococcal colonization may be a major determinant in salivary antibody maintenance.

Introduction

Protein-conjugated pneumococcal vaccines (PCVs) are effective against vaccine serotype invasive pneumococcal disease (IPD), as well as pneumonia and acute otitis media (AOM).[1-3] Besides protection against disease, systemic administration of PCV results in a reduction of nasopharyngeal vaccine serotype acquisition and colonization.[4;5] Vaccine-induced systemic anticapsular IgG antibodies, which activate complement and enhance phagocytosis, are presumed to mediate protection against IPD.[6] For nasopharyngeal colonization systemic serotype-specific IgG levels are reported to be inversely related to new nasopharyngeal acquisition of the given serotype[7;8], and serological IgG levels as 'correlates of protection' against AOM and carriage have been suggested.[9;10]

At the mucosal surface, anti-capsular IgA antibodies have been shown to support complement-dependent opsonophagocytosis, and agglutination of the pneumococcus.[11;12] IgA antibodies against pneumococcal surface proteins also have been described as major contributor in protection against mucosal disease.[13] The role of mucosal antibodies after systemic PCV immunization in protection against pneumococcal disease and carriage is however less clear. PCVs induce also IgG and IgA antibodies at the mucosal level, but the magnitude and dynamics of these mucosal antibodies are largely unknown.[14-18] Most studies on mucosal antibodies lack unvaccinated control groups which hampers full estimation of vaccine impact because of mucosal antibody responses are also enhanced by natural pneumococcal carriage.[11;13;14] Furthermore, studies were often restricted to few serotypes[14;15] with limited data on persistence and boostability of mucosal antibody levels.[17;18] Finally, in most published studies mucosal antibody levels were difficult to measure, possibly due to the used ELISA detection-method. This restricted the study observations therefore allowed only the description of very rough vaccine effects.[14;15;18]

In this study, we applied highly sensitive, fluorescent bead-based multiplex immuno assay (MIA) using LUMINEX technology[19] to determine salivary IgG and IgA antibody levels. Responses against 11 vaccine and non-vaccine serotypes were measured in a large group of children participating in a randomized controlled trial on reduced-dose schedules with the 7-valent CRM197-conjugated pneumococcal vaccine (PCV7).[4] Paired salivary samples were collected at the age of 12 and 24 months from vaccinees and unvaccinated controls. Besides studying the mucosal antibody responses, we explored the association between serum and saliva antibody levels. Also, we studied the effect of natural exposure to pneumococcal carriage on homologous mucosal IgG and IgA levels in unvaccinated children.

Methods

Study design

Between July 2005 and February 2006, before nationwide implementation of PCV7 in June 2006 in the Netherlands, 1005 infants were enrolled in a randomized controlled trial investigating the effects of reduced-dose PCV7 schedules on pneumococcal carriage during

the first two years of life (NCT00189020).[4] Healthy infants younger than 12 weeks of age, not yet having received any infant vaccination were eligible for inclusion. Groups of infants received various vaccination schedules, (a) two primary doses of PCV7 at 2 and 4 months of age (2-dose group); (b) two primary doses at 2 and 4 months followed by a booster dose at 11 months of age (2+1-dose group); (c) no PCV7 vaccination (control group). Paired saliva samples were taken from approximately 60 participants per group at 12 and 24 months. At home visits children were asked to chew a sponge swab (Malvern, Worchester UK). Salivary fluid was collected and immediately stored on dry ice (carbon dioxide -56°C), whereafter it was stored within 8 hours at -80°C. From 15 children blood samples were obtained next to saliva samples. Serum was separated within 24 hours and stored at -20°C until assayed. From all children nasopharyngeal swabs were taken consecutively at the age of 6 weeks and at 6, 12, 18 and 24 months. Carriage results were described earlier [4]. Identification of *S pneumoniae* nasopharyngeal carriage was based on colony morphology and conventional methods of determination, as described earlier.[4] Informed consent was obtained from the parents or guardians of all study participants. The study was approved by a national medical ethics committee (Stichting Therapeutische Evaluatie Geneesmiddelen, http://www.stegmetc.org) and undertaken in accordance with the European Statements for Good Clinical Practice, which includes the provisions of the Declaration of Helsinki of 1989. Laboratory personnel were unaware of treatment allocation, and the randomization key was not disclosed until the study was completed.

Study Vaccines

The licensed 7-valent CRM197-conjugated pneumococcal vaccine (Prevenar™ Pfizer/Wyeth), containing pneumococcal polysaccharides 4, 6B, 9V, 14, 18C, 19F and 23F, was administered during regular well baby-clinic visits, together with routine DTaP-IPV-Hib immunizations according to the Dutch NIP.[20]

Multiplex immunoassay

IgG and IgA antibody levels in serum and saliva were measured by a multiplex fluorescent bead-based immunoassay (MIA) using LUMINEX technology.[19] Eleven sets of microspheres were coated with the pneumococcal polysaccharide antigens 4, 6B, 9V, 14, 18C, 19F and 23F (serotypes covered by PCV7) and 1, 3, 5, 7F (non-vaccine serotypes) (ATCC; Manassas, VA). Antigens were conjugated to Poly-L-lysine, after which the complex was attached to the microspheres by a reaction using EDC with sulpho-NHS. Standard reference serum (89SF-5; FDA) with known antibody concentrations for 23 pneumococcal capsular polysaccharides was used for standard serial dilutions in duplo. Salivary samples were thawed and centrifuged. Supernatants were diluted 1:1 using 5% antibody-depleted human serum containing cell wall polysaccharide and 22F polysaccharide (ADHS-CWPS Multi; Statens Serum Institut), and incubated at 4°C. Samples were tested in duplo with a minimum of 2 blank wells per run. Sera samples were diluted 1:100 and 1:1000 using 5% ADHS-CWPS Multi and incubated at 4°C with shaking. From each diluted sample 2 times 25 µl was mixed with an equal volume of beads. Goat-anti-human-IgG-PE or goat-anti-human-IgA-

PE solution 1:200 (Jackson Immuno Research) was added. After a final wash, analysis of the beads was performed on a BioPlex 100 apparatus (Bio-Rad) using the BioPlex software package (version 4.1.1; Bio-Rad). Antibody levels were expressed in ng/ml IgA or IgG. The cut-off for positivity (2 SD of 20 blank wells) varied for IgG antibodies between 0.15 ng/ml (serotype 7F) and 1.35 ng/ml (serotype 9V) and for IgA antibodies between 0.03 ng/ml (serotype 23F) and 0.49 ng/ml (serotype 3). Samples below the cutt-off were assigned half the detection limit for the given serotype.

Statistical Analysis

Salivary IgG and IgA antibody levels are expressed in geometric mean concentrations (GMC; ng/ml) with 95% CI. Statistical differences between IgG and IgA GMC values were assessed by log transformed unpaired t test. Antibody concentrations with paired saliva samples taken at 12 and 24 months were compared by log transformed paired t test. Correlations between salivary and serum antibody levels were assessed by Spearman correlation. For analyses of potential correlations between previous carriage and consecutive antibody responses in unvaccinated controls, we focused on the 3 most frequently carried serotypes: 6B, 19F and 23F. Children were defined as colonized when one or more positive cultures were obtained for that serotype up to the moment of saliva sampling (i.e. 12 or 24 months of age). The children with serotype-negative nasopharyngeal samples up to the moment of saliva sampling were defined as non-colonized. All reported p-values are 2-sided, p-values smaller than 0.05 were considered significant. Analyses were performed with SPSS 15.0.

Results

Study participants

Of the 1005 enrolled children in the main carriage trial, 187 children participated in the saliva immunogenicity study. Paired samples at both 12 and 24 months were obtained in 165 participants. Because of limited volumes of saliva obtained of some individuals, salivary IgG antibodies were measured in 177 and 175 samples and salivary IgA antibodies in 166 and 158 samples at 12 and 24 months, respectively (see Table 1 for baseline characteristics and sample numbers per group). No major differences in demographic characteristics and risk factors for pneumococcal carriage were found between groups of children participating in the main trial and the saliva subset.[4] We analysed natural and vaccine-induced mucosal antibodies as plain titers (ng/ml) and titers corrected for dilution factor by normalizing for total protein concentration in saliva. The data did not differ after correction for salivary total protein compared to uncorrected salivary antibody levels (data not shown). We further present data using plain antibody concentrations for our analyses.

Salivary vaccine serotype IgG antibody levels

In unvaccinated control children at the age of 12 months, salivary IgG GMC antibody values varied between 1.1 ng/ml (serotype 18C) and 11.2 ng/ml (serotype 19F) (Table 2).

Table 1. Baseline characteristics of participants receiving 2 doses, 2+1-doses or no PCV7 vaccinations (controls).

	Study group		
	Controls	2-dose	2+1-dose
	n = 61	n = 61	n = 65
Male (%)	29 (48)	26 (43)	37 (57)
Age at vaccination; mean (SD), mo			
PCV 2 mo	-	2.1 (0.2)	2.1 (0.2)
PCV 4 mo	-	4.3 (0.4)	4.2 (0.3)
PCV 11 mo	-	-	11.1 (0.3)
Age at saliva sampling; mean (SD), mo			
12 mo	11.9 (0.3)	12.0 (0.3)	12.1 (0.3)
24 mo	24.5 (0.8)	24.4 (0.6)	24.2 (0.5)
Saliva sample taken (%)			
12 mo	60 (98)	54 (89)	63 (97)
24 mo	55 (90)	58 (95)	62 (95)
Siblings present (%)			
12 mo	28 (46)	36 (59)	36 (55)
24 mo	35 (58)	40 (66)	40 (64)
Daycare attendance* (%)			
12 mo	40 (66)	34 (56)	31 (48)
24 mo	44 (72)	37 (61)	40 (62)
Passive tobacco smoke exposure (%)			
12 mo	3 (5%)	5 (8%)	3 (5%)
24 mo	2 (3%)	5 (8%)	5 (8%)

*Defined as more than 4 hours per week with at least 1 child from a different family.

Significantly higher IgG GMC values were found for all 7 vaccine serotypes in the 2-dose group compared to the control group ($p<0.001$); where GMC values varied between 8.2 ng/ml (serotype 23F) to 49.7 ng/ml (serotype 14). A further increase of IgG GMC values was observed in the 2+1-dose group compared to the 2-dose group for all serotypes ($p<0.001$). Lowest GMC values were found for serotype 23F.

In controls at the age of 24 months, salivary vaccine serotype IgG GMC values had increased for the serotypes 6B, 18C, 19F and 23F compared with values at 12 months (Figure 1); highest GMC values were found for serotype 19F (19.7 ng/ml) (Table 2). In the 2-dose group at 24 months, IgG GMC values also had increased for serotypes 6B, 19F and 23F but decreased for vaccine serotypes 4, 9V and 14 compared with values at 12 months (Figure 1). Still GMC values against all vaccine serotypes remained higher in the 2-dose group compared to controls. In the 2+1-dose group at 24 months, IgG GMC values had declined for all vaccine serotypes ($p<0.01$). However, GMC values at 24 months remained significantly higher after 2+1 doses compared to the 2-dose group, except for serotypes 6B and 19F, which were higher compared to other serotypes and similar in both vaccine groups.

Table 2. Salivary IgG antibody levels (GMC; ng/ml) in children after 2 doses, 2+1-doses of PCV7 or no PCV7 vaccinations (controls) at the age of 12 and 24 months.

	12 months				24 months			
	Controls	2-dose	2+1-dose		Controls	2-dose	2+1-dose	
Serotype	n = 60	n = 54	n = 63	p-Values	n = 55	n = 58	n = 62	p-Values
4	1.8	11.3	124.0	α, β, γ	1.5	6.0	14.8	α, β, γ
6B	4.4	12.7	188.2	α, β, γ	7.1	40.4	67.0	β, γ
9V	2.6	18.7	177.9	α, β, γ	2.7	11.8	34.3	α, β, γ
14	3.1	49.7	360.3	α, β, γ	3.2	15.7	52.3	α, β, γ
18C	1.1	11.5	137.3	α, β, γ	2.0	8.8	19.0	α, β, γ
19F	11.2	33.8	147.2	α, β, γ	19.7	72.3	85.7	β, γ
23F	1.2	8.2	112.4	α, β, γ	2.0	12.8	39.7	α, β, γ
1	4.6	3.0	3.7		4.3	4.1	5.7	
3	1.2	1.3	0.7		1.0	1.4	1.5	
5	8.6	7.3	9.1		11.2	10.7	12.9	
7F	5.1	3.3	3.5		4.2	3.1	5.3	

α p-Values <0.05; 2+1 vs. 2-dose schedule
β p-Values <0.05; 2+1-dose vs. controls
γ p-Values <0.05; 2-dose vs. controls
Calculated using log transformed unpaired t test, p-values are 2 sided

Salivary vaccine serotype IgA antibody levels

At the age of 12 months, salivary IgA GMC antibody values in unvaccinated control children varied between 0.8 ng/ml (serotype 18C) to 7.2 ng/ml (serotype 19F) (Table 3). Compared to the control group, significantly higher IgA GMC values were found in the 2-dose group for 4 of the 7 vaccine serotypes. However, no differences were observed for serotype 18C, 19F and 23F. GMC values varied between 0.9 ng/ml (serotype 18C) and 17.5 ng/ml (serotype 14). In the 2+1-dose group IgA GMC values further increased for 6 of the 7 vaccine serotypes compared to the 2-dose group. Again highest response was observed for serotype 14 (78.3 ng/ml). For serotype 19F IgA levels were high in all study groups and did not significantly differ between the 2+1-dose group, 2-dose and control group.

In the control group at the age of 24 months, salivary IgA GMC values against all vaccine serotypes had increased compared with 12 months (Figure 1). In the 2-dose group at 24 months IgA values had increased for the serotypes 6B, 19F and 23F. However, no significant differences in IgA values against vaccine serotypes were observed between the 2-dose group and controls, except for serotype 6B (Table 3). In the 2+1-dose group at 24 months IgA GMC values for serotypes 4 and 14 declined compared with 12 months, while serotypes 6B, 9V, 18C and 23F remained at the same levell. Overall, this resulted in higher IgA values against 5/7 vaccine serotypes in the 2+1-dose group compared with controls, except for serotypes 18C and 19F. Compared to the 2-dose group, only higher responses were observed for the serotypes 9V and 18C after 2+1-doses. Like at 12 months of age, serotype 19F showed similar salivary IgA GMC values in all 3 randomization groups at the age of 24

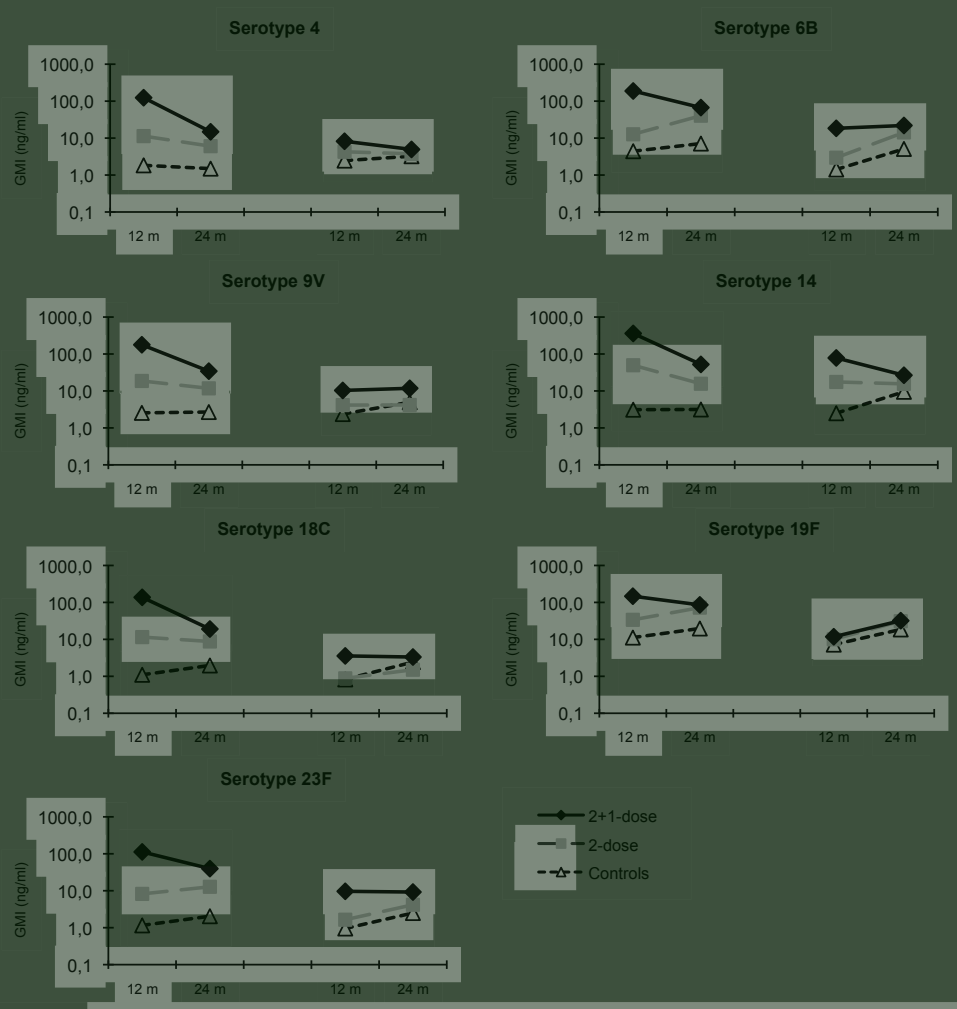

Figure 1. Salivary IgG and IgA antibody levels (GMC; ng/ml) against vaccine serotypes in children after 2 doses, 2+1-doses of PCV7 or no PCV7 vaccinations (controls) at the age of 12 and 24 months.

months (Table 3).

Salivary non-vaccine serotype antibody levels

At the age of 12 months salivary IgG GMC values against non-vaccine serotypes did not differ between the different randomization groups (Table 2). At the age of 24 months, IgG GMC values for the non-vaccine serotypes increased in the 2-dose group for serotype 5 and in the 2+1-dose group for all 4 non-vaccine serotypes compared with values at 12 months. However, these increases did not result in significant differences between the 3 randomization groups (Table 2).

IgA GMC values against non-vaccine serotypes at the age of 12 months did not differ

Table 3. Salivary IgA antibody levels (GMC; ng/ml) in children after 2 doses, 2+1-doses of PCV7 or no PCV7 vaccinations (controls) at the age of 12 and 24 months.

	12 months				24 months			
	Controls	2-dose	2+1-dose		Controls	2-dose	2+1-dose	
Serotype	n = 57	n = 51	n = 58	p-Values	n = 49	n = 50	n = 59	p-Values
4	2.5	4.3	8.2	α, β, γ	3.2	3.8	4.9	β
6B	1.4	2.9	18.4	α, β, γ	5.1	14.2	22.0	β, γ
9V	2.4	4.1	10.3	α, β, γ	4.9	4.2	11.8	α, β
14	2.5	17.5	78.3	α, β, γ	9.5	15.6	26.6	β
18C	0.8	0.9	3.6	α, β	2.4	1.5	3.3	α
19F	7.2	10.0	11.8		18.6	31.0	32.0	
23F	1.0	1.6	9.6	α, β	2.5	4.2	9.2	β
1	4.4	3.9	3.1		4.8	4.0	4.2	
3	8.6	12.0	8.3		9.7	11.9	14.9	
5	4.8	4.1	4.3		6.2	5.2	6.8	
7F	4.7	3.7	3.4		6.4	4.8	6.3	

α *p*-Values <0.05; 2+1 vs. 2-dose schedule
β *p*-Values <0.05; 2+1-dose vs. controls
γ *p*-Values <0.05; 2-dose vs. controls
Calculated using log transformed unpaired t test, p-values are 2 sided

between the 3 randomization groups (Table 3). IgA GMC values increased in the control group for serotypes 5 and 7F and in the 2+1-dose group for the serotypes 3, 5 and 7F. Like salivary IgG, no significant differences in IgA GMC values were observed between the 3 randomization groups (Table 3).

Serum vs. salivary antibodies

Simultaneous saliva and serum samples were available for 15 PCV7 vaccinated children. A positive correlation was found between serum and salivary IgG antibody levels for all vaccine serotypes (range r=0.54 for serotype 14 to r=0.88 for serotype 6B) (Table 4). Also positive correlations between serum and salivary IgA antibody levels were observed (range r=0.57 for serotype 4 to r=0.92 for serotype 6B). For the non-vaccine serotypes no positive correlation between serum and saliva IgG or IgA antibody levels existed. For vaccine serotypes 11.0 to 26.2-fold higher IgG levels were observed in serum compared to saliva (Table 5). In contrast, for IgA the highest serum/saliva-ratio was 2.0 (serotype 23F). For serotype 19F even higher salivary IgA levels were observed compared to serum levels (serum/saliva-ratio 0.4).

Table 4. Relation between serum and salivary IgG and IgA antibody levels in the PCV7 vaccinated groups.

	IgG Serum-Saliva (n=15)		IgA Serum-Saliva (n=15)	
Serotype	r	p	r	p
4	0.63	0.011	0.57	0.032
6B	0.88	0.000	0.92	0.000
9V	0.64	0.010	0.71	0.005
14	0.55	0.035	0.66	0.010
18C	0.79	0.000	0.65	0.012
19F	0.57	0.027	0.77	0.001
23F	0.87	0.000	0.69	0.007
1	0.32	0.248	0.40	0.160
3	-0.06	0.824	0.26	0.375
5	0.38	0.164	0.20	0.483
7F	0.31	0.254	0.30	0.296

Correlations between salivary and serum antibody levels were assessed by Spearman correlation.
All reported p-values are 2-sided, p-values smaller than 0.05 were considered significant and are depicted in bold.

Development of salivary antibodies in relation to carriage in unvaccinated controls

We analysed nasopharyngeal pneumococcal carriage at the age of 6 weeks and 6, 12, 18 and 24 months. Until 12 months of age, nasopharyngeal carriage was found in the PCV7 unvaccinated control group in 9 of the 60 children (15%) for serotype 6B, in 11 children (18%) for 19F and 9 children (15%) for 23F. At this age, higher homologous salivary IgG and IgA GMC values were found in previously colonized children for serotype 19F and 23F compared to children with serotype-negative swabs (Figure 2).

Table 5. Serum / saliva ratio for serotype-specific IgG and IgA antibodies in the PCV7 vaccinated groups.

	Serum/Saliva ratio*	
Serotype	IgG	IgA
4	26.2	1.8
6B	11.0	1.4
9V	17.0	1.1
14	22.7	0.9
18C	18.8	1.8
19F	11.6	0.4
23F	24.5	2.0
1	27.8	1.7
3	141.7	1.4
5	5.8	0.6
7F	15.9	0.8

*Serum/saliva ratio's presented in geomeans

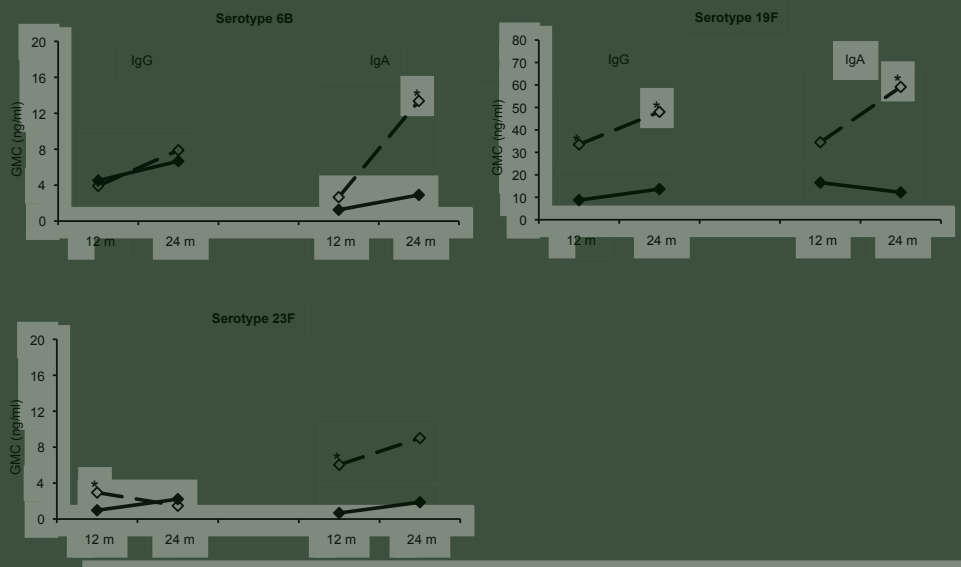

Figure 2. Salivary IgG and IgA levels (GMC; ng/ml) at 12 or 24 months of age in unvaccinated children after previously being colonized with the homologous serotype at 6 weeks, 6, 12, 18 or 24 months of age. Closed squares: children with serotype-negative swabs, open squares: serotype-positive children. *Significant difference; $p<0.05$.

After additional swabs at 18 and 24 months, the proportion of carriers in this control-group had risen to 21 (38%) of the 55 participants for serotype 6B, 16 children (29%) for 19F and 11 children (20%) for 23F at 24 months of age. Higher homologous salivary IgG GMC values were observed for serotype 19F carriers at 24 months compared to children with serotype-negative swabs (Figure 2). With respect to IgA, higher homologous levels were found in previously colonized children with serotypes 6B or 19F. In the 2-dose and 2+1-dose group numbers of serotype-positive samples were too small for comparative analyses.

Discussion

We studied mucosal IgG and IgA antibody responses in PCV7 vaccinated and non-vaccinated children in a randomized controlled setting before nationwide PCV7 introduction in the Netherlands. We showed that in unvaccinated controls mucosal antibodies were induced by natural boosting. Earlier it was already observed by Simell *et al* that natural exposure to pneumococci induced salivary IgA antibody responses.[21] Our study confirms this observation. However, we also observed that in unvaccinated controls besides salivary IgA salivary IgG antibody levels were boosted by nasopharyngeal carriage. Until now, this association between mucosal IgG antibodies and carriage was not observed, most likely because of low detectable mucosal IgG levels.[21] Carriage-induced mucosal IgA levels against serotype 19F in controls even equalled mucosal IgA levels in both vaccinated groups at 12 and 24 months.

Systemic administration of 2 primary doses of PCV7 resulted in increased salivary IgG antibody responses against all vaccine serotypes compared to unvaccinated controls. An additional PCV7 booster dose at 11 months increased salivary IgG antibody levels one month later compared to 2 primary dose only, showing pneumococcal conjugate vaccines contribute to salivary IgG antibodies.[17] For mucosal IgA different dynamics in salivary antibody responses were observed. At 24 months of age the parallel increase in serotype-specific salivary IgA levels in the 2-dose and unvaccinated control group resulted in less pronounced differences between randomization groups, suggestive for natural boosting. In vaccinees less vaccine serotype-specific carriage occurred.[4] This might have lead to less natural boosting of IgA antibody levels compared to the control group, and can explain why also most previous studies did not observe a difference in mucosal IgA levels between PCV7 vaccinated and unvaccinated children.[15-17]

Furthermore, we observed that mucosal IgG antibody levels correlated well with serum antibody levels, supporting the hypothesis of IgG transport to the mucosal site.[14;21] In PCV7 vaccinated children systemic IgG levels proved to be 10-20 fold higher than salivary IgG levels. When compared to serum IgG levels between the different randomization groups, no differences were observed at 24 months for 5 of 7 vaccine serotypes (Chapter 7). In salivary IgG levels the benefit of an additional booster remained for most serotypes and suggests active IgG production at the mucosal site as well. For IgA the serum/saliva-ratio's are suggestive for a stronger mucosal response. For serotype 19F even higher mucosal IgA levels were observed compared with serum levels, and clearly both IgG and IgA salivary antibody levels increased with serotype 19F carriage.

No significant differences were observed in non-vaccine serotype mucosal antibody responses between controls and vaccinees, which is in correspondence with earlier reports.[17] Increases between 12 and 24 months of age, especially in the 2+1-dose group, may represent natural boosting. Although there is potentially more carriage of non-vaccine serotypes in the vaccinated groups, none of these serotypes were frequently encountered in conventional cultures in our carriage study[4]. Polyreactivity on the same antigenic stimulus may also be possible [8], as well as cross-reactivity with other bacteria.[22;23]

The exact contribution of PCV7 induced salivary IgA and IgG antibody levels in protection against nasopharyngeal carriage and disease is not known yet. We reported a 58% and 60% reduction in vaccine serotype pneumococcal carriage at the age of 24 months in the 2-dose and 2+1-dose group compared to unvaccinated controls, respectively.[4] In the present study however at this age no difference in mucosal IgA antibody levels between the 2-dose and controls could be observed for most vaccine serotypes. In contrast to mucosal IgG antibodies where a stepwise increase was observed between the control, 2-dose and 2+1-dose group. This may suggest that vaccine induced anti-capsular mucosal IgG antibodies have a stronger contribution to protection against pneumococcal colonization then IgA. However, level of antibodies may not represent functionality[3] and IgA antibodies have been shown to support anti-capsular complement-dependent opsonophagocytosis, and agglutination of the pneumococcus at the mucosal surface.[11;12]

This study was performed well before herd effects after PCV7 introduction in the Dutch

NIP. In the coming years the impact of decreased vaccine serotype nasopharyngeal colonization on mucosal antibody responses have to be evaluated, as natural boosting seems important in mucosal antibody persistence. Mucosal vaccine responses may vary between regions and continents. Also primary doses at older ages, more doses or broader intervals between doses can impact vaccine immunogenicity, as well as other factors like concomitant childhood vaccinations or ethnic background variability.[24-27] Some limitations of this study should be addressed. As IgA is susceptible to cleavage through bacterial IgA1 proteases, sample collection, processing and storage can have impact on mucosal IgA levels.[28;29] To prevent this, samples were immediately frozen after collection and analyzed directly after thawing. The randomized controlled setting also contributes to the reliability of the observed differences between groups. Since IgA antibody levels strongly depend on the secretion flow rate of the participant during sample collection[28], antibody levels were also corrected for total salivary protein, which did not change results. Furthermore, due to the large 6-months interval of nasopharyngeal sampling, in between we will have missed carriage episodes in individuals. Therefore not at all time points higher mucosal antibody levels in colonized children could be observed compared with non-colonized children. Also the single colony method for serotyping may have resulted in missed multiple serotype carriage. Still for all of the 3 tested serotypes boosting of the immune system after natural exposure could be shown. Strengths of our study include the randomized controlled study design with an unvaccinated control group. This made it possible to estimate the effect of PCV7 administration and pneumococcal carriage on mucosal responses without the influences of temporal or geographical trends in distribution of circulating pneumococcal serotypes. Also the highly sensitive new multiplex technique (MIA) allowed to obverse less robust differences between the different time points and groups. In conclusion, systemic administration of PCV7 proved to induce both salivary IgG and IgA antibodies. However, we observed differences in IgG and IgA antibody levels between serotypes after PCV7 administration and nasopharyngeal carriage. IgG antibody levels remained higher after 2+1-doses at 24 months, while for IgA a strong increase was observed in the 2-dose group and unvaccinated controls. Nasopharyngeal carriage proved to be a major contributor to mucosal antibody maintenance. Immunological protection against pneumococcal disease after herd effects of PCV7 implementation should be monitored.

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Part Three

Effect of pneumococcal carriage on immune responses to pneumococcal conjugate vaccine after nationwide implementation

10

Lower challenge responses to pneumococcal conjugate vaccination after previous carriage of *Streptococcus pneumoniae* in the first 2 years of life

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Abstract

Objective

To determine whether nasopharyngeal pneumococcal carriage with serotypes 6B, 19F or 23F interferes with IgG serum antibody responses towards administration of the 7-valent pneumococcal conjugate vaccine (PCV7) at age of 24 months in previously PCV7-vaccinated children.

Study design

Blood samples were collected before and after a PCV7 challenge 24 months from subsets of children participating in a randomized controlled trial. Children had previously received 2 doses of PCV7 at 2 and 4 months; 2+1 doses of PCV7 at 2, 4 and 11 months or no dosage until 24 months (controls). Nasopharyngeal swabs were cultured for *S. pneumoniae* at age 6 weeks, and at 6, 12, 18 and 24 months. IgG antibodies against PCV7 serotypes were determined by ELISA.

Results

Lower serotype-specific IgG responses upon PCV7 challenge at 24 months of age were found in both previously vaccinated groups. Relative antibody decreases were found in case of previous colonization for serotype 6B (0.31; 95% CI 0.19-0.50; p<0.0001), 19F (0.65; 95% CI 0.43-0.97; p=0.035), and 23F (0.30; 95% CI 0.19-0.47; p<0.0001) compared with non-carriers, both groups pooled together. Furthermore, lower non-homologous responses were observed.

Conclusions

Pneumococcal colonization up to the second year of life is associated with diminished immune-responses to PCV7. Underlying mechanisms deserve further investigation.

Introduction

Streptococcus pneumoniae is a major cause of pneumonia, bacteremia and meningitis, especially in children in the first years of life.[1]. Pneumococcus is a polysaccharide encapsulated bacterium and a common but transient colonizer of the human nasopharynx. In young children asymptomatic nasopharyngeal carriage rates may be as high as 70-80%.[2] Pneumococcal capsular polysaccharides are poorly immunogenic in infants up till 2 years of age and the 23-valent pneumococcal polysaccharide vaccine failed to show efficacy against invasive diseases in most clinical trials in children.[3] By recruitment of CD4+ T-helper cells, pneumococcal polysaccharide-protein-conjugate vaccines proved to be immunogenic in infants as early as a few weeks of age.[4] Clinically, the 7-valent pneumococcal conjugate vaccine (PCV7) showed over 90% protection against vaccine serotype invasive pneumococcal disease (IPD) and to a lesser extent protection against pneumococcal respiratory disease and nasopharyngeal acquisition by vaccine serotypes.[2;5] IPD in infants has been reported to cause serotype-specific and non serotype-specific hyporesponsiveness to later vaccination with PCV7 [6]. Also repeated immunization with bacterial polysaccharide vaccines has been associated with immune hyporesponsiveness to polysaccharide antigens.[3] Recently, nasopharyngeal carriage of the most commonly carried pneumococcal serotypes shortly before administration of the primary series in infants was also reported to result in serotype-specific hyporesponsiveness towards different pneumococcal conjugate vaccines.[7;8]

In this report we describe that even after the primary series with PCV7, subsequent carriage with pneumococci in first 2 years of life has impact on later immune responses to a PCV7 challenge at 24 months of age, resulting in lower homologous, but also non-homologous IgG antibody responses.

Methods

Study design

Between July 2005 and February 2006, 1005 infants were enrolled in a randomized controlled trial investigating the effects of reduced-dose PCV7 schedules on pneumococcal carriage during the first two years of life (NCT00189020).[2] Healthy infants were randomly allocated to receive various vaccination schedules, (1) PCV7 at 2 and 4 months of age (2-dose group; n = 333), (2) PCV7 at 2 and 4 months followed by a booster dose at 11 months of age (2+1-dose group; n = 336) or (3) no PCV7 (control group; n = 336). At 24 months of age, participants of all three study groups were offered a PCV7 immunization on voluntary basis. Exclusion criteria for the study were known immunodeficiency, craniofacial or chromosomal abnormalities, language barrier, or expected relocation within the follow-up period. The study vaccine was the 7-valent CRM197-conjugated pneumococcal vaccine (Prevenar™ Pfizer). The study was approved by a national medical ethics committee. Informed consent was obtained from the parents or guardians of all study participants.

Measurements

Nasopharyngeal swabs were taken at the age of 6 weeks before first PCV7 vaccination, and at 6, 12, 18 and 24 months of age. The last swab was collected immediately before PCV7 challenge vaccination. Identification of *S. pneumoniae* nasopharyngeal carriage was based single colony morphology and conventional methods of determination, as described earlier [2]. Blood samples were obtained at 24 months of age from approximately 80 children per study group before challenge PCV7 vaccination and from approximately 80 children per group 28-42 days after challenge. Serum IgG antibody levels to the 7 vaccine serotypes were determined by double adsorption ELISA with cell wall polysaccharide and 22F polysaccharide, as described before.[9] In all measurements the operator was blinded of the study group of samples being processed.

Statistical Analysis

Pre- and post-challenge antibody levels are expressed as geometric mean concentration (GMC) with 95% confidence intervals (95% CI). For analyses of potential correlations between carriage and consecutive antibody responses to PCV7, we focused on the 3 most frequently carried vaccine serotypes in the study population: 6B, 19F and 23F. Infants were defined as prior carriers when positive for that serotype at 6 weeks or 6, 12 18 and 24 months of age. The children with serotype-negative nasopharyngeal samples up to 24 months of age were defined as non-carriers. Statistical differences in IgG GMC values for the serotypes 6B, 19F and 23F were assessed by log transformed unpaired t test when >5 blood samples were available per group. With infants primed at 2 and 4 months pooled together, a multivariable linear regression model was used to assess the relationship between the different moments of carriage (6 weeks, 6, 12, 18 or 24 months) and log-transformed IgG antibody levels, and were adjusted for vaccination group (2-dose and 2+1-dose schedule) and prior carriage of non-homologous serotypes. All reported p-values are 2-sided, p-values smaller than 0.05 were considered significant. Analyses were performed with SPSS 15.0.

Results

Study subjects

Blood samples were collected from 231 infants before and from 230 children 28-42 days after the PCV7 challenge, respectively. The cumulative proportion of children with pneumococcal carriage during the total study period was found 91% (418/461) of the children, with carriage prevalence ranging with age from 16% at 6 weeks to 67% at 18 months. Serotype 6B carriage was found in 109 (24%) infants in one or more consecutive nasopharyngeal samples, followed by serotype 19F in 96 (21%) subjects and serotype 23F in 78 (17%) infants. No major differences in demographic characteristics and risk factors for pneumococcal carriage were found between children in the main trial and the immunogenicity subset.[2]

IgG antibody levels before challenge at 24 months

Before the PCV7 challenge at 24 months of age, no differences in homologous IgG GMC values could be observed between non-carriers and prior carriers in both the 2-dose and 2+1-dose group (Table 1). In unvaccinated controls, prior carriage of the serotypes 19F and 23F led to higher homologous antibody levels at 24 months compared with non-carriers (Table 1).

IgG antibody responses after PCV7 challenge at 24 months

Overall, higher post-challenge responses were observed in the PCV7 primed groups compared to infants who had a first PCV7 at 24 months. After the PCV7 challenge, prior carriage resulted in lower homologous GMC values for serotype 6B, 19F and 23F in the 2-dose group and for serotype 6B in the 2+1-dose group compared with non-carriers. In the controls lower homologous GMC values were observed for serotype 23F in prior carriers after a first PCV7 at 24 months (Table 1).

No significant differences were observed in post-challenge antibody levels between the 2-dose and 2+1-dose group. With the 2-dose and 2+1-dose group pooled, prior homologous carriage was associated with lower IgG challenge responses: relative difference compared with non-carriers was 0.31 (95% CI 0.19-0.50; p<0.0001) for serotype 6B, 0.65 (95% CI 0.43-0.97; p=0.035) for serotype 19F and 0.30 (95% CI 0.19-0.47; p<0.0001) for serotype 23F. When corrected for different moments of pneumococcal carriage, inverse relations could be shown for serotype-specific carriage at most sample moments and later IgG challenge responses (Table 2).

Moreover, with the 2-dose and 2+1-dose group pooled together, also non-serotype-specific inhibition of IgG responses was observed. Prior 19F carriage was associated with lower post-challenge serotype 6B IgG responses (0.51; 95% CI 0.29-0.89; p=0.02) as compared to non-carriers. Prior 23F carriage was associated with lower serotype 19F post-challenge responses (0.60; 95% CI 0.38-0.85; p=0.007).

Table 1. Homologous serotype-specific IgG antibody responses after prior pneumococcal nasopharyngeal carriage of the serotypes 6B, 19F and 23F before and after PCV7 challenge vaccination in infants 24 months of age.

	Non-carriers [a] GMC (95% CI)	Prior carriers [b] GMC (95% CI)	No. non-carriers/ prior carriers	p-value
Before PCV7 challenge at 24 months				
Serotype 6B				
2-dose group	0.68 (0.46-0.99)	1.23 (0.63-2.45)	60 / 20	0.13
2+1-dose group	1.10 (0.80-1.54)	1.03 (0.43-2.45)	61 / 13	0.88
Controls	0.22 (0.18-0.26)	0.33 (0.18-0.62)	63 / 10	0.10
Serotype 19F				
2-dose group	1.40 (1.01-1.93)	1.99 (1.25-3.17)	70 / 10	0.43
2+1-dose group	1.84 (1.38-2.45)	1.78 (1.08-2.95)	62 / 12	0.93
Controls	**0.76 (0.58-1.00)**	**1.31 (0.99-1.72)**	49 / 28	**0.01**
Serotype 23F				
2-dose group	0.40 (0.29-0.55)	0.46 (0.23-0.91)	72 / 8	0.78
2+1-dose group	0.59 (0.44-0.78)	0.47 (0.15-1.40)	67 / 7	0.63
Controls	**0.14 (0.12-0.17)**	**0.22 (0.14-0.35)**	55 / 22	**0.03**
Post-PCV7 challenge at 24 months				
Serotype 6B				
2-dose group	**8.85 (6.48-12.10)**	**2.59 (1.32-5.11)**	59 / 20	**<0.001**
2+1-dose group	**10.70 (8.09-14.17)**	**3.67 (1.63-8.27)**	55 / 16	**0.003**
Controls	0.62 (0.44-0.86)	0.49 (0.33-0.73)	54 / 26	0.44
Serotype 19F				
2-dose group	**7.02 (5.54-8.91)**	**3.63 (2.26-5.83)**	62 / 17	**0.01**
2+1-dose group	n.a.	n.a.		
Controls	1.95 (1.59-2.40)	2.08 (1.47-2.93)	56 / 24	0.75
Serotype 23F				
2-dose group	**6.24 (5.03-7.74)**	**1.24 (0.69-2.25)**	67 / 12	**<0.001**
2+1-dose group	5.40 (4.29-6.79)	2.95 (1.73-5.02)	63 / 8	0.08
Controls	**0.91 (0.64-1.29)**	**0.40 (0.23-0.67)**	59 / 21	**0.01**

n.a. not applicable, ≤5 samples/group [a] Non-carrier: given serotype not detected at any moment of sampling.
[b] Prior carrier: given serotype sampled at 6 weeks or at 6, 12, 18 or 24 months of age. GMC; Geometric Mean Concentration.
Significant differences are depicted in bold; Calculated using log transformed unpaired t test, p-values are 2-sided.

Table 2. Relationship between pneumococcal carriage at different time-points and homologous serotype specific IgG antibody responses after PCV7- challenge vaccination at 24 months of age. Children previously received a 2-dose or 2+1-dose schedule. Associations are presented in relative homologous antibody decreases/increases of serotype-specific carriers vs. children with sero-negative samples.

	Relative IgG antibody changes; post-PCV7 at 24 months					
	Serotype 6B		Serotype 19F		Serotype 23F	
Carriage homologous serotype at	ß	P-value	ß	P-value	ß	P-value
6 weeks	**0.07**	**0.03**	1.38	0.76	1.44	0.59
6 months	0.58	0.16	0.55	0.51	**0.18**	**0.001**
12 months	**0.29**	**0.001**	0.54	0.053	**0.35**	**0.003**
18 months	**0.42**	**0.02**	**0.40**	**0.03**	**0.30**	**0.002**
24 months; PCV7 challenge*	**0.38**	**0.02**	1.11	0.73	1.63	0.40
All sample moments	**0.31**	**<0.0001**	**0.65**	**0.03**	**0.30**	**<0.0001**

ß; relative antibody decrease (<1.0) / increase (>1.0) compared to children with serotype-negative samples for serotype 6B, 19F or 23F. Significant differences are depicted in bold. The effect of carriage at different sample moments on log-transformed antibody levels was adjusted for vaccination group (2-dose and 2+1-dose schedule) and different carriage moments of homologous and non-homologous serotypes. *nasopharyngeal swab taken concomitant with PCV7 challenge vaccination.

Discussion

Induction of serotype-specific anti-capsular IgG antibody responses has been shown after immunization with the 7-valent pneumococcal conjugate vaccine, but also after natural nasopharyngeal acquisition of the encapsulated pneumococcal serotypes.[10] Recently it was shown that pneumococcal carriage shortly before a first PCV administration at 6 weeks to 2 months of age was associated with serotype-specific hyporesponsiveness upon vaccination in Fillipinian and Bedouin/Jewish children.[7;8] The proposed underlying mechanism for this hyporesponsiveness was B cell exhaustion by circulating polysaccharide antigens shortly before vaccination, hampering activation and priming of B cells after PCV7 administration.[3;7]

We now describe that also after completing a primary 2-dose PCV7 schedule, subsequent nasopharyngeal pneumococcal carriage leads to diminished serotype-specific B cell antibody responses for the commonly carried serotypes 6B, 19F and 23F. Even carriage in the second year of life lowers IgG immune responses upon a challenge-PCV7 at 24 months of age. This is an interesting finding, since at the age of 24 months, the B cell compartment is considered to be more mature.(3) One potential explanation for the lower challenge responses would be that existing memory B cells differentiate into plasma cells upon polysaccharide exposure by carriage, but without replenishment of the memory compartment. In children with 2 or more serotype-positive swabs, even lower homologous responses were observed compared to children cultured only once for that given serotype (serotypes 6B and 23F; data not shown). The prolonged impact on B cell responses following exposure to pneumococcal polysaccharides was also found in a Fijian study in infants primed with PCV7 and boostered with a 23-valent pneumococcal polysaccharide

booster at 12 months of age.[11] This polysaccharide booster resulted in lower antibody responses to a small challenge dose of pneumococcal polysaccharides 5 months later, compared to children who had not received the booster.[11] Hyporesponsiveness to PCV7 has also been reported in several infants following IPD who received first vaccination more than 2 months after infection.[6]

In correspondence with the previous studies, the underlying mechanism appeared mostly capsule-specific, because most outspoken influence on vaccine responses were seen for the homologous serotype.[7;8] However, non-capsule specific influences cannot be excluded, since we found in this study that also previous pneumococcal carriage of non-homologous serotypes was also correlated with lower IgG challenge responses for serotypes 6B and 19F. The possibility of non-serotype dependent hyporesponsiveness to PCV7 has also been suggested by 2 infants following IPD with serotype 7F and 14, who were incapable of mounting a protective serotype-specific antibody response against serotype 6B.[6] IgG responses by the host to pneumococcal polysaccharides might be genetically controlled; SNPs in the alleles coding for IL-4 and IL-13 have been shown to induce lower PCV7 responses in infants with previous otitis media, although the exact mechanisms are still unknown.[12] But since in our study prior carriers before the challenge PCV7 at the age of 24 months showed similar or even higher antibody levels compared with non-carriers, a genetic inability to mount high antibody response to specific capsular polysaccharides is unlikely. One could hypothesize that since use of PCV7 in children is associated with disappearance of circulating vaccine strains in the community, eradication of commonly carried serotypes may lower serotype-specific vaccine responses due to a lack of booster effects. On the other hand, we now have shown that natural carriage may also diminish vaccine booster responses; therefore disappearance of circulating strains might also be beneficial to vaccine potency. It is therefore important to study the final impact of widespread vaccination on circulating antibody levels in children, also after the age of 2 years.

A limitation of this study is the limited number of samples which only allowed analyses for the 3 mostly carried vaccine serotypes. Hierarchy of carriage serotypes is partly predicted by polysaccharide structure and thickness of the capsular serotypes, which could also result in different host-pathogen interactions.[13] Furthermore, heavily encapsulated serotypes 6B, 19F and 23F are known to have longer carriage duration, possibly leading to more impact on immune responses as well as memory B cell development.[14;15] We thus do not know the influence of carriage on more immunogenic polysaccharide capsules or of those infrequently encountered in carriage like serotypes 1, 3, or 7F. Furthermore, because of the 6-months sampling interval we might easily have missed homologous serotype carriage in individuals. Moreover, single colony–method for serotyping may have resulted in missed multiple serotype carriage. Both can potentially bias the measured impact of carriage on the observed homologous and non-homologous IgG levels. The study concerns a post-hoc analysis with a limited number of samples. Therefore no firm conclusions can be drawn of the influence of carriage on each specific time point. However, the impact of carriage at 6, 12 and 18 months on later challenge responses was found consistently in all groups. Lastly, effects of early nasopharyngeal carriage before the first PCV7 administration at 6

weeks could not be calculated, because the observed carriage at this time point in our study population was very low (≤1% per serotype).

In conclusion, in infants already primed with PCV7 and colonized with pneumococcal vaccine serotypes 6B, 19F or 23F, lower homologous, but also non-homologous, IgG responses were observed after PCV7 challenge vaccination compared to non-carriers of these serotypes. How immunological mechanisms (capsule-specific or generalized non-capsule specific) and host-related factors influence the immune response to PCV7 needs further investigation.

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11

Impact of decreased nasopharyngeal colonization of vaccine-serotype *S. pneumoniae* on antibody persistence in children after introduction of 7-valent pneumococcal conjugate vaccination

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Abstract

IgG antibody levels towards vaccine-serotype pneumococci were compared between cohorts of 11- and 24-month-old children who received 7-valent pneumococcal conjugate vaccination (PCV7) before or after routine implementation. Coinciding with strongly decreased vaccine-serotype carriage, IgG-levels against serotypes 6B and 19F were significantly lower after PCV7-implementation.

Introduction

After licensure, 7-valent CRM197-conjugated pneumococcal vaccine (PCV7) has been implemented in most developed countries in a recommended scheme of 2 or 3 primary vaccinations before 6 months of age followed by a booster vaccination around or in the second year of life.[1] Besides direct protection against vaccine-serotype invasive pneumococcal disease (IPD), infant PCV7 vaccinations result in a reduction in nasopharyngeal colonization leading to a gradual eradication of circulating vaccine strains and subsequent herd effects in the unvaccinated population.[2;3]

Following nationwide introduction in the United Kingdom of other conjugate vaccines against *Haemophilus influenzae* type b (Hib) and *Neisseria meningitidis* serogroup C (MenC), a rapid decline in IgG antibody levels within one year after the primary series was observed in infants who did not receive a booster vaccination, accompanied with waning disease protection.[4] This has been attributed to loss of natural boosting of the immune system as a result of carriage eradication.[5] For this reason, many countries recommend a booster vaccination in the second year of life for MenC, Hib and also pneumococcal conjugate vaccines.[1] Data on maintenance of serotype-specific antibody levels against pneumococcal vaccine serotypes after herd effects are scarce.

In the Netherlands, PCV7 was implemented in the national immunization programme (NIP) for all newborns born after March 31, 2006 without a further catch-up program for older children. Infants were vaccinated at 2, 3 and 4 months of age followed by an 11-month booster vaccination. In a carriage surveillance study between February and July 2009, a very strong reduction in vaccine-serotype colonization in 11-month and 24-month-old children was shown with vaccine-serotype prevalence carriage rates of 4% - 8% (Spijkerman *et al*, submitted), together with herd effects in other age groups. We explored the impact of decreased circulation of vaccine serotypes on anticapsular IgG antibody levels in cohorts of children vaccinated before and 3 years after PCV7 introduction in the Netherlands, at 11 months of age before the booster vaccination, and 1 year after the PCV7 booster at 24 months of age.

Methods

Study design

Between February and July 2009, blood samples were collected from 11-month-old and 24-month-old children, having received PCV7 administrations in a 3+1-dose schedule. IgG antibody levels of pre-booster 11-month-old children in this herd period with strongly decreased natural circulation were compared with IgG levels of pre-booster children that had started primary immunizations in the first 3 months of PCV7 introduction in 2006 (ISRCTN97785537), with natural circulation of vaccine strains still present (pre-herd period).[3] IgG levels from the 24-month-old cohort in 2009 (i.e. herd-period) after a 3+1-dose schedule were compared with age-matched children born in 2005 who had received a 2+1-dose PCV7 schedule with 2 primary doses at 2 and 4 months and an 11-month

booster. These children participated in a randomized controlled trial on reduced-dose PCV7 schedules (NCT00189020) and reached the age of 24 months in winter 2007 before herd effects.[3] Written informed consent was obtained for all study participants. Studies were approved by a national ethics committee and undertaken in accordance with the European Statements for Good Clinical Practice, which includes the provisions of the Declaration of Helsinki of 1989.

Study vaccines and laboratory measurements

In all studies children were vaccinated with 7-valent CRM197-conjugated pneumococcal vaccine (Prevenar™, Wyeth/Pfizer), containing pneumococcal polysaccharides 4, 6B, 9V, 14, 18C, 19F and 23F), concomitantly with DTP-IPV-Hib (Infanrix-IPV-Hib™ or Pediacel™).[6] IgG antibody levels to the 7 vaccine pneumococcal polysaccharides were determined with double adsorption ELISA by the same staff in the same laboratory.[7]

Statistical analysis

IgG antibody levels are expressed in geometric mean concentration (GMC) with 95% confidence interval (95% CI). Statistical differences were assessed by log transformed unpaired t tests. Differences in percentages of subjects reaching threshold values were calculated using Fisher's exact test or chi-square when appropriate. Post-primary antibody levels ≥0.35 µg/ml are estimated as a threshold for protection against IPD and used for assessing non-inferiority of novel PCVs.[8] No threshold values have been defined for ages 1 and 2 years, but levels ≥1.0 µg/ml have been used as indication of protection.[7;9] All reported p-values are 2-sided, p-values <0.05 were considered significant. Analyses were performed with SPSS 15.0.

Results

Pre-booster IgG antibody levels

Twenty-eight pre-booster blood samples from 11-month-old children from the herd-period in 2009 were compared with 98 samples from children vaccinated in the pre-herd period in 2006 (Table). In 2009 lower IgG GMC values were observed for serotype 6B (0.18 vs. 0.40 µg/ml; p=0.004) compared to children vaccinated in 2006 (Table), as were proportions of children with antibody levels ≥0.35 µg/ml; 52% vs. 29% (p=0.028). In contrast, higher GMC values were found for serotypes 4 (0.69 vs. 0.30 µg/ml; p<0.001) and 18C (0.37 vs. 0.22 µg/ml; p=0.003), resulting in higher proportions of children with antibody levels ≥0.35 µg/ml for serotypes 4 (75% vs. 42%; p=0.002) and 18C (50% vs. 19%; p=0.001) in the herd period compared to the pre-herd period. For serotype 4 also higher proportions reached threshold levels ≥1.0 µg/ml (39% vs. 6%; p=0.001).

Table. Specific IgG antibody levels against vaccine serotypes before the booster dose and at 24 months of age in different PCV7-vaccinated cohorts before and after herd effects of nation-wide PCV7 implementation in the Netherlands.

	Pre-booster; GMC µg/ml (95% CI)			Age 24 months; GMC µg/ml (95% CI)		
	Pre-herd period	Herd period		Pre-herd period	Herd period	
	3-dose (n=98)	3-dose (n=28)	p-value*	2+1-dose (n=74)	3+1-dose (n=24)	p-value*
Serotype 4	**0.30** (0.26-0.34)	**0.69** (0.44-1.08)	**<0.001**	**0.26** (0.22-0.30)	**0.28** (0.19-0.41)	0.600
Serotype 6B	**0.40** (0.31-0.49)	**0.18** (0.10-0.33)	**0.004**	**1.09** (0.80-1.48)	**0.35** (0.19-0.62)	**0.001**
Serotype 9V	**0.31** (0.27-0.36)	**0.35** (0.26-0.46)	0.551	**0.30** (0.24-0.37)	**0.48** (0.33-0.71)	**0.035**
Serotype 14	**1.56** (1.23-1.81)	**1.03** (0.73-1.46)	0.053	**1.41** (1.15-1.71)	**1.25** (0.81-1.93)	0.584
Serotype 18C	**0.22** (0.18-0.25)	**0.37** (0.27-0.50)	**0.003**	**0.24** (0.20-0.29)	**0.37** (0.24-0.58)	**0.029**
Serotype 19F	**0.96** (0.74-1.24)	**0.75** (0.41-1.38)	0.399	**1.83** (1.42-2.36)	**0.70** (0.39-1.26)	**0.001**
Serotype 23F	**0.22** (0.18-0.26)	**0.26** (0.18-0.37)	0.353	**0.57** (0.44-0.76)	**0.72** (0.46-1.10)	0.433
Date of Birth	March-July 2006	May-Aug 2008		May-Nov 2005	March-June 2007	
Period of sampling	March-June 2007	April-July 2009		May 2007-Jan 2008	March-June 2009	
Age at blood drawing, months (SD)	12.1 (0.6)	11.0 (0.3)		24.0 (0.4)	24.2 (0.3)	

GMC; Geometric mean concentration. *p-value pre-herd vs. herd period. Calculated using log transformed unpaired t test, p-values are 2 sided. Significant differences (*p*-Values <0.05) in IgG antibody levels are depicted in bold, highest levels are underlined.

IgG antibody levels at the age of 24 months

Twenty-four samples from 24-month-old children vaccinated with a 3+1-dose schedule in 2009 were compared with 74 samples from children in the pre-herd period that had received a 2+1-dose schedule (Table). Despite more PCV7 vaccinations, 24-month-old children in 2009 showed lower IgG GMC values for serotypes 6B (0.35 vs. 1.09 µg/ml; p=0.001) and 19F (0.70 vs. 1.83 µg/ml; p=0.001) compared with children born in the pre-herd period that had received a reduced 2+1-dose schedule (Table). Likewise, the percentages of children with GMC ≥0.35 µg/ml at 24 months were lower for serotype 6B (54% vs. 84%; p=0.003) and serotype 19F (63% vs. 96%; p<0.001), as were the proportions of children reaching IgG levels ≥1.0 µg/ml: serotype 6B (13% vs. 45%; p=0.006) and 19F (29% vs. 70%; p<0.001) in the herd- vs. pre-herd period. In contrast, higher GMC values were found for serotypes 9V (0.48 vs. 0.30 µg/ml; p=0.035) and 18C (0.37 vs. 0.24 µg/ml; p=0.029). Subsequently, higher proportions reaching ≥0.35 µg/ml were found for serotype 9V (58% vs. 31%; p=0.017), as well as ≥1.0 µg/ml threshold for serotypes 4 (13% vs. 1%; p=0.044) and 9V (29% vs. 9%; 0=0.017).

Discussion

In correspondence with previous experience after conjugate vaccine implementation for Hib and MenC, we now found lower antibody levels after PCV7 implementation before administration of the booster dose for serotype 6B at 11 months and for serotypes 6B and 19F at 24 months, compared to children vaccinated before decreased carriage of vaccine serotypes following PCV7 implementation. Serotypes 6B and 19F are both shown to be among the least immunogenic serotypes in the CRM197-conjugated 7-valent pneumococcal vaccine and were most frequently found in carriage studies in the Netherlands before PCV7 implementation.[3;7] At 24 months of age IgG levels against serotype 19F in the herd period were even had reduced to levels of unvaccinated children in the pre-herd era (data not shown). This suggests that serotype-specific IgG levels against these frequently carried serotypes heavily depend on natural contact by vaccine-serotype carriage and that loss of circulation of these serotypes result in lower antibody levels.

However, opposite to the lower IgG levels of these frequently colonizing serotypes, serotypes 4, 9V and 18C showed higher IgG antibody levels after PCV7 implementation. Since in the pre-herd period the 24-month-old children were vaccinated with a reduced-dose schedule, this may contribute to the difference observed in antibody levels, although for the serotype 4 and 9V no inferiority of a 2+1-dose schedule compared to a 3+1-dose schedule has been observed.[7;9] Diminished, serotype-specific vaccine responses have been reported after primary series when children had been colonized with a homologous serotype before start of PCV7 vaccinations.[10] Possibly this B cell hyporesponsiveness resolved in the post-PCV7 era due to reduction of circulating vaccine strains like serotype 4, 9V and 18C.

The clinical consequences of lower IgG antibody levels for serotypes 6B and 19F need to be awaited. Decreased exposure to vaccine-serotype pneumococci will obviously reduce the risk of infection by vaccine strains. Up till now, vaccine failures are become scarce to absent in the Netherlands.[2] Furthermore, antibody levels for clinical protection seem serotype-dependent and low 6B levels were shown to be highly effective in carriage and disease reduction.[3;11] Functional antibody activity is also determined by other antibody characteristics like affinity and complement susceptibility and natural clinical protection against IPD and carriage may not depend on anti-capsular antibodies alone.[12;13] However, in the future antibody persistence against *S. pneumoniae* should be closely monitored in relation to disease also in older age groups. Our study also shows that altered immune responses can complicate the interpretation of immunogenicity studies with novel PCVs that are performed in countries well after herd effects of PCV7 implementation.

This report is meant to be a first alert of potential implications of eradication of pneumococcal vaccine serotypes. The results should however be interpreted with caution. Because of the small sample sizes in the herd period minor differences with the pre-herd cohorts might have been missed. Also there is the risk of potential confounders. First cohorts received different lot numbers of PCV7. Second, the 24-month-old children in the pre-herd period were partly primed with another acellular DTaP-IPV-Hib vaccine.[6]

However, pertussis booster was identical and a study assessing the compatibility of PCV7 with acellular pertussis vaccine showed no differences in vaccine responses between groups receiving the vaccines concomitantly or separately.[14] Also differences in antibody levels were consistently found for both pre-booster and 24-month-old cohorts. Third, pre-booster blood samples in the herd period were collected at a slightly younger age than in the pre-herd period. Therefore the lower pre-booster antibody levels found for serotype 6B can be underestimated.

In conclusion, we observe different kinetics of antibody persistence after nationwide PCV7 implementation, probably due to decreased natural vaccine-serotype circulation. Clinical consequences should be awaited. Currently used non-inferiority threshold levels may not be adequate after nationwide PCV implementation. Next to disease-surveillance, also sero-surveillance seems mandatory.

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12

B cell memory in childhood after 3 primary doses with the 7-valent pneumococcal conjugate vaccine after nationwide introduction

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Abstract

Background

Following nationwide implementation of the 7-valent pneumococcal conjugate vaccine (PCV7), we found decreased circulation of vaccine strains to result in lower antibody levels for serotypes 6B and 19F. As sustained protection may depend on antibody persistence and immunological memory, we assessed the memory B cell responses in children of 11 and 24 months of age following nationwide PCv7 implementation.

Methods

All children had received PCV-7 in a 3+1-dose schedule following the national immunization programme in the Netherlands. Blood samples were taken before or 7-9 days after the scheduled PCV7 booster at 11 months or before or after an additional PCV7 challenge at 24 months of age. IgG antibody levels were measured against all 7 vaccine serotypes by ELISA. Plasma and memory B cells were measured by ELISPOT for serotypes 6B, 14, 19F and 23F.

Results

Despite low antibody levels against serotypes 6B, 19F and 23F, a high proportion of participants showed serotype-specific plasma and memory B cells after the booster PCV7 at 11 months. At 24 months of age, after the PCV7-challenge, in all participants serotype-specific plasma/memory B cell frequencies were detected for all tested serotypes. Correlations between post-challenge B cell frequencies and IgG responses could be shown for all serotypes. Compared to age-matched children born in 2005 with natural circulation present, the 24-month-old cohort showed comparable post-challenge memory responses for the serotypes 6B and 19F.

Conclusions

Although antibody levels declined rapidly for the serotypes 6B and 19F without natural boosting by vaccine-strains, PCV7 induced proper B cell memory. Consequences of waning antibody levels for long term clinical protection should be awaited.

Introduction

The CRM197-conjugated 7-valent pneumococcal vaccine (PCV7) has shown to be effective against invasive pneumococcal disease (IPD) after a 3+1-dose vaccination schedule with 3 primary doses and a booster dose as well as after a reduced 2+1-dose schedule.[1-4] Besides providing direct protection in vaccinees, nationwide PCV7 immunization of the youngest age-categories resulted in decreased natural circulation of vaccine-type pneumococci and subsequent indirect protection for the unvaccinated populations (herd effects).[5-8] In the Netherlands, PCV7 was implemented in the national immunization programme (NIP) for all newborns born after March 31, 2006 without a further catch-up program for older children. Infants were vaccinated at 2, 3 and 4 months of age followed by an 11-month booster vaccination. Recently, we observed lower IgG antibody levels against serotypes 6B and 19F at the age of 11 or 24 months in PCV7-vaccinated children after nationwide PCV7 implementation compared to age-matched children vaccinated before herd effects (Chapter 11). This was in correspondence with previous experience after conjugate vaccine implementation for *Haemophilus influenzae* type b (Hib) and *Neisseria meningitidis* serogroup C (MenC).[9;10] In the UK decreased exposure by vaccine strains after nationwide introduction of these conjugate vaccines resulted in waning antibody levels rapidly after the primary series and decreased clinical protection.[9;10] For this reason, a booster injection around 12 months of age for both MenC and Hib conjugate vaccines was implemented in the UK in 2006 to prevent breakthrough cases in the second year of life [11]. Besides boosting of serum antibodies by vaccination or exposure to naturally circulating antigens, serotype-specific plasma and memory B cells may contribute to long-term immunity.[12] With the loss of natural boosting, serotype-specific protection may become more dependent on vaccine-induced memory, since correlations between memory B cells and antibody maintenance have been described.[13] Long-term antibody persistence is thought to occur via differentiation of memory B cells into serotype-specific antibody-producing plasma cells.[14] Most of the plasma and memory B cell compartment is assumed to be hosted in the spleen and bone marrow niches.[15] Therefore, administration of a polysaccharide or conjugate vaccine can be used to evaluated memory by the height of the serum IgG antibody increase and detection of plasma and memory B cells shortly after immunization.[14;16;17] In this report we described the serotype-specific memory B cell compartment of the vaccinated cohorts with waning antibody levels against serotypes 6B and 19F after PCV7 implementation. Also we compared memory B cell frequencies and IgG responses of the 24-month-old cohort with post-challenge responses of an age-matched cohort vaccinated before herd effects.

Methods and Materials

Study participants

We collected blood samples from children 11 and 24 months of age between February and July 2009, 3 years after nationwide PCV7 implementation in the Netherlands. All children

were vaccinated with PCV7 according to the national immunization program (NIP). Blood was drawn from: healthy 11-month-old children before or 7-9 days after the 11-month-booster and healthy 24-month-old children before or 7-9 days after an additional PCV7 challenge dose. The participants were born between April-August 2008 (11 months old) and March-June 2007 (24 months old). Eight ml blood samples were collected using 4 ml Vacutainer cell preparation tubes (CPT; Becton Dickinson). Exclusion criteria for children were (1) acute fever (>38.5 °C), (2) administration of plasma products within three months of study enrolment, (3) presence of a known or suspected immunological disorder, (4) bleeding disorders, (5) allergy/hypersensitivity against one of the vaccine ingredients, (6) presence of a serious disease that requires medical care that could interfere with the results of the study, (7) administration of other pneumococcal vaccines or another PCV7 schedule then Dutch NIP. Written informed consent was obtained for all study participants. The study was approved by a national medical ethics committee (Central Committee on Research Involving Human Subjects, www.CCMO.nl) and undertaken in accordance with the European Statements for Good Clinical Practice, which includes the provisions of the Declaration of Helsinki of 1989. In all laboratory measurements the operator was blinded. Data of the post-challenge 24-month-old vaccinated cohort were compared with post-challenge data from age-matched children born in 2005, who had received a reduced 2+1-dose PCV7 schedule with doses at 2 and 4 months and an 11-month booster followed by a 24-month PCV7 challenge vaccination. These children participated in a randomized controlled trial on reduced-dose PCV7 schedules (NCT00189020) and reached the age of 24 months in winter 2007 before herd effects had set in.[7]

Study vaccines

All infants received the 7-valent CRM197-conjugated pneumococcal vaccine (Prevenar™ Wyeth), containing serotypes 4, 6B, 9V, 14, 18C, 19F and 23F, according to the Dutch NIP. The PCV7 challenge vaccinations at 24 months were administered during a home visit by authorized medical staff.

Measurement of IgG antibodies

Plasma was stored at -80°C after density gradient centrifugation. Plasma IgG antibody levels were measured to all 7 vaccine pneumococcal polysaccharides by ELISA using double absorption as described before.[18]

Preparation of PBMCs

Specific B cell frequencies were measured for the serotypes 6B, 14, 19F and 23F. PBMCs were isolated by density gradient centrifugation. Washes were done using PBS + 5% fetal calf serum (FCS; HyClone). PBMCs were resuspended in AIM-V medium containing 10% FCS and supplemented with penicillin (100 U/ml), streptomycin (100 µg/ml) and L-glutamine (200nM) (Gibco BRL).

B cell stimulation in vitro

For the indirect ELISPOT, PBMCs were resuspended and cultured at a concentration of $2x10^6$ cells/ml in AIM-V culture medium in 24-wells plates. PBMCs were stimulated polyclonally with 3 µg/ml CpG-C, PTO modified (5'-TCG TCG TCG TTC GAA CGA CGT TGA T-3') (Isogen) in the presence of 10 ng/ml IL-2 (Strathmann), 10 ng/ml IL-10 (Calbiochem) and 2 ng/ml polysaccharides 6B, 14, 19F and 23F (Statens Serum Institute) for 5 days at 37˚C and 5% CO_2. Cells were harvested by centrifugation, washed with culture medium and tested in antigen-specific ELISPOT assays.

ELISPOT assay

Multiscreen Filtration plates were pre-incubated with 35% ethanol for 1 minute, washed and coated with 100 µl PBS containing either 10 µg/ml goat-anti human IgG (SBA), 25 µg/ml polysaccharides 6B, 14, 19F or 23F or PBS only as negative control. All plates were incubated at 4˚C overnight, washed and blocked for at least 30 minutes with PBS containing 5% FCS. Afterwards 3-fold dilutions of the PBMCs suspensions (Direct ELISPOT, plasma B cells) were added to the plates at a starting concentration of $3x10^5$ cells/well, or stimulated PBMCs (Indirect ELISPOT, memory B cells) at a starting concentration of at least $0.5x10^5$ cells/well up to $2x10^5$ cells/well in AIM-V culture medium and incubated overnight at 37˚C and 5% CO_2. After washing with 0.05% Tween 20/PBS, plates were incubated with alkaline phosphatise (AP)-labelled goat-anti human IgG (1:5000) (SBA) for 2-4 hrs at 37˚C. After washes (last wash in PBS), plates were incubated with 50 µl substrate solution (1 mM 5-bromo-4-chloro-3-indolyl phosphate in H_2O; Sigma) for 30-60 minutes. Reaction was stopped by washing and dried. Plaques appearing as blue spots were measured as antigen-secreting cells by using an ELISPOT reader and software (CTL Europe).

Statistical Analysis

B cell frequencies are shown in medians and were compared using non-parametric Mann-Whitney test. Results of antibody levels are expressed in geometric mean concentration (GMC) with 95% confidence interval (95% CI). Statistical differences IgG GMC values were assessed by log transformed unpaired t test. Correlations between IgG levels and circulating B cells were assessed by Spearman correlation. All reported p-values are 2-sided, p-values smaller than 0.05 were considered significant. Analyses were performed with SPSS 15.0 and PRISM4.

Results

All analyses (measurements of plasma and memory B cells and IgG antibody levels) were performed in 103 of the 129 (80%) participants. No major differences in baseline characteristics (gender, age at PCV7 administrations, number of siblings, day-care attendance) were found between the cohorts before and after vaccination (Supporting information).

Serotype-specific antibody and B cell responses in 11-month-old infants

At the age of 11 months, before the booster-vaccination, lowest antibody levels were observed for serotype 6B (0.18 µg/ml) and 23F (0.26 µg/ml) (Table 1). Pre-booster circulating plasma and memory B cells were low at 11 months for all tested serotypes (Figure 1). Seven to 9 days after the administration of the booster dose at 11 months of age, IgG GMC values against all serotypes increased significantly compared to pre-booster values. Also circulating plasma and memory B cells increased for all serotypes, with the exception of serotype 19F (plasma B cells). Serotype-specific memory B cell responses could be observed in 83-92% of the participants. Lowest B cell frequencies were found for serotype 19F (Figure 1).

Table 1. IgG antibody levels (GMC) in infants at 11 and 24 months of age after 3 primary doses before and 7-9 days after a booster or challenge PCV7.

	11 months		24 months		p-values 11 vs. 24 months	
Serotype	Pre-booster n=28	Post-booster n=31	Pre-challenge n=24	Post-challenge n=27	Pre-PCV7	Post-PCV7
4	0.69 (0.44-1.08)	10.84 (7.80-15.07)	0.28 (0.19-0.41)	16.24 (11.22-23.52)	0.005	0.114
6B	0.18 (0.10-0.33)	6.23 (3.45-11.25)	0.35 (0.19-0.62)	22.11 (14.83-32.94)	0.140	0.001
9V	0.35 (0.26-0.46)	7.04 (5.07-9.79)	0.48 (0.33-0.71)	11.06 (8.51-14.38)	0.170	0.044
14	1.03 (0.73-1.46)	18.87 (13.30-26.79)	1.25 (0.81-1.93)	19.97 (13.02-30.62)	NS	NS
18C	0.37 (0.27-0.50)	8.55 (6.74-10.84)	0.37 (0.24-0.58)	12.16 (9.25-16.00)	NS	0.061
19F	0.75 (0.41-1.38)	4.17 (3.04-5.69)	0.70 (0.39-1.26)	7.73 (5.12-11.68)	NS	0.021
23F	0.26 (0.18-0.37)	12.29 (9.08-16.64)	0.72 (0.46-1.10)	24.02 (16.43-35.11)	0.001	0.008

Pre-PCV7; blood drawing before PCV7 administration, Post-PCV7; blood drawing 7-9 days after PCV7 booster or challenge.
Significant p-values (<0.05) are depicted in bold; calculated by Mann Whitney U test, p-values are 2 sided.

Serotype-specific antibody and B cell responses in 24-month-old infants

Before the challenge vaccination at the age of 24 months of age lowest IgG GMC values were observed for serotype 4 (0.28 µg/ml) (Table 1), with highest pre-challenge B cell frequencies for serotype 6B and 23F (Figure 1). After the challenge vaccination at 24 months IgG GMC values of all serotypes increased significantly. Highest challenge responses were observed for serotype 6B (22.11 µg/ml) and 23F (24.02 µg/ml). Also circulating B cells increased after the challenge vaccination for all serotypes, with again highest frequencies for serotype 6B and 23F. In all children specific memory B cells against the 4 tested serotypes could be detected.

In comparison to pre-vaccination frequencies at the age of 11 months, pre-challenge at 24 months higher number of circulating memory B cells were observed for the serotypes 6B (p=0.001), 14 (p=0.037) and 19F (p=0.053) (Figure 1). After challenge vaccination, IgG GMC values for all serotypes were higher at 24 months compared to the post-booster at 11 months of age (Table 1), except for serotype 14. Also higher serotype-specific plasma B cell responses were found for all tested serotypes after the 24-month challenge (Figure 1). With respect to memory B cells higher post-challenge responses were found at 24 months for serotype 6B (p=0.010), 19F (p<0.001) and 23F (p=0.054).

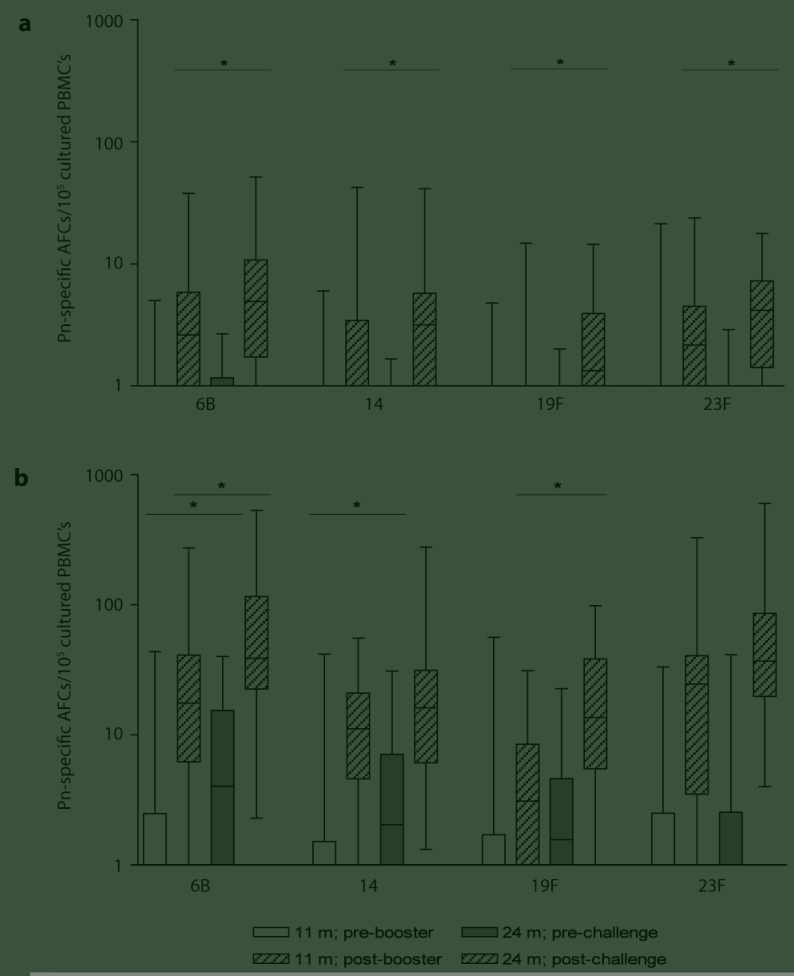

Figure 1. Plasma and Memory B Cell responses in infants at 11 and 24 months of age after 3 primary doses (1a) Serotype-specific plasma B cells; responses were measured by direct Elispot. (1b) Serotype-specific memory B cells; responses were measured by cultured Elispot before and 7-9 days after a booster or challenge PCV7 (medians). *Significant difference; p<0.05.

Correlations serum B cells – IgG antibody levels

Before PCV7 administration no correlations were found at 11 or 24 months of age between memory B cells, plasma B cells and IgG antibodies, except for serotype 19F (plasma B cells vs. IgG levels; r=0.48). After the booster at 11 months of age, correlations between IgG-levels and circulating B cells were found for serotype 6B and 23F. After the challenge-dose at the age of 24 months correlations could be observed for all 4 tested serotypes and were highest for serotype 6B (r=0.73 and r=0.44) and 14 (r=0.66 and r=0.71), IgG antibody levels vs. plasma B cells and memory B cells respectively.

Challenge responses at 24 months; herd period vs. pre-herd period

In 2007 (i.e. pre-herd period), 16 samples were collected 7-9 days after a PCV7 challenge vaccination from 24-month-old children that previously had received PCV7 in a 2+1-dose schedule at 2, 4 and 11 months. No significant differences were observed in post-challenge IgG responses for the serotypes 6B (22.11 vs. 15.42 µg/ml) and 19F (7.73 vs. 7.29 µg/ml) between children in 2009 after previous 3+1-doses or children in 2007 after previous 2+1-doses (Figure 2). Higher post-challenge IgG GMC values were observed for serotypes 4 (16.24 vs. 5.13 µg/ml; p<0.001), 9V (11.06 vs. 4.60 µg/ml; p<0.001), 18C (12.16 vs. 3.29 µg/ml; p<0.001) and 23F (24.02 vs. 8.50 µg/ml; p=0.002) in 2009 compared to 2007 respectively. Memory B cell frequencies were comparable 7-9 days after the challenge vaccination between the two periods for all 4 tested serotypes, despite the difference in number of previous vaccinations (Figure 2). Lowest responses were observed for serotype 19F (13.6 and 10.7 ASCs/10^5 cultured PBMCs) in both periods, 2009 and 2007 respectively.

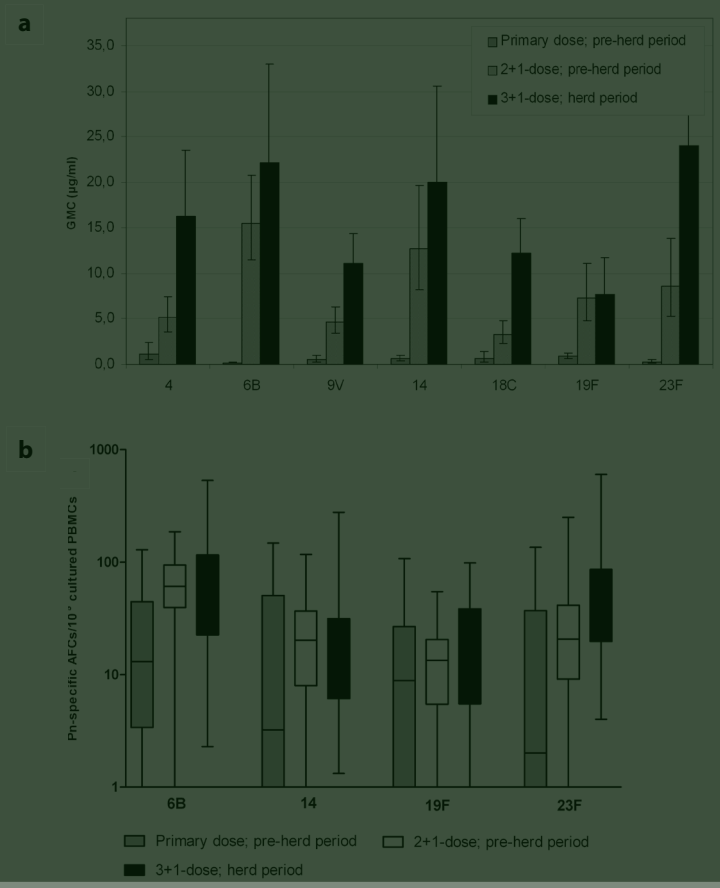

Figure 2. (a) Post-challenge IgG antibody responses in 24-month-old children after immunization with PCV7 schedules in the pre-herd and herd period (GMCs; blood samples taken 7-9 days after PCV7 challenge) (b) Post-challenge Memory B cell frequencies in 24-month-old children after immunization with PCV7 schedules in the pre-herd and herd period (medians).

Discussion

After nationwide implementation, vaccine-serotype disease is prevented in the population by (a) so called herd effects by decreased exposure to the pathogen and (b) the immunological protection of vaccinated individuals by functional circulating antibodies and the capacity to mount a proper memory response.[12] In the Netherlands similar herd effects following PCV7 implementation were observed as reported elsewhere.[19;20] In a carriage surveillance study between February and July 2009, a very strong reduction in vaccine-serotype carriage in 11-months and 24-months-old children was shown with vaccine-serotype colonization rates of 4% - 8% (Spijkerman et al, submitted). This decreased circulation of vaccine strains resulted in lower antibody levels before administration of the 11-month-booster dose for serotype 6B and at 24 months for serotypes 6B and 19F, compared to children vaccinated with a reduced-dose schedule before herd effects (Chapter 11).

Here we show that this decreased antibody persistence occurred despite the presence of the vaccine-induced memory induction. Compared to the pre-herd period, similar memory responses were observed for the serotypes 6B and 19F in 24-month-old children vaccinated in the herd period. Above finding suggest that serotype-specific IgG serum levels against serotype 6B and 19F heavily depend on natural contact by vaccine-serotype carriage, as antibody levels in the pre-vaccination era did not necessary reflected vaccine induced B-cell memory. Without natural circulation of vaccine strains we now showed relations between serotype-specific plasma and memory B cells and IgG antibody responses after PCV7 administration. Earlier it has been shown that without natural boosting after the booster dose for MenC and Hib, the antibody decay is quite similar for both antigen-specific IgG antibody levels and antibody maintenance seems primarily dependent of the magnitude of the booster-responses and the presence of B cell memory.[21] For the MenC conjugate vaccine post-primary IgG antibody levels and memory B cells are correlated with later antibody maintenance and the height of booster responses in the second year of life.[13] At 11 months of age, after 3+1-doses, children showed high post-booster antibody responses and in a high proportion of children memory B cells could be detected. After a full 3+1-dose schedule at 24 months the memory B cell pool increased further with higher challenge-responses and in all children detectable memory B cells against the 4 tested serotypes. Yet the ability to mount a proper memory response does not necessarily prevent infection, since the pace of pathogenesis after initial colonization is thought to be rapid.[22] First rises in serum plasma cells and antibody levels have been described not until 4-6 days after boosting.[13;23] Investigation of MenC and Hib vaccine failures confirmed that despite immune memory these children developed invasive disease.[24;25] However, also lower antibody avidity has been described in Hib-vaccine failures, which can indicate less immunological priming and memory B cell induction has occurred.[26]

At this moment data on serotype-specific long-term immunity after PCV7 administration is scarce. Until now, despite waning antibody levels, PCV7 vaccine breakthroughs are scarce in herd protected populations.[3;27] However, contribution of immunological memory, remaining circulating serum antibodies or herd effects in protection against pneumococcal disease is currently unknown. Interestingly, in the USA 40% of the cases

of breakthrough infections in infants having received ≥3 doses were serotype 19F[28], for which we observed lowest memory B cell responses. We earlier found a distinct pattern in booster and challenge-responses for serotype 19F with only a temporary rise in antibody-avidity suggesting different and less successful B cell priming or may be related to the composition of the vaccine (Chapter 7).[29]

If diminished antibody levels result in declined clinical protection, addition of booster doses at later ages may be required. This may be especially the case in resource poor countries where incidence- and mortality-rates of IPD remain high after infancy and waning immunity can have major consequences for vaccine effectiveness.[30;31] Here we show that at later age higher vaccine responses can be expected with better memory induction.[10] At 24 months repeated administrations and older age even resulted in highest responses for the low-immunogenic serotypes 6B and 23F. Another option would be to delay the booster-dose in the second year of life, since herd effects will provide protection through indirect effects in the period between primary dose and booster dose.[1] Clinically, in Australia, major reductions in vaccine-serotype IPD are observed in the vaccinated and unvaccinated population, after a 3 primary dose schedule without a booster dose, that was implemented with a catch-up campaign assuring rapid onset of herd effects.[32;33] Also the first post-implementation surveillance data from Canada confirm the clinical effectiveness of a late booster dose at 18 months after 3 primary doses.[34;35] However, long-term surveillance data are necessary to better assess the option of delaying the booster dose.

Some potential limitations should be addressed. Translation of our results to other countries or vaccine schedules is difficult since the age of the infants at the primary injections, number of primary doses, as well as other factors like concomitant childhood vaccinations or ethnic background variability may have impact on vaccine immunogenicity.[36;37] Also no longitudinal data were available to correlate pre-vaccination serum antibody levels with post-vaccination memory B cells and antibody responses. Although carriage surveillance-data showed strong reductions in circulating vaccine-serotypes in 2009, exposure to vaccine-strains in the first year of life cannot be excluded for the 24-month old children.

In summary, this study confirms the capability of a 3-dose primary schedule to induce B cell memory in vaccinated infants in the post-PCV7 era, also for the low-immunogenic serotypes 6B and 19F. However, antibody maintenance was limited and clinical consequences should be awaited. Until now, herd effects contribute to protection and vaccine failures are rare. Next to disease-surveillance, also sero-surveillance seems mandatory. If waning antibody levels result in declined clinical protection, administration of booster dose at later age may be necessary.

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Supporting information

Table. Baseline characteristics of the different cohorts aged 11 and 24 months.

	11 months		24 months	
	Pre-booster	Post-booster	Pre-challenge	Post-challenge
	n=36	n=35	n=25	n=29
Male, n (%)	22 (61%)	21 (60%)	11 (44%)	15 (52%)
Age at primary dose, months (SD)				
PCV1	2.1 (0.2)	2.0 (0.3)	2.1 (0.2)	2.1 (0.2)
PCV2	3.2 (0.3)	3.1 (0.3)	3.3 (0.4)	3.2 (0.3)
PCV3	4.4 (0.4)	4.3 (0.4)	4.5 (0.4)	4.3 (0.3)
Age at booster dose, months (SD)	-	11.1 (0.3)	11.3 (0.5)	11.4 (0.6)
Age at 24 month challenge, months (SD)	-	-	-	24.3 (0.2)
Age at blood drawing, months (SD)	11.0 (0.3)	11.3 (0.26)	24.2 (0.3)	24.5 (0.2)
No. of siblings, median (IQR)	0.5 (0-1)	2 (0-2)	1 (1-1)	1 (1-2)
Daycare attendance, n (%)	29 (81%)	27 (77%)	17 (68%)	20 (69%)
Tobacco smoke exposure indoors, n (%)	2 (6%)	1 (3%)	1 (4%)	1 (3%)

13

Summarizing discussion

Summarizing Discussion

The 7-valent pneumococcal conjugate vaccine (PCV7) has been introduced in the Netherlands in June 2006 in a 3+1-dose schedule with 3 primary doses at 2, 3 and 4 months of age followed by an 11-month booster vaccination. The aims of this thesis were: (1) To determine the burden of invasive pneumococcal disease (IPD) in the Netherlands before implementation of PCV7 in the national immunization program, and to evaluate the impact of PCV7 implementation on prevention of IPD in the first 2 years. (2) To compare antibody responses after a reduced 2+1-dose schedule with a full-dose 3+1-dose PCV7 schedule as currently introduced in the Netherlands. Furthermore to evaluate reduced-dose vaccination schedules on vaccine-serotype specific immune responses *i.e.* to determine antibody responses, B cell memory development, antibody functionality and mucosal antibody responses after 2 primary doses of PCV7 with or without a booster dose in young children. (3) To explore the effects of pneumococcal carriage on antibody responses and persistence following pneumococcal conjugate vaccinations in young children.

In this chapter the main findings of this thesis are summarized and discussed. Recommendations are presented concerning future vaccine policies, surveillance programs and future research.

Part 1. IPD in the Netherlands before and after implementation of PCV7

Vaccine serotype IPD

In this thesis we showed that in the years before nationwide PCV7 implementation in the Netherlands the burden of invasive disease caused by *S. pneumoniae* was high, especially in young children, elderly and immunocompromised patients (**Chapter 2**). In the Netherlands, around 2500 patients per year developed IPD which resulted in an estimated 8 deaths in children under the age of 2 years and approximately 375 deaths in the other age-groups within 30 days after IPD infection. In the first 2 years (June 2006 to June 2008) following nationwide PCV7 implementation for all newborns born after April 1, 2006, PCV7 immunizations resulted in a 67% reduction of vaccine serotype IPD in all children under the age of 2 years. A 90% reduction was observed in those children that were born after April 1, 2006 and thus age-eligible for PCV7 (**Chapter 3**).

This high 90% effectiveness against vaccine-serotype IPD is observed in all western countries where PCV7 has been implemented in a 3 primary dose schedule with or without a booster vaccination (Australia) around or in the second year of life.[1-5] At present more than half of the European countries have introduced reduced 2+1-dose schedules with 2 rather than 3 primary injections before the age of 6 months with a booster dose in the second year of life.[6;7] First surveillance reports also confirm high effectiveness of reduced-dose schedules that are similar to the impact of PCV7 in the Netherlands after a full-dose 3+1-dose schedule.[8-10] In the United Kingdom, a 79% reduction in vaccine serotype IPD was observed in all children under 2 years of age in the first 3 years after implementation of a 2+1-dose PCV7 schedule with a catch-up campaign for all children under the age of 2

years (Figure 1).[9] At present, in 2010, almost no cases of PCV7 vaccine-serotype IPD occur anymore in children under 2 years of age in the UK.[11]

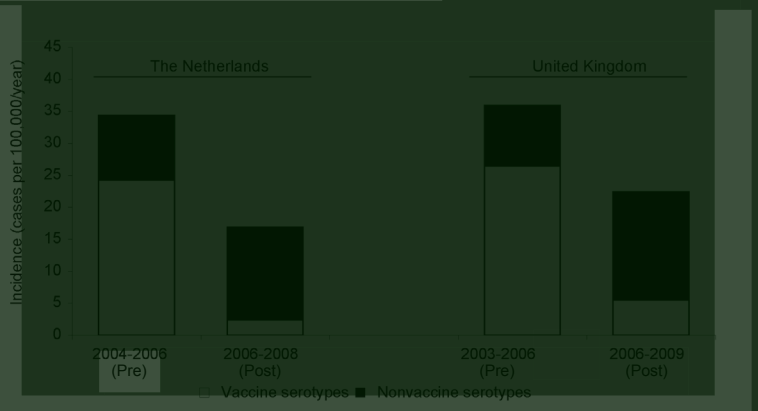

Figure 1. Incidences of invasive pneumococcal disease in children under 2 years of age before and afternationwide vaccine implementation in the Netherlands (3+1-dose schedule; 2 years pre- vs. post-implementation) and the United Kingdom (2+1-dose schedule with catch-up program; 3 years pre- vs. post-implementation. Adapted from Foster *et al*).[9]

Non-vaccine serotype IPD

In the Netherlands, the impact of PCV7 on IPD was partly offset by a 71% increase of non-vaccine serotype IPD, resulting in a net 44% reduction of overall IPD incidence in children age-eligible for PCV7 in the first 2 years post-implementation. These findings are very similar to those observed in other European countries as well. In the United Kingdom 3 years after nationwide PCV7 implementation, non-vaccine serotype IPD also had increased by 78% and resulted in an overall 38% reduction of IPD in children under 2 years of age (Figure 1).[9;12] There is much debate about the discrepancy between the net European effectiveness data and the more favorable results of PCV7 implementation reported in the United States. In the United States a 76% overall reduction in IPD is observed, due to the apparently far more limited increase of nonvaccine serotype IPD compared to Europe.[1] Factors like pre-implementation coverage of PCV7, genetic variability and strain-selection by antibiotic pressure might have affected effectiveness (Chapter 2). However, when looking more carefully at the surveillance data from the United States, only 31% of the children under the age of five years were hospitalized for IPD.[1] In European surveillance systems far lower incidence rates of childhood pneumococcal bacteremia are observed, since in Europe virtually all blood cultures are restricted to hospitalized children with much more severe disease. This explains the difference in pneumococcal bacteremia between Europe and the United States, whereas the incidence rates for meningitis are rather similar. The large proportion of non-hospitalized, outpatient cases of younger than 5 years of age in the United States showed no increase of nonvaccine serotypes. This has obscured the extent of emerging nonvaccine disease in the more severe and hospitalized cases constituting the patient population of most other countries' IPD surveillance. When IPD rates from only

hospitalized patients from the United States and Europe are compared, also in the United States a more European-alike picture of upcoming nonvaccine serotype IPD emerges: an 102% increase of nonvaccine serotype IPD that results in an overall ~60% reduction of IPD in hospitalized children under 5 years of age.[1] The absence of nonvaccine serotype increase in the outpatient children in the United States may be due to a change in blood culture practices with fewer cultures taken in children who are not critically ill and need not to be hospitalized. These data emphasize the need for detailed and high-quality surveillance systems for the coming years to better compare and predict public health benefits of the different pneumococcal vaccination programs in the long term. Surveillance data from short post-introduction periods like the 2 year post-implementation data in our study must be handled with caution. Trends in time before the introduction should be measured over longer periods since serotype distribution have been known to vary with time and differences or changes in clinical care and blood culture practices can influence results.[13-15]

Indirect vaccine effects and herd protection

Besides providing high protection against vaccine serotype pneumococcal disease in vaccinated children, nationwide PCV7 implementation in the United States lead to widespread reduction in vaccine serotype IPD also in non-immunized individuals, so called indirect effects or herd protection.[16-18] These indirect effects have been attributed to reduced carriage of vaccine serotype pneumococci in vaccinated infants that result in reduced transmission and therefore decreased spread of vaccine serotypes in the community. However, in the Netherlands, within the first 2 years after implementation, herd effects were not observed yet (**Chapter 3**). This was not unexpected since PCV7 was implemented in the Netherlands without a catch-up campaign for children born before April 1, 2006 which resulted in a relatively small vaccinated cohort (2.25%) compared to the total Dutch population.[19] Likely, a catch-up program in the Netherlands in 2006 for the ~385.000 unvaccinated children less than 2 years of age in this period would have resulted in a much earlier onset of herd effects in all other age categories. Most other countries in Europe implemented PCV7 for newborns together with a catch-up campaign for older children and confirmed early herd effects by lower vaccine serotype IPD incidence rates in non-vaccinated age groups as notified in the United States.[20] In United Kingdom and Norway after the implementation of a 2+1-dose schedule including a catch-up campaign, decreases in vaccine serotype IPD incidences in unvaccinated age-categories have been observed within 2 years after vaccine implementation.[9;21;22] The herd protection observed in these countries are in correspondence with results from our randomized controlled trial on pneumococcal carriage after reduced-dose schedules.[23] In this trial, we observed that after both a 2-dose PCV7 schedule and a 2+1-dose schedule, an adequate reduction of vaccine serotype carriage up to 60% was achieved at the age 24 months compared with unvaccinated controls, which is not different from the reported carriage reductions after 3+1-dose schedules.[23-25] Herd effects in other age groups are therefore expected to be similar after a reduced 2+1-dose schedule for infants.

Serotype-dependent disease characteristics and severity

By evaluating serotype-associated course of disease and outcome, we found that the rise of nonvaccine serotype IPD (e.g. serotypes 19A and 22F) is not expected to lead to a milder course of disease compared with the vaccine serotypes now included in the current PCV7 (**Chapters 4, 5**). We showed that the polysaccharide capsule that defines the serogroup or serotype appears to be associated with invasiveness and disease severity, which is in correspondence with several other epidemiological studies.[26;27] However, the high proportion of the 7 vaccine serotypes among pathogenic IPD isolates from children before PCV7 implementation, might suggest that these serotypes have certain advantageous characteristics for causing invasive disease compared with nonvaccine serotypes. Recently, it was shown by Weinberger and colleagues that pneumococcal strains expressing capsular polysaccharides that are metabolically less costly produce a thicker capsule, therefore be more resistant to opsonophagocytosis and more likely to dominate in carriage in children, like serotypes from serogroup 19 and 6.[28;29] Besides carriage hierarchy, these more heavily encapsulated serotypes also tend to cause more severe disease once they invade the bloodstream, as are nonvaccine serotypes like 6A/C, 9N and 19A.[26]

Part 2. Immune responses after reduced-dose schedules with PCV7 before nationwide implementation

Comparison of a primary 2-dose versus a 3-dose PCV7 schedule

Vaccine-induced systemic anticapsular IgG antibodies, which activate complement and enhance phagocytosis, are presumed to mediate protection against IPD.[30] For IPD an antibody level of 0.35 µg/ml as measured by a standardised ELISA one month after the primary series, is defined by WHO as post-primary correlate of protection assumed to be associated with clinical efficacy against IPD.[31] Later in infancy, around the age of the booster vaccinations, higher values like 1.0 µg/ml are used as possible correlate of protection.[32] Following a 2+1-dose or 3+1-dose schedule with the CRM197- conjugated 7-valent vaccine, we observed comparable IgG antibody responses after the booster dose at 11 months for 5 of the 7 vaccine serotypes. It has to be noticed that this study was performed in a period with vaccine serotypes still circulating in the population and possibly adding to natural priming and boosting of serotype-specific immune responses (Chapter 6). Furthermore we showed that high proportions of infants reached the antibody thresholds ≥0.35 µg/ml and ≥1.0 µg/ml after the booster dose following both schedules. The only exceptions were the 2 most frequently carried serotypes, *i.e.* serotype 6B that showed lower pre- and post-booster antibody IgG levels after 2 primary doses instead of 3 primary doses and 19F with somewhat lower post-booster antibody levels after a 2+1-dose schedule. Shortly after introduction of a reduced-dose schedule in the United Kingdom in 2006, several IPD cases by vaccine serotypes in the first year of life concerned these frequently encountered serotypes 6B and 19F.[10;33] Serotype 6B is a known low-immunogenic serotype and antibody levels against serotype 6B depend on the number of administrated doses.[32-34] However, the lower antibody levels found for serotype 6B might still be protective against disease,

as was shown for both IPD and acute otitis media (AOM).[35;36] In our carriage study we also observed for serotype 6B a dose-dependent decline in carriage.[23] However, despite lower IgG levels, after a 2+1-dose schedule an 80% decline in 6B carriage was achieved at 24 months of age compared with unvaccinated controls. This indicates that herd effect by strong carriage reductions can also be achieved by less doses and protection against disease will be realised as soon as the strain is eradicated from circulation in the population.[23] For serotype 19F it was already shown that high antibody levels were needed for prevention of acute otitis media with the current CRM197-conjugated vaccine.[36] This serotype accounted for ~30% of the PCV7-breakthrough infections in the United States after nationwide implementation.[37] In our study, significant carriage reduction of serotype 19F after PCV7 was observed only shortly after the booster vaccination at 11 months, when the highest antibody levels were present. However, in the United Kingdom IPD caused by serotypes 6B and 19F declined after herd effects had set in.[9;11]

Induction of long-term immunity

For long-term protection in vaccinated children, serum antibody maintenance and immunological memory seem mandatory, especially when diminished natural boosting is expected after nationwide vaccine implementation and herd effects.[38] Data on long-term protection after PCV7 schedules are scarce.[32;39;40] Also the effect of vaccine-serotype carriage eradication on immune responses are scarce to absent. Before herd effects and with vaccine serotypes still circulating, we found that in children with 2 primary doses before 6 months of age higher antibody levels at the age of 12 and 24 months are induced than in PCV7-unvaccinated controls. An 11-month booster dose resulted in higher IgG antibody levels at 12 months of age compared to 2 primary doses only. However, when comparing antibody levels at 24 months of age, the benefit of this booster dose had much diminished for most serotypes (**Chapters 7, 8**). Furthermore, 2 primary doses also induced a satisfying B cell memory response at 24 months of age. We observed a higher IgG antibody increase following a challenge vaccination compared to unvaccinated controls with higher antibody avidity and a higher number of memory B cells (**Chapter 7**).[31;41] Noteworthy, for the 11-month booster dose we did not observe an additional benefit on post-challenge antibody and memory B cell responses at 24 months for any vaccine serotype, except for serotype 6B. Probably after 2 primary doses already a certain upper threshold of primed B cells is reached for these serotypes and the level of antigen-specific B cells cannot increase further at that moment.[42;43] However, in case of serotype 6B, antibody responses seemed dose-dependent (**Chapter 6**)[32-34] and also the 11-month booster vaccination resulted in higher avidity indices and higher antibody responses 7-9 days after PCV7 challenge at 24 months compared to 2 doses without a booster. Also for serotype 19F a different pattern in booster and challenge-responses was observed (**Chapter 7**). Serotype 19F was the only vaccine serotype where already one month after the 11-month booster dose a rise in antibody avidity was observed. However, this difference was shown to be temporary At 24 months of age antibody avidity had decreased to similar levels as after 2 primary doses only. This suggests a different and less successful B cell priming for serotype 19F by PCV7.[44;45]

Functionality of antibody responses

At the moment, quantitative serotype-specific IgG responses as determined by a standardized ELISA are used as the primary tool to compare different pneumococcal conjugate vaccines.[31] However, the height of serotype-specific IgG antibody levels does not necessary reflect antibody functionality and is serotype dependent, as was recently shown for PCV7-induced antibodies against the vaccine-related serotype 19A.[46] Since anticapsular immunity in the host is thought to be mediated by opsonin-dependent phagocytosis, *in vitro* opsonophagocytic assays may be a more appropriate to estimate protection.[30] We showed that after PCV7 immunization quantitative IgG antibody levels are correlated to *in vitro* opsonophagocytic activity (OPA) (**Chapter 8**). Kinetics of OPA titers obeyed similar patterns as did IgG antibody levels. However, after PCV7 administration antibody levels waned rapidly and differences were observed between quantitative IgG GMC and functional OPA GMT values. After 2 primary doses no benefit in OPA titers could be proven for 4 vaccine serotypes at 12 months and 24 months compared to unvaccinated children, whereas there were significant differences in serotype-specific quantitative IgG levels. Also the 2+1-dose group showed waning OPA titers at 24 months, especially for serotypes 4, 9V and 19F. In addition, the antibody levels needed for 50% killing differed between the vaccine serotypes and lower antibody levels sufficed for *in vitro* killing for serotypes 18C and 23F, while higher levels were required for 4 and 19F.[29;36] We showed the IgG threshold ≥0.35 µg/ml underestimated participants reaching OPA threshold of ≥1:8 dilution for serotypes 18C and 23F, however overestimated participants with OPA titers ≥8 for serotypes 4, 9V and 19F. However, it should be noted that OPA responses can be influenced by other factors like other serotype specific antibody isotypes, like IgM and IgA antibodies. Both IgM and IgA have been reported to impact opsonic capacity of IgG antibodies.[47;48] Also antibodies to pneumococcal surface proteins and polyreactive antibodies may have had an influence on OPA titers since serum contains a wide variety of antibodies to different antigens.[49] Further investigation of different-dose schedules and the effect of decreased natural boosting on antibody functionality seems warranted.[50] However, when interpreting these results for clinical practise however we need to realise the limitation of the *in vitro* opsonophagocytic assay, which is dependent on many variable factors as used bacterial strains, complement and cell line.[30;51;52]

Mucosal responses

At the mucosal surface, anti-capsular IgA antibodies have been shown to support complement-dependent opsonophagocytosis, and agglutination of the pneumococcus.[53;54] IgA antibodies against pneumococcal surface proteins also have been described as major contributor in protection against mucosal disease.[55] Although several studies showed that systemic administration of PCVs also induce salivary IgG and IgA antibodies against pneumococcal serotypes, their role at the mucosal surface has still to be determined. We applied a highly sensitive, bead-based multiplex assay (LUMINEX)[56] that allowed us to determine salivary IgG and IgA antibody levels in PCV7 vaccinated and unvaccinated children in our randomized study (**Chapter 9**). We confirmed that PCV7 induced both salivary IgG

and IgA serotype-specific antibodies. High correlations were observed between salivary and serum antibody levels, suggestive for transfusion of serum antibodies to the mucosal site. We showed however that at the age of 24 months, salivary IgG levels differed between children after 2 doses or 2+1 doses. This is in contrast to serum IgG antibody levels where no significant differences were observed for 5 out of 7 vaccine serotypes at 24 months after 2 or 2+1 doses. This suggests that also locally salivary IgG is produced by serotype-specific IgG B cells at the mucosal level, probably boosted by natural contacts with more benefit for the 2+1-dose schedule compared to the 2-dose schedule. Homologous nasopharyngeal carriage of serotypes contributed strongly to higher mucosal anticapsular serotype-specific IgG antibody levels as was shown for serotypes 6B, 19F and 23F. Nasopharyngeal carriage contributed even more for serotype-specific salivary IgA levels. Although PCV7 vaccinations clearly resulted in higher salivary IgA levels at the age of 12 months, at 24 months of age strong increases in the 2-dose and unvaccinated control-group resulted in less pronounced differences with the 2+1-dose group in salivary IgA levels. The exact contribution of PCV7-induced salivary IgA and IgG antibody levels in protection against nasopharyngeal carriage and disease is not determined yet. We earlier reported a 58% reduction in vaccine serotype pneumococcal carriage at the age of 24 months in the 2-dose group compared to unvaccinated controls.[23] We observed at this age however no difference in mucosal IgA antibody levels between the 2-dose and controls (**Chapter 9**). In contrast, mucosal IgG antibodies were higher in the 2-dose group compared with controls. This may suggest that vaccine-induced anticapsular mucosal IgG antibodies have a stronger attribution in protection against pneumococcal colonization than IgA.

Part 3. Effect of pneumococcal carriage on immune responses to PCV7 after nationwide implementation

Carriage and vaccine responses

As mentioned earlier, all data presented in part 2 of this thesis were collected with natural circulation of vaccine strains. In a carriage surveillance study 3 years after PCV7 implementation (spring 2009) a very strong reduction in vaccine-serotype carriage in 11-months and 24-months-old children was shown with vaccine-serotype prevalence rates of 4% - 8% (Spijkerman et al, submitted). Considering IgG antibody development, we found that this decreased natural circulation of vaccine serotypes in the population can affect serotype-specific antibody responses upon PCV7 vaccinations as well as antibody maintenance (**Chapters 10, 11**). Natural exposure in carriage by frequently carried serotypes indeed enhanced antibody IgG levels as demonstrated for the serotypes 6B and 19F (**Chapters 7-9**). As a consequence, eradication of these most frequently carried and least-immunogenic serotypes may result in lower antibody levels upon PCV7 vaccination after nationwide implementation compared to levels of children that were vaccinated when vaccine serotypes still circulated (**Chapter 11**). Interestingly, in contrast to lower antibody levels, we found no differences in the memory induction at 24 months for the serotypes 6B and 19F. After a challenge PCV7 similar IgG antibody levels and memory B

cell responses were observed in the post-PCV7 implementation period compared with the pre-vaccination period (**Chapter 12**). These findings suggest that maintenance of higher serum serotype-specific IgG antibody levels against serotype 6B and 19F may depend on natural contact by vaccine serotype carriage. This may be relevant for immediate clinical protection as was shown for *Neisseria meningitidis* serogroup C (MenC) in the United Kingdom.[57] Investigation of MenC vaccine failures confirmed that despite immune memory these children were infected.[58] However, in Hib-vaccine failures also lower antibody avidity has been described, which can indicate less immunological priming and memory B cell induction.[59]

The interplay between carriage and impact on antibody responses upon vaccination is however complex. Opposite to the lower IgG levels of these frequently carried serotypes, infrequently detected serotypes 4, 9V and 18C showed higher IgG antibody levels 3 years after PCV7 implementation. Since in the pre-herd period the 24-month-old children were vaccinated with a reduced 2+1-dose schedule, this may have contributed to the difference observed in antibody levels at 24 months. For the serotype 4 and 9V however earlier no inferiority of a 2+1-dose schedule compared to a 3+1-dose schedule was observed (**Chapter 6**).[32] In Chapter 10 we described other effects of carriage on vaccine-serotype IgG levels. PCV7-vaccinated children who were previous carriers of serotypes 6B, 19F or 23F showed diminished IgG responses towards a challenge PCV7 vaccination at the age of 24 months. An underlying mechanism for this hyporesponsiveness would be that existing memory B cells differentiate into plasma cells producing antibodies upon natural polysaccharide exposure by carriage, but without replenishment of the memory compartment.[60-62] Therefore, eradication of these circulating vaccine strains may result in more adequate memory induction by PCV7 as measured by a challenge vaccination at 24 months **(Chapter 12)**.[61;62]

Timing and need of the booster dose

At present we do not know what the loss of natural boosting on serotype-specific antibody maintenance will mean for protection against disease in the future. Protection against IPD may also depend on other (non-serotype dependent) mechanisms rather than anticapsular antibodies that mature with age.[63;64] The current opinion is that we need a PCV7 booster vaccination in the second year of life for clinical protection by enough circulating serotype-specific antibodies, based on previous experience after implementation of Hib and MenC conjugate vaccines in the United Kingdom without a booster dose.[33;65] However, once vaccine strains have been eradicated from the population, vaccine breakthroughs will be scarce to absent and lower antibody thresholds may be sufficient for protection. (Figure 2). In the United Kingdom and Norway, high vaccine effectiveness was observed despite the observed rapid waning of functional antibody levels after 2 primary doses until the booster dose (**Chapter 8**).[8;9] Also after 3 primary doses without a booster dose, high PCV7 effectiveness is observed in Australia.[4;5] After introduction of the MenC conjugate vaccine in the Netherlands in 2002 as single vaccination at 14 months with a large catch-up program

for all children and adolescents, herd effects attributed to high clinical protection.[66] Despite rapidly waning antibody levels within 1-2 years after the MenC vaccination at 14 months, no vaccine failures have been reported with limited disease incidence in the unvaccinated population.[66] As anticapsular antibody maintenance without natural boosting seems majorly dependent of the magnitude of the booster-response, delaying the booster dose may be an option.[43;67] We observed significantly higher antibody responses after late booster vaccination at 24 months compared to an early booster dose at 11 months in children who earlier received 2 primary doses, which underlines the relevance of timing of the booster vaccination (**Chapters 7, 8**). This opens opportunities for exploring optimal vaccination schedules in times of herd protection of young children, like delaying primary vaccinations or booster doses with perhaps room for later additional boosting when required.[38]

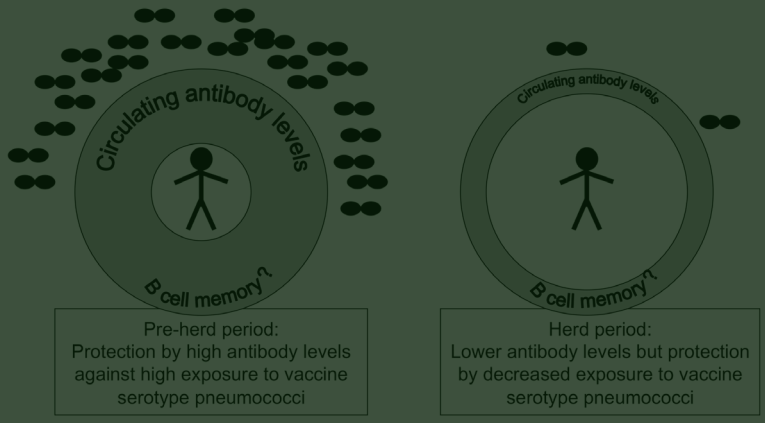

Figure 2. Schematic representation of the vaccine-mediated protection against disease caused by frequently encountered, heavily encapsulated serotypes like 6B and 19F. (a) Before herd effects protection is dependent of immunological protection of individuals by circulating serum antibodies and possibly the capacity to mount a proper memory response. Natural circulating pneumococci boost antibody levels.
(b) In the herd period for some serotypes antibody levels decrease because of less natural boosting. However, vaccine failures are rare since protection is maintained by decreased natural circulation; i.e. herd effects.

Conclusions

In summary, the major findings of this thesis were:

Part 1

- Before nationwide PCV7 implementation in the national immunization program, the burden of vaccine serotype IPD was high in the Netherlands (Chapters 2,3).
- PCV7 showed to be highly effective against vaccine serotype IPD. However, coinciding increases in non-vaccine serotype IPD reduced net vaccine benefits in the Netherlands (Chapter 3).
- Increase by nonvaccine serotype IPD is not expected to show a milder disease course or a better outcome compared with vaccine serotype IPD (Chapters 3-5).

Part 2

- Serotype-specific anticapsular IgG antibody levels were comparable pre- and post-booster for 5 out of the 7 vaccine serotypes following a 2+1 or a 3+1-dose PCV7 schedule (Chapter 6).
- Two primary doses of PCV7 at the age of 2 and 4 months resulted in induction of higher antibody levels and B cell memory compared to unvaccinated age-matched controls. Functionality of the antibodies however seemed to wane rapidly within 1 year after vaccination (Chapters 7-9).
- An early 11-month booster did not result in better induction of B cell memory at age 24 months compared to 2 primary doses only, although a serotype-dependent vaccine benefit was observed in serum and mucosal antibody maintenance (Chapters 7-9).
- Higher quantitative and functional antibody responses were observed after a booster at 24 months compared with a booster at 11 months of age in children who both had received 2 primary doses (Chapter 8).

Part 3

- Pneumococcal colonization proved a major contributor to circulating IgG levels, in particular for heavy encapsulated strains prominent in carriage (Chapters 7-10).
- Previous pneumococcal colonization was found to be associated with diminished IgG antibody responses to challenge PCV7 vaccination at 24 months of age (Chapter 10).
- Decreased natural circulation of vaccine strains lead to strongly decreased antibody levels against serotypes 6B and 19F at 24 months of age, but vaccine-induced memory seemed preserved (Chapters 11,12).

Future perspectives

PCV schedules in the Netherlands

At present, pneumococcal conjugate vaccination proved to be highly effective in reducing vaccine serotype IPD in the vaccinated children. However, the rapid increase in non-vaccine serotype IPD reduced the net vaccine benefit. Even though herd effects in all other age groups are becoming clearly present now in the Netherlands, these effects also seem to be largely offset by increases in non-vaccine serotype disease (personal communication A. van der Ende). Like in the United Kingdom the herd effects and decreases in vaccine serotype IPD in the unvaccinated age-categories are almost fully counterbalanced by non-vaccine serotype increases in IPD.[9;68]

For cost-effectiveness, broader coverage by future vaccines and implementation of a reduced-dose schedules are opportunities to reach cost-neutral or cost-saving pneumococcal vaccine schedules.[69] Protection against in particular serotypes 1, 7F as well as serotypes 3 and 19A would further decrease the incidence of pneumococcal disease at all ages. In a recent advice the Dutch Health Council suggested broader coverage by the recently licensed multivalent vaccines, namely the 10-valent *non-typeable Haemophilus influenzae* protein D (PD)-conjugated vaccine and the 13-valent CRM197-conjugated vaccine.[70] These vaccines would theoretically have covered an estimated additional 52 to 64 IPD cases in the vaccine-eligible cohorts in the first 2 years after PCV7 implementation (Chapter 3). Since these 10- and 13-valent vaccines would also substantially improve coverage of pneumococcal disease in other age groups, this may have positive impact on herd effects and potentially less replacement IPD.

Besides broader coverage, reducing vaccine prices or implementation of a reduced-dose schedule can be opportunities to reach more cost-saving pneumococcal vaccine schedules. One injection less for every newborn child will save burden for the child and several million euro every year in the Netherlands.[69] Rapid and strong decreases in vaccine serotype IPD were observed in all countries after widespread implementation of reduced-dose PCV7 schedules, in particular after a catch-up strategy for children with highest transmission.[9;21] Based on experience with PCV7, the 13-valent CRM197-conjugated vaccine is also licensed for a 2+1-dose schedule. For the 7 vaccine serotypes currently in PCV7 eradication of carriage in children has been obtained and maintenance of herd effects can be expected with reduced 13-valent CRM197-conjugated vaccine schedules after nationwide implementation.[71] The coming years also the PD-conjugated 10-valent vaccine in a 3+1-dose schedule will likely preserve herd effects for the 7 vaccine serotypes. However, results of reduced doses on carriage and thus herd effects need to be awaited for this vaccine and clinical studies are still ongoing in Finland. Also the results for the additional serotypes (1, 5, 7F in the 10-valent and 1, 3, 5, 6A, 7F and 19A in the 13-valent vaccine) remain to be awaited. Also for antibody maintenance and long-term memory there remain many questions for both vaccines, as described earlier.

Long-term protection

In the first years after vaccine implementation an early booster dose after 2 primary doses might be necessary. Despite high vaccine effectiveness, in Canada in the first years after implementation of a 3+1-primary dose schedule, some vaccine failures have been reported just before the late 18-month booster dose.[2] In resource poor countries with much higher carriage rates at lower age and where incidence- and mortality-rates of IPD remain high after infancy, both sufficient early vaccinations as well as later booster doses may be relevant.[72;73] Recently, PCV7 has been implemented in the resource poor countries with a 2-dose schedule and a very early booster at the age of 9 months (Rwanda, South Africa).[74] Considering the likely limited additional value of such an early booster dose on long-term immunity an additional later booster dose may probably be advantageous for long-term protection.[38] In Australia, major reductions in vaccine serotype IPD are observed in the PCV7 vaccinated and unvaccinated population in the first years after implementation of a 3 primary dose schedule without a booster dose. However, this schedule was implemented with a catch-up campaign so herd effects might have contributed to this success in eradicating rapidly vaccine strain circulation.[4;5] When waning antibody levels result in decreased clinical protection, another option would be to add booster doses at later ages. Considering 4 doses for the total schedule, a 2+1-dose schedule in the first 2 years of life in these countries followed by an additional booster between the age of 24 and 36 months may be best option. However logistics and contact moments may prove difficult. Long-term surveillance data are needed and comparison between countries and vaccines are necessary before one could opt for an optimal vaccine schedules after wide spread implementation of the vaccine and eradication of circulating vaccine serotypes.

Vaccine development

Although results presented in this thesis have shown that PCV7 is effective in preventing vaccine serotype pneumococcal disease, future research is needed for further success in protection against this pathogen and to counteract emerging non-vaccine serotypes. Although multivalent conjugate vaccines have the potential to prevent an additional proportion of disease globally, it seems unlikely these will eventually include all pneumococcal serotypes. One other approach is to only prevent the vaccine-serotype pneumococci to become invasive and keep occupation of the ecological niche in the nasopharynx intact, hereby preventing replacement by nonvaccine serotypes or other bacteria and remain natural boosting of vaccine serotype antibody levels. However, major disadvantages will be the potential loss of protection against mucosal disease like pneumonia and the loss of herd protection for the unvaccinated population. Another approach would be identification of serotype-independent antigens to develop protein-vaccines or pneumocccal whole-cell vaccines, since naturally acquired protection majorly depends on non-serotype specific CD4+ Th17 cells and pattern recognition receptors/Toll-like receptors.[64;75-77] Because the availability of such a vaccine however appears to be some years off, current vaccine policies in the nearby future should be directed to implement conjugate vaccines with broader coverage. We showed that anticapsular antibody and

memory development are strongly serotype-dependent and further elucidating the mechanism of B cell activation for the different serotypes after vaccination or natural exposure would be interesting. Also the exact role of anticapsular mucosal responses deserves further attention, as conjugate vaccines proved highly immunogenic in salivary fluids, use of alternative non-parental routes of immunization might be optional.

Changing target

The findings in the thesis underscore the importance of post licensure surveillance studies. Serotype-specific pneumococcal disease incidences should be closely monitored by continued high quality disease surveillance. We showed that emerging non-vaccine serotypes are also invasive and result in serious burden of disease. Detailed disease surveillance will be essential for good estimations of vaccine effectiveness in different patient populations and clinical syndromes. Costs of such in-dept IPD surveillance are still minor compared to the total costs of the PCV7 vaccination program. Also other forms of monitoring like carriage- and immune-surveillance studies will be essential to fully interpret serotype-specific disease incidences. Carriage studies in pre- and post-PCV7 implementation period have shown widespread changes in colonization patterns and marked increases in non-vaccine serotypes. More sensitive serotype-specific detection methods need to be developed, as conventional tend to methods underestimate pneumococcal carriage diversity and miss most low-density serotypes and multiple-strain colonization.[78;79] Carriage surveillance studies are informative given that colonization is a necessary precondition for IPD. As reduction of vaccine serotype colonization correlates with (herd) protection against IPD, increased nasopharyngeal carriage with potentially virulent, non-vaccine serotypes reasonably predicts increased risk of non-vaccine IPD. Still much is unknown about the ´healthy ecological environment´ in the nasopharynx and its development with age and the impact of environmental factors. By conventional culture methods we observed small shifts in colonization with other colonizing bacteria in infants after reduced-dose PCV7 schedules. *S. aureus* for instance doubled in infants after a 2+1-dose PCV7 schedule compared to unvaccinated controls. The clinical consequences of this observed increase however require further evaluation. More precise data about composition of the nasopharyngeal flora by novel unbiased high-throughput approaches are needed.[80] Vaccine serotype carriage data are also needed to interpret changes in antibody persistence and vaccine responses over time, as observed for serotypes 6B and 19F.

The relatively homologous population, the high vaccine-uptake, the well organized health system, and the low bacterial resistance to penicillin make the Netherlands particularly suitable for these surveillance programs and estimations of vaccine effects. We showed in this thesis protection against a pathogen like *S. pneumoniae* requests a dynamic approach in prevention and vaccine policies, as the bacteria proved to be a moving target, adjusting to its environment. Continuous revision of vaccines strategies is therefore needed as currently used vaccine schedules may not be optimal some years after implementation and in the future.

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Nederlandse Samenvatting

Streptococcus pneumoniae is een belangrijke bacteriële verwekker van hersenvlies-ontsteking (meningitis), bloedvergiftiging en van luchtweginfecties zoals longontsteking en middenoorontsteking. Naar schatting van de Wereldgezondheidsorganisatie (WHO) sterven er wereldwijd gemiddeld elk jaar ten minste 1,6 miljoen mensen aan infecties veroorzaakt door pneumokokken, waarvan 0,7 tot 1 miljoen kinderen jonger dan 5 jaar. Het betreft vooral kinderen in ontwikkelingslanden waar onvoldoende medische zorg voorhanden is. De mens draagt regelmatig een pneumokok in de neus en keelholte en is het natuurlijk reservoir voor deze bacterie. Meestal leidt deze kolonisatie in de neus en keelholte niet tot een infectie, maar is er sprake van asymptomatische 'kolonisatie' of dragerschap. Vrijwel alle kinderen maken regelmatig perioden van pneumokokkendragerschap door. Het hoogste percentage dragerschap bij kinderen onder de 2 jaar is bij kinderen die regelmatig naar een kinderdagverblijf gaan of die contact hebben met andere kinderen binnen het gezin. Bij kinderen duurt de periode van kolonisatie met pneumokokken enkele weken tot maanden. Deze periode van dragerschap neemt af met het ouder worden van het kind en het rijpen van de afweer. Kinderen zijn de belangrijkste dragers en verspreiders van pneumokokken in de samenleving.

De pneumokok is een bacterie die wordt omringd door een suikerkapsel bestaande uit polysacchariden. Er zijn meer dan 90 verschillende typen suikerkapsels (serotypen) bekend, elk met een unieke samenstelling. Het suikerkapsel is een belangrijke virulentie factor voor de bacterie; het bepaalt of de bacterie zich kan beschermen tegen de afweer van de mens, kan koloniseren in de neus en keel en of de bacterie ziekte kan veroorzaken. Een pneumokok zonder kapsel veroorzaakt over het algemeen geen ziekte. Pneumokokkeninfecties komen met name voor bij kinderen jonger dan 5 jaar vanwege het nog onrijpe immuunsysteem, bij oudere patiënten en bij patiënten met een gestoorde afweer. Functionele afweerstoffen gericht tegen het suikerkapsel blijken tegen ziekte te beschermen. Vanwege de hoge ziektelast werd er al vroeg in de 19^{e} eeuw gestart met het ontwikkelen van een vaccin tegen de meest voorkomende kapseltypen van de pneumokok. Deze vaccins bestonden uit de polysaccharide kapselcomponenten. Echter, bij jonge kinderen bleken deze polysaccharide vaccins nauwelijks effectief, omdat op deze leeftijd nog geen afweerstoffen opgebouwd worden tegen pure polysacchariden. Een oplossing werd gevonden door de ontwikkeling van polysaccharide-eiwit-geconjugeerde vaccins, de zogenaamde conjugaatvaccins, waarbij de polysacchariden zijn gekoppeld aan een dragereiwit. Een kind kan vlak na de geboorte al goed afweerstoffen tegen eiwitvaccins maken. Als men een polysaccharide aan een eiwit koppelt, lijken bepaalde cellen van de afweer betrokken te worden bij de opbouw van afweerstoffen tegen polysacchariden en is

ook bij jonge kinderen na vaccinatie een goede kapselspecifieke antistofreactie zichtbaar. Voor invoering van de pneumokokkenconjugaatvaccins werden 1993 en 2002 eerst de polysaccharide-eiwit conjugaatvaccins tegen respectievelijk *Haemophilus influenzae type b* (Hib) en *Neisseria meningitidis type C* (meningokokken type C), beide belangrijke verwekkers van hersenvliesontsteking, ingevoerd in het Nederlandse rijksvaccinatieprogramma. Beiden waren een groot succes. Naast een verminderde ziektelast bij gevaccineerde kinderen (direct effect), was er ook een afname van Hib en meningokokken C infecties bij ongevaccineerden in alle leeftijdsgroepen. Conjugaatvaccins leiden namelijk tot een afname van kolonisatie van de bacterie waartegen wordt gevaccineerd in de neus en keelholte. Gevaccineerde kinderen dragen minder vaak de bacterie bij zich waardoor deze minder wordt verspreid in de populatie en uiteindelijk nagenoeg verdwijnt. Daardoor verdwijnt ook de ziekte door deze bacterie. Dit fenomeen wordt ook wel indirect effect of herd-effect van de conjugaatvaccins genoemd (voor uitgebreide uitleg http://www.health.harvard.edu/video/herd-immunity).

Een 7-valente pneumokokkenconjugaatvaccin (vanaf hier 7-valente vaccin of PCV7 genoemd) gericht tegen de zeven meest voorkomende pneumokokken serotypen bij kinderen jonger dan 5 jaar, werd in 2000 met succes ingevoerd in de Verenigde Staten. Het aanbevolen schema voor zuigelingen was 3 vaccinaties onder de leeftijd van 6 maanden gevolgd door een vierde vaccinatie in het tweede levensjaar (3+1-schema). Tevens werd een inhaalcampagne aanbevolen voor kinderen onder de 5 jaar. Na invoering van het 7-valente vaccin zag men bij gevaccineerde kinderen een >90% daling van invasieve pneumokokkenziekte (bloedvergiftiging en meningitis) veroorzaakt door de 7 pneumokokken serotypen die opgenomen waren in dit vaccin (vaccintypen). De daling in vaccintype invasieve ziekte ging echter gepaard met een lichte toename van invasieve ziekte door pneumokokken serotypen die niet in het vaccin waren opgenomen (niet-vaccintypen). De stijging van invasieve ziekte door niet-vaccintypen leek beperkt, waardoor in 2007, zeven jaar na invoering van het 7-valente vaccin in de Verenigde Staten, de netto reductie van alle invasieve pneumokokkenziekte bij kinderen jonger dan 5 jaar ongeveer 75% bedroeg. Ook werd na introductie van het 7-valente vaccin in de ongevaccineerde leeftijdsgroepen een sterke daling van de vaccintype pneumokokkenziekte gezien, in lijn met ervaringen van de eerdere ingevoerde conjugaatvaccins tegen Hib en Meningokokken C. Deze indirecte vaccineffecten droegen dermate bij aan de kosteneffectiviteit berekeningen van het 7-valente vaccin voor Nederland, dat deze in juni 2006 werd ingevoerd in het rijksvaccinatieprogramma. Alle zuigelingen geboren na 1 april 2006 krijgen sindsdien op de leeftijd van 2, 3, 4 en 11 maanden het 7-valente vaccin aangeboden.

Deel 1. IPD in Nederland voor en na landelijke invoering van PCV7

In het eerste deel van dit proefschrift beschreven we het vóórkomen van invasieve pneumokokkenziekte op alle leeftijden in Nederland en vervolgens de effectiviteit van de invoering van het 7-valente pneumokokkenconjugaatvaccin in het rijksvaccinatieprogramma. We toonden aan dat ook in Nederland de ziektelast van invasieve pneumokokken infecties voor de invoering van het 7-valente vaccin groot is (**hoofdstuk 2**). Groepen die het meeste risico lopen voor pneumokokkenziekte zijn vooral jonge kinderen, ouderen en patiënten met afweerstoornissen. Hoewel de incidentie het hoogst is bij jonge kinderen, gevolgd door ouderen, vormen ook mensen met afweerproblemen een grote groep. Voor invoering van het 7-valente vaccin in Nederland ontwikkelden per jaar ruim 2500 patiënten een invasieve pneumokokkenziekte waarvan bijna 400 gevallen met een dodelijke afloop.

In **hoofdstuk 3** beschreven we dat in de eerste 2 jaar na invoering van het 7-valente vaccin in Nederland (juni 2006-juni 2008) een zeer sterke afname (90%) zichtbaar was van vaccintype invasieve pneumokokkenziekte bij kinderen jonger dan 2 jaar, geboren na april 2006. Echter, in deze groep werd ook een directe 70% toename gezien van invasieve ziekte door pneumokokken serotypen die niet in het vaccin waren opgenomen. Hierdoor viel de uiteindelijke daling van alle invasieve pneumokokkenziekte bij jonge kinderen met 44% aanmerkelijk lager uit in dit cohort dan verwacht. Deze snelle opkomst van niet-vaccintypen en de lagere totale effectiviteit tegen invasieve pneumokokkenziekte is in overeenstemming met met data vanuit het Verenigd Koningrijk (Engeland en Wales). Ook in het Verenigd Koninkrijk beschermt het vaccin uitstekend tegen de zeven vaccintypen maar eveneens wordt hier het netto effect deels teniet gedaan door de snelle opkomst van ziekte door de niet-vaccintypen. Deze observaties rechtvaardigen de vraag waarom in Nederland en Verenigd Koninkrijk het netto-effect minder positief uitvalt dan de zeer gunstige uitkomsten in de Verenigde Staten na invoering van deze vaccinatie? Deze discrepantie lijkt vooral veroorzaakt door de grote verschillen in opzet van het gezondheidszorgsysteem en de surveillance van invasieve pneumokokkenziekte. De incidentie van pneumokokken bloedvergiftiging bij jonge kinderen in de Verenigde staten ligt zeker drie keer hoger dan in Nederland. Dit komt omdat in de Verenigde staten bij een kind met koorts meestal een bloedkweek wordt afgenomen, waarbij men dan een pneumokok kan vinden. Het kind is echter niet ziek genoeg om te hoeven worden opgenomen in het ziekenhuis. Bijna 70% van alle kinderen die zijn geregistreerd in het Amerikaanse surveillance systeem met een invasieve pneumokokkenziekte hoefden niet te worden opgenomen in een ziekenhuis. In Nederland echter (en naar het lijkt in heel Europa), wordt bij minder zieke kinderen die niet hoeven worden opgenomen, helemaal geen bloedkweek afgenomen omdat het geen verdere consequenties heeft. In Nederland ziet de huisarts deze kinderen. Dit verschil in bloedkweek afnames verklaart ten eerste het grote verschil in incidentie van invasieve

pneumokokkenziekte vóór invoering van het vaccin, te weten 188 gevallen per 100.000 bij kinderen jonger dan 2 jaar in de Verenigde Staten versus 35 per 100.000 in Nederland. Maar vervolgens verklaart het ook deels het verschil in effectiviteit van de invoering van het 7-valente vaccin. Als we in de Verenigde Staten enkel kijken naar de effectiviteit van het 7-valente vaccin bij patiënten waarbij een opname in het ziekenhuis nodig was, dan zien we een meer vergelijkbaar vaccineffect met Nederland: een 60% afname van alle invasieve pneumokokkenziekte met een 102% toename van invasieve ziekte door niet-vaccintype pneumokokken.

Met de gegevens in **hoofdstuk 4** en in **hoofdstuk 5** toonden we aan dat infectie veroorzaakt door niet-vaccintype pneumokokken niet leidt tot een milder verloop van de ziekte in vergelijking tot vaccintype pneumokokken. Dit heeft tot gevolg dat als typevervanging plaatsvindt, dit waarschijnlijk ook niet gepaard zal gaan met een mindere mortaliteit of morbiditeit na pneumokokken infecties. Van belang is echter te benadrukken dat de surveillance data die in dit proefschrift worden gepresenteerd slechts de eerste 2 jaar na invoering van het 7-valente vaccin vertegenwoordigen. Om tot een duidelijkere uitspraak te komen over de vaccin effectiviteit en mogelijke typevervanging zijn data van een langere, aaneengesloten periode nodig.

Na invoering van het 7-valente vaccin in de Verenigde Staten werd ook bij de ongevaccineerde populatie een daling van vaccintype pneumokokken infecties gezien. In Nederland werden deze indirecte effecten echter in de eerste 2 jaar nog niet waargenomen: in alle ongevaccineerde leeftijdscategorieën bleven incidenties van invasieve pneumokokken ziekte stabiel. Dit kan verklaard worden door het feit dat in 2006 het vaccin in Nederland is ingevoerd zonder inhaalcampagne voor oudere kinderen. Hierdoor was na 2 jaar maar een relatief klein deel van de bevolking (~2,25%) gevaccineerd.

In Nederland en de Verenigde Staten wordt momenteel gevaccineerd met een schema bestaande uit 3 inentingen in de eerste 6 levensmaanden (primaire vaccinaties) en een herhalingsinjectie, ook wel boostervaccinatie genoemd, rond of net na het eerste levensjaar (3+1-schema). In een groot deel van Europa zijn echter vaccinatieschema's ingevoerd met een gereduceerd aantal inentingen, te weten 2+1-schema's met 2 doses in de eerste 6 levensmaanden en een boostervaccinatie later in het tweede levensjaar. Ten opzichte van landen met 3+1-schema's is na invoering van deze gereduceerde vaccinatieschema's een vergelijkbare effectiviteit te zien in de preventie van invasieve pneumokokkenziekte door de vaccintypen, bijvoorbeeld in het Verenigd Koningrijk. Ook in ongevaccineerde leeftijdsgroepen werd in het Verenigd Koningrijk al eerder een daling van vaccintype pneumokokken infecties waargenomen. Waarschijnlijk heeft hier de inhaalcampagne voor alle kinderen onder de twee jaar hier bijgedragen aan de snellere indirecte effecten in de gehele populatie. Het optreden van goede effectiviteit en van deze indirecte effecten in de ongevaccineerde populatie ook na een 2+1-schema zijn in overeenstemming met

de resultaten van onze MINOES studie, waarbij we hebben gekeken naar de afname van dragerschap door pneumokokken serotypen in de neus en keelholte bij gevaccineerde kinderen en hun ouders na een 2+1-schema en zelfs na alleen 2 prikken voor de leeftijd van 6 maanden. We zagen dat zowel na alleen 2 primaire inentingen op 2 en 4 maanden als na een 2+1-schema op 2, 4 en 11 maanden een vermindering van het vaccintype dragerschap tot 60% op de leeftijd van 24 maanden werd bereikt in vergelijking met een ongevaccineerde controlegroep. Dit lijkt vergelijkbaar met de daling in andere studies na een 3+1-schema. Een 2+1-schema is dus een goed alternatief, zeker in combinatie met een inhaalcampagne voor alle kinderen onder de twee jaar bij de aanvang van dit schema.

Deel 2. Immunologische responsen na gereduceerde vaccinatieschema's met PCV7 voor landelijke invoering

In het tweede deel van dit proefschrift werden de resultaten van dit MINOES onderzoek op de ontwikkeling van afweerstoffen (antistoffen) en geheugencellen van de afweer gepresenteerd. We onderzochten diverse kapselspecifieke antistofresponsen bij een vaccinatieschema van 2 primaire inentingen op de leeftijd van 2 en 4 maanden al dan niet gecombineerd met een boostervaccin op 11 maanden, in vergelijking met ongevaccineerde controle kinderen.

Vaccingeïnduceerde IgG antistoffen tegen kapsels van de pneumokok activeren en versterken fagocytose door afweercellen en worden gezien als het primair mechanisme voor bescherming door vaccinatie tegen invasieve ziekte. Op dit moment wordt door de WHO de hoogte van de kapselspecifieke IgG antistofconcentratie gebruikt als maat voor bescherming tegen invasieve pneumokokkenziekte en als primair criterium om verschillende pneumokokkenconjugaatvaccins te vergelijken. In **hoofdstuk 6** beschreven we dat in het bloed op de leeftijd van 12 maanden, 1 maand na de boostervaccinatie, even hoge IgG antistofconcentraties werden gezien voor 5 van de 7 vaccintypen na een 2+1-schema vergeleken met een 3+1-schema. De twee uitzonderingen waren de 2 serotypen die het meest worden aangetroffen in de dragerschapstudie MINOES voor de PCV7 introductie in Nederland, de serotypen 6B en 19F. Serotype 6B is een bekend laag-immunogeen kapseltype, wat de kapselspecifieke antistofconcentratie sterk afhankelijk maakt van het toegediende aantal vaccinaties. Deze kapselspecifieke antistoffen tegen 6B blijken dermate effectief dat ook een lage IgG antistofconcentratie nog goede bescherming bood in diverse studies. Ook bleken ze zeer effectief tegen serotype 6B dragerschap in de MINOES studie. Voor serotype 6B lijkt dus met lagere antistofconcentraties volstaan te kunnen worden. Voor serotype 19F is dit niet het geval. Hiervoor is aangetoond dat er een hoog gehalte aan antistoffen nodig is met het huidige 7-valente vaccin voor optimale bescherming tegen ziekte. In onze dragerschapstudie MINOES werd ook alleen kort na de

boostervaccinatie op 11 maanden een duidelijke afname van serotype 19F dragerschap gezien ten opzichte van controle kinderen, maar later niet meer. In het Verenigd Koninkrijk zijn na invoering van een gereduceerd 2+1-schema in 2006 enkele invasieve infecties beschreven met serotypen 6B en 19F in het eerste levensjaar na PCV7 vaccinatie. Op het moment van schrijven van dit proefschrift, enkele jaren later na invoering van PCV7, zijn door indirecte effecten / verminderde circulatie van vaccintype pneumokokken de incidenties van invasieve ziekte veroorzaakt door serotype 6B en 19F geminimaliseerd.

In gevaccineerde kinderen is het persisteren van de kapselspecifieke antistoffen noodzakelijk voor bescherming op de meer lange termijn, minimaal liefst tot 5 jaar. We toonden aan dat kinderen die 2 primaire vaccinaties hadden gekregen al dan niet gevolgd door een boostervaccinatie, hogere kapselspecifieke antistofconcentraties hadden op de leeftijd van 12 en 24 maanden in vergelijking met de ongevaccineerde controlegroep (**hoofdstuk 7)**. Het aantonen van immunologisch geheugen kan worden beoordeeld door het meten van antistofconcentraties na toediening van een PCV7 herhalingsdosis. Na toediening van een herhalingsvaccinatie met PCV7 op 24 maanden werden duidelijk hogere antistofresponsen gemeten tegen de 7 vaccintypen met meer geheugen B cellen na 2 vaccinaties op 2 en 4 maanden in vergelijking met ongevaccineerde controle kinderen. De kinderen die na de twee vaccinaties op 2 en 4 maanden nog een boostervaccinatie op 11 maanden hadden gekregen, lieten opvallend genoeg vrijwel dezelfde antistofresponsen en aantallen geheugen B cellen zien dan de kinderen na enkel 2 vaccinaties, op serotype 6B na. Voor serotype 6B was er wel een duidelijk effect van de boostervaccinatie meetbaar. Ook voor aviditeit van de antistoffen, dat beschouwd mag worden als een maat voor rijping van het B cel compartiment, was er maar een geringe bijdrage van deze boostervaccinatie zichtbaar. Voor serotype 19F was er na vaccinatie maar een kortdurend en tijdelijk verschil in aviditeit zichtbaar ten opzichte van ongevaccineerde controle kinderen. Dit zou kunnen duiden op een mogelijk andere en minder succesvolle B cel inductie door het 7-valente vaccin voor serotype 19F.

In **hoofdstuk 8** beschreven we de functie van de antistoffen met een opsonofagocytose assay. Met deze assay wordt gemeten met welke verdunning van het bloed fagocytose van de pneumokok nog steeds meetbaar is. De hoogte van de kapselspecifieke antistoffen hoeft noodzakelijkerwijs niet overeen te komen met functionaliteit. We toonden aan dat fagocytose tot op zekere hoogte gerelateerd is aan de hoeveelheid IgG antistoffen, maar er waren ook diverse verschillen zichtbaar bij de verschillende serotypen; de IgG antistof concentraties die nodig waren voor 50% fagocytose verschilden sterk tussen de serotypen uit het 7-valente vaccin. Belangrijk is te noemen dat deze functionaliteitsassay ook door andere factoren beïnvloed kan worden, zoals andere isotypen van antistoffen (IgM of IgA). Ook is deze test sterk afhankelijk van variabele factoren zoals de gebruikte bacteriestammen, de gebruikte afweercellen en het complement. Het precieze verband tussen functionaliteit en de werkzaamheid tegen invasieve pneumokokkenziekte is nog

niet vastgesteld en behoeft verder onderzoek.

In **hoofdstuk 9** beschreven we de kapselspecifieke IgA en IgG antistoffen in het speeksel van kinderen uit de MINOES studie en we vergeleken weer de gevaccineerde kinderen met ongevaccineerde controle kinderen. Verschillende studies rapporteerden dat pneumokokkenconjugaatvaccinatie resulteerde in IgG antistoffen in speeksel, maar de rol van de antistoffen in bescherming tegen dragerschap en ziekte is nog onduidelijk. In dit hoofdstuk toonden we aan dat er zeer goede correlaties zijn tussen de hoeveelheid IgG antistoffen in speeksel en in bloed. Dit duidt op transfusie van IgG antistoffen van het bloed naar de slijmvliezen, al zijn er ook aanwijzingen voor een locale, mucosale productie van IgG antistoffen op slijmvliesniveau met name voor IgA antistoffen. Dragerschap van pneumokokken serotypen bleek een sterke bijdrage te leveren aan het in stand houden van antistoffen op slijmvliesniveau. Dragerschap van serotypen 6B, 19F en 23F resulteerde in hogere kapselspecifieke antistofconcentraties in het speeksel tegen deze kapseltypen. Voor IgA antistoffen was deze natuurlijke inductie van antistoffen het meest evident. Op de leeftijd van 24 maanden werd tegen een aantal vaccintypen zelfs geen verschil meer gezien in IgA antistoffen tussen ongevaccineerde controle kinderen met een hoog dragerschap van vaccintypen en kinderen die 2 primaire vaccinaties hadden gehad. De rol van IgA antistoffen in protectie tegen dragerschap is mogelijk erg beperkt, omdat we ondanks eenzelfde IgA antistofniveau bij gevaccineerde en controle kinderen een 58% reductie in vaccintype dragerschap konden waarnemen in de gevaccineerde groep ten opzichte van de controle groep.

Deel 3. Effect van pneumokokken dragerschap op immunologische PCV7 responsen na landelijke invoering

Alle beschreven fenomenen in deel 2 van dit proefschrift komen uit een tijd (2005-2008) dat vaccintypen nog volop circuleerden in de bevolking omdat het verdwijnen van dragerschap met vaccintypen pas later optrad na introductie van PCV7. Van indirecte bescherming tegen invasieve ziekte door een verminderde circulatie in de bevolking was in die periode ook nog geen sprake. In het 3^{e} deel van dit proefschrift onderzochten we de impact van verminderd dragerschap en circulatie op het behoud van kapselspecifieke antistofconcentraties na invoering van de pneumokokkenconjugaatvaccinatie in het rijksvaccinatieprogramma. Met de gegevens in **hoofdstuk 11** toonden we aan dat 3 jaar na invoering van het 7-valente vaccin in Nederland de antistofconcentraties tegen serotype 6B en 19F bij gevaccineerde kinderen sterk was gedaald in vergelijking met de kinderen in de MINOES studie die waren gevaccineerd voor invoering van het vaccin in het RVP. De mate van inductie van geheugen B cellen bleek echter ongewijzigd na invoering van PCV7 (**hoofdstuk 12**). Deze bevindingen suggereren dat het handhaven van hogere kapselspecifieke antistofconcentraties in het bloed sterk afhankelijk is van

natuurlijk contact met serotypen, zoals we lieten zien voor de 6B en 19F. Natuurlijke boostering zorgde voor behoud van de circulerende antistoffen en met de verminderde circulatie van deze vaccintypen valt dit weg. De wisselwerking tussen dragerschap en de pneumokokkenconjugaatvaccinatie blijkt echter nog meer complex dan enkel het wegvallen van natuurlijk boostering door dragerschap. De belangrijkste bevinding in **hoofdstuk 10** was dat gevaccineerde kinderen die eerder drager waren geweest van een bepaald vaccintype pneumokok na PCV7 toediening minder antistoffen maakten dan niet-dragers. Deze verlaagde vaccinatieresponsen komen mogelijk doordat natuurlijk dragerschap leidt tot uitputting van de kapselspecifieke geheugen B cellen.

Op dit moment weten we niet wat het verlies van natuurlijke boostering en het effect op het in bloed circuleren van kapselspecifieke antistoffen zal betekenen voor de bescherming tegen invasieve pneumokokkenziekte. Het huidige idee is dat we een herhalingsvaccinatie in het tweede levensjaar nodig hebben voor het houden van klinische bescherming zeker na het verdwijnen van de vaccintypen uit de populatie. Dit berust op de ervaring met Hib en MenC in het Verenigd Koninkrijk. In het Verenigd Koninkrijk werd na de invoering van conjugaatvaccins tegen Hib en MenC als uitsluitend primaire vaccinaties onder de leeftijd van 6 maanden zonder een latere boostervaccinatie na een paar jaar een toename van doorbraakinfecties gezien bij gevaccineerde kinderen door een afname van circulerende antistoffen. Vervolgens werd een boostervaccinatie geïntroduceerd. Het tijdstip van de boostervaccinatie mag waarschijnlijk wel variëren. In het algemeen geldt dat hoe later je vaccineert, hoe beter de respons op het vaccin. Zeker ten tijde van het eradicatie van de vaccintype pneumokokken zou een latere booster van belang kunnen zijn voor een langer en beter profijt van deze vaccinatie. In **hoofdstuk 7 en 8** beschreven we dat het op latere leeftijd toedienen van de boostervaccinatie mogelijk bij kan dragen tot langere bescherming door hogere responsen. Een late boostervaccinatie op de leeftijd van 24 maanden geeft significant meer en betere functionele kapselspecifieke antistoffen dan een vroege boostervaccinatie op 11 maanden. Met name door de indirecte bescherming na nationale invoering van PCV7 zijn er de mogelijkheden om een dergelijk vaccinatieschema uit te voeren in combinatie met gereduceerd primair schema. Men moet zich echter realiseren dat al bovengenoemd onderzoek is uitgevoerd met een conjugaatvaccin met een CRM197 dragereiwit en nieuwe conjugaatvaccins met een andere samenstelling en conjugaateiwit opnieuw onderzocht zullen moeten worden.

Conclusies

Deel 1

- Voor de introductie van het 7-valente pneumokokkenconjugaatvaccin in het rijksvaccinatieprogramma was de ziektelast van invasieve pneumokokkenziekte in Nederland hoog bij zuigelingen en peuters, senioren en mensen met een verminderde weerstand.
- Kapseltypevervanging door niet-vaccintype pneumokokken zal waarschijnlijk niet resulteren in een milder ziektebeloop of daling van sterfte na infectie door deze kapseltypes.
- Het 7-valente pneumokokkenconjugaatvaccin was in Nederland in de eerste twee jaar na invoering in het rijksvaccinatieprogramma zeer effectief tegen vaccintype invasieve infecties. Er bleek tegelijkertijd een snelle toename van niet-vaccintype pneumokokkenziekte op te treden wat het netto rendement verminderde. Er is behoefte aan een bredere dekking met méér-valente vaccins.

Deel 2

- Kapselspecifieke IgG antistofconcentraties zijn vergelijkbaar voor en na de boostervaccinatie voor 5 van de 7 vaccintypen na een 2+1 of een 3+1-schema met het 7-valente pneumokokkenconjugaatvaccin. Een 2+1-schema lijkt een goed alternatief voor een 3+1-schema met het onderzochte 7-valente vaccin.
- Twee primaire vaccinaties met het 7-valente vaccin op de leeftijd van 2 en 4 maanden resulteert in de inductie van hogere antistofconcentraties en geheugen B cellen ten opzichte van ongevaccineerde kinderen. De functionaliteit van deze antistoffen lijkt mogelijk af te nemen binnen 1 jaar na vaccinatie, waardoor een boostervaccinatie gewenst lijkt.
- Een boostervaccinatie met het 7-valente vaccin op 11 maanden na 2 primaire vaccinaties op 2 en 4 maanden resulteert niet in een betere inductie van geheugen B cellen op de leeftijd van 24 maanden ten opzichte van enkel 2 primaire vaccinaties zonder booster. Voor bepaalde vaccintypen is deze booster echter wel van waarde voor het behoud van kapselspecifieke antistoffen in bloed en speeksel.
- Een boostervaccinatie met het 7-valente vaccin op de leeftijd van 24 maanden in vergelijking met een booster op 11 maanden resulteert in hogere kwantitatieve en functionele antistofresponsen na eerder 2 primaire vaccinaties. Met het verdwijnen van de bacterie uit de circulatie na nationale introductie van een conjugaatvaccin kan een latere booster van voordeel zijn.

Deel 3

- Pneumokokkendragerschap levert een belangrijke bijdrage aan het behoud van circulerende kapselspecifieke IgG antistoffen zoals aangetoond voor serotype 6B en 19F. Pneumokokkendragerschap resulteert echter ook in een verminderde respons op een daarop volgende pneumokokkenconjugaatvaccinatie voor sommige serotypen.

Aanbevelingen

Vaccinatiebeleid in Nederland

In de eerste 2 jaar na invoering in Nederland was het 7-valente pneumokokkenconjugaatvaccin zeer effectief in het verminderen van vaccintype pneumokokkenziekte in gevaccineerde kinderen. Echter, de toename van ziekten door pneumokokken die niet gedekt worden door het huidige 7-valente vaccin verlagen het netto-effect van de vaccinatie. Indirecte effecten in ongevaccineerde mensen waren in de eerste 2 jaar na invoering van het 7-valente vaccin in het RVP nog niet zichtbaar. Op het moment van schrijven van dit proefschrift is er echter ook een duidelijke daling in ziektelast door de 7 serotypen uit het vaccin zichtbaar bij ongevaccineerde bevolkingsgroepen. De netto gezondheidswinst wordt echter gedeeltelijk teniet gedaan door opkomst van ziekte door niet-vaccintypen. Dit maakt het huidige 7-valente vaccin minder kosteneffectief dan eerder geschat voor invoering in 2006. Het verlagen van de kosten per vaccinatie, het verminderen van het aantal toegediende vaccins of het overgaan op nieuwe conjugaatvaccins werkzaam tegen meer pneumokokken kapseltypen zijn opties om te komen tot een gunstigere kosteneffectiviteitsverhouding. In een recent advies van de Nederlandse gezondheidsraad wordt een bredere dekking door nieuwe méér-valente pneumokokkenconjugaatvaccins voorgesteld, werkzaam tegen respectievelijk 10 en 13 pneumokokken kapseltypen.
Het 13-valente vaccin (PCV13) bevat 6 additionele pneumokokken kapseltypen (serotypen 1, 3, 5, 6A, 7F en 19A). In dit vaccin zijn alle kapselsuikers gekoppeld aan hetzelfde conjugaat-eiwit als dat van het huidige 7-valente vaccin (CRM197, een eiwit afgeleid van difterietoxoid). Het 10-valente vaccin (PCV10) bevat 3 additionele pneumokokken kapseltypen (serotypen 1,5 en 7F). In dit vaccin zijn 8 van de 10 serotypen gekoppeld aan een geconserveerd eiwit dat voorkomt bij de niet-typeerbare *Haemophilus influenzae* bacterie. Theoretisch zouden deze nieuwe en bredere vaccins nog 52 tot 64 gevallen van invasieve pneumokokkenziekte hebben voorkomen in de periode tussen 2006 en 2008 bij kinderen die in aanmerking kwamen voor pneumokokkenconjugaatvaccinatie.
Behalve een bredere dekking door een méér-valent vaccin, zou een gereduceerd vaccinatieschema met minder inentingen voor elke pasgeborene zowel tot minder belasting voor het kind als minder kosten voor het RVP leiden. In de landen waar na 2006 een 2+1-schema is ingevoerd werd een vergelijkbare en sterke daling in vaccintype

pneumokokkenziekte gezien als in Nederland en snellere indirecte bescherming als ook een inhaalcampagne was ingevoerd. Van het 13-valente vaccin mag na introductie van een 2+1-schema nu een even groot direct en indirect effect verwacht worden voor de serotypen van het huidige 7-valente vaccin. Voor de 6 additionele kapseltypen, met name voor de toegevoegde serotypen 1, 3, 5 en 7F moet dit in de komende jaren blijken uit gegevens van landen als het Verenigd Koninkrijk waar het 13-valente vaccin in een 2+1-schema inmiddels in ingevoerd. Voor het 10-valente vaccin, met een ander dragereiwit, zijn er op dit moment nog onvoldoende gegevens beschikbaar over het effect van de een gereduceerd vaccinatieschema op dragerschap en het vervolgens optreden van indirecte bescherming in de bevolking. Deze gegevens worden de komende jaren uit lopende onderzoeken verwacht.

Het is onwaarschijnlijk dat conjugaatvaccins alle pneumokokken serotypen zullen kunnen dekken, omdat dit er meer dan negentig zijn. Een alternatieve benadering zou kunnen zijn om eiwitvaccin met gemeenschappelijke pneumokokkeneiwitten voor alle serotypen of een "whole-cell" vaccin te ontwikkelen. Omdat de beschikbaarheid van dergelijke vaccins voor een nationaal vaccinatieschema in de nabije toekomst echter onwaarschijnlijk is, zal in de komende jaren verlaging van pneumokokken ziektelast bewerkstelligd moeten worden door conjugaatvaccins met een bredere dekking tegen meer pneumokokkentypen.

Bescherming op lange termijn

In Australië worden alleen 3 primaire doses met het 7-valente vaccin gegeven aan alle kinderen zonder een latere boostervaccinatie in het tweede levensjaar. Ook hier wordt een hoge vaccineffectiviteit waargenomen, zij het dat er een inhaalcampagne voor alle kinderen heeft plaatsgevonden. Het is interessant om de effecten op wat langere termijn in dit land te zien. We weten immers dat zonder natuurlijke circulatie van vaccintypen, de IgG antistofconcentraties in het bloed snel te dalen. Indien zou blijken dat op termijn alleen primaire vaccinaties leiden tot doorbraakinfecties, dan moet men inderdaad ook voor pneumokokken een boostervaccinatie geven. Voor een optimaal effect zou dit zo laat als kan in het tweede levensjaar moeten worden gegeven. Als men vast wil houden aan 4 vaccinaties, dan zou een 2+1-schema met een additionele booster op 24-36 maanden mogelijk een betere optie dan het huidige 3+1-schema. Surveillance data van verschillende vaccinatieschema's van landen voor langere, aaneengesloten perioden zullen duidelijk moeten maken wat het optimale schema is na nationale vaccininvoering en verminderde circulatie van vaccintypen. Het loont zeker de moeite dit te doen voor zowel effectiviteit als kosten in het RVP.

Changing target

De bevindingen in dit proefschrift benadrukken het belang van gedegen surveillance. We toonden door gedetailleerd statusonderzoek aan dat opkomende niet-vaccintypen ook ernstige invasieve ziekte veroorzaken. Betrouwbare en gedetailleerde ziektesurveillance is van essentieel belang voor een goede inschatting van de effectiviteit van vaccinatie voor verschillende klinische syndromen en vooral ook in verschillende patiëntengroepen. Ook andere vormen van monitoring, zoals dragerschap- en antistof-surveillance studies zijn van essentieel belang om kapselspecifieke ziekteincidenties goed te kunnen duiden. De relatief homogene bevolkingssamenstelling, het hoge percentage gevaccineerde zuigelingen (~95%) en de goed georganiseerde gezondheidszorg maken Nederland in het bijzonder geschikt voor studie van vaccinatie-effecten. We toonden in dit proefschrift aan dat de bescherming tegen een bacterie als *S. pneumoniae* vraagt om een dynamisch vaccinatiebeleid, aangezien dit pathogeen zich voortdurend blijkt aan te passen aan zijn omgeving. Continue herziening van vaccinatiestrategieën is dan ook nodig. Het huidige vaccinatieschema tegen pneumokokkenziekte in Nederland lijkt 4 jaar na invoering niet meer optimaal. De kosten van dergelijke surveillance programma's zijn zeer gering in vergelijking met de totale kosten van het rijksvaccinatieprogramma en hierin moet blijvend geïnvesteerd worden. Op basis van surveillance en monitoring kan het Rijksvaccinatieprogramma worden aangepast tot een minimaal belastend maar maximaal effectief schema.

Dankwoord

Het zijn mooie jaren geweest, met dit proefschrift als meest concrete eindproduct. De mensen en goede contacten zijn minder tastbare resultaten van deze periode die het benoemen waard zijn. Door de volgende woorden hoop ik iets van mijn dank hiervoor weer te geven. De samenwerking en inzet van dit team aan mensen vormden de basis van dit werk. Voordat ik dit doe wil ik echter allereerst al de kinderen en hun ouders bedanken voor hun trouwe deelname aan onze diverse studies. Dank jullie wel voor jullie bijdrage aan het verder uitbreiden van onze kennis over de pneumokok en het effect van de pneumokokkenconjugaatvaccinatie.

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Curriculum Vitae

Gerwin Rodenburg werd geboren op 1 december 1981 te Zwijndrecht. In 2000 behaalde hij op het Streeklyceum te Ede zijn atheneumdiploma inclusief 2 additionele vakken. In hetzelfde jaar begon hij aan de studie geneeskunde aan de Universiteit Utrecht. In 2004 ging hij voor een coschap tropische kindergeneeskunde naar Kagando Hospital, Oeganda. Onder begeleiding van prof. dr. ir. H. Jochemsen, Universiteit van Amsterdam en prof. dr. J.J.M van Delden, UMC Utrecht deed hij in 2005 onderzoek naar de medisch-ethische kanten van pre-symptomatische screening bij volwassen. Na zijn artsexamen in 2006 werkte hij als arts-assistent kindergeneeskunde in het Wilhelmina Kinderziekenhuis te Utrecht. Hierna startte hij met het promotieonderzoek resulterend in dit proefschrift, onder supervisie van prof. dr. Lieke Sanders, Wilhelmina Kinderziekenhuis in nauwe samenwerking met het Nationaal Referentie Laboratorium Bacteriële Meningitis (NRLBM) Amsterdam, het Nederlands Vaccin Instituut (NVI), Rijksinstituut voor Volksgezondheid en Milieuhygiëne (RIVM) te Bilthoven en het Spaarne ziekenhuis Hoofddorp. Na afronding van zijn promotieonderzoek startte hij in januari 2011 met de klinische opleiding tot kinderarts vanuit het UMC Utrecht–Wilhelmina Kinderziekenhuis, opleider dr. J. Frenkel. Op dit moment is hij werkzaam als arts-assistent in het Sint Antonius ziekenhuis te Nieuwegein, opleider dr. W.A.F. Balemans. Hij is getrouwd met Shiri Verkruijssen en samen hebben zij twee kinderen, Emme en Mats.

List of Publications

This thesis

(1) Rodenburg GD*, de Greeff SC*, Jansen AGSC, de Melker HE, Schouls LM, Hak E, Spanjaard L, Sanders EAM, van der Ende A. Effects of pneumococcal conjugate vaccine 2 years after its introduction, the Netherlands. *Emerging Infectious Diseases* 2010; 16 (5):816-823.

(2) Rodenburg GD, van Gils EJM, Veenhoven RH, Jones N, Tcherniaeva I, Hak E, van Alphen L, Berbers GA, Sanders EAM. Comparability of antibody response to a booster dose of 7-valent pneumococcal conjugate vaccine in infants primed with either 2 or 3 doses. *Vaccine* 2010; 28 (5):1391-1396.

(3) Jansen AGSC*, Rodenburg GD*, de Greeff SC, Hak E, Veenhoven RH, Spanjaard L, Schouls LM, Sanders EAM, van der Ende A. Invasive pneumococcal disease in the Netherlands: Syndromes, outcome and potential vaccine benefits. *Vaccine* 2009; 27 (17):2394-2401.

(4) Jansen AGSC, Rodenburg GD, van der Ende A, van Alphen L, Veenhoven RH, Spanjaard L, Sanders EAM, Hak E. Invasive pneumococcal disease among adults: associations among serotypes, disease characteristics, and outcome. *Clinical Infectious Diseases* 2009; 49 (2):e23-e29.

(5) Jansen AGSC, Hak E, Rodenburg GD, van der Ende A, Spanjaard L, Veenhoven RH, Sanders EAM. Outcome of childhood invasive pneumococcal infection by serotype: retrospective case series. *Vaccine*, Accepted for publication.

(6) Rodenburg GD, Sanders EAM, van Gils EJM, Veenhoven RH, Tcherniaeva I, van Gaans-van den Brink JAM, van Dijken H, van Westen E, Berbers GA, van den Dobbelsteen GPJM. Lower antibody responses to pneumococcal conjugate vaccination at the age of 2 years after previous nasopharyngeal carriage of *S. pneumoniae*. Submitted.

(7) Rodenburg GD, Sanders EAM, van Gils EJM, Veenhoven RH, Tcherniaeva I, van Gaans-van den Brink JAM, van Dijken H, van Westen E, Berbers GA, van den Dobbelsteen GPJM. Induction of long-term immunity after reduced-dose schedules with 7-valent pneumococcal conjugate vaccine in children; antibody responses, avidity maturation and memory B cells. Submitted.

(8) Rodenburg GD, Sanders EAM, van Gils EJM, Veenhoven RH, Zborowski T, Bloem A, Berbers GA, van den Dobbelsteen GPJM, Bogaert D. Mucosal immune responses to the 7-valent pneumococcal conjugate vaccine in the first 2 years of life. Submitted.

(9) Rodenburg GD, Berbers GA, van Gils EJM, Veenhoven RH, Tcherniaeva I, G van den Dobbelsteen GPJM, Sanders EAM. Impact of decreased nasopharyngeal colonization of vaccine-serotype *S. pneumoniae* on antibody persistence in children after introduction of heptavalent pneumococcal conjugate vaccination. Submitted.

(10) Rodenburg GD, Sanders EAM, Veenhoven RH, Wijmenga-Monsuur A, Berbers GA, van den Dobbelsteen GPJM. B cell memory in childhood after 3 primary doses with the 7-valent pneumococcal conjugate vaccine after nationwide introduction. In preparation.

(11) Rodenburg GD, van Westen E, van Gils EJM, Bogaert D, Zborowski T, Veenhoven RH, Tcherniaeva I, van Dijken H, Berbers GA, van den Dobbelsteen GPJM, Sanders EAM. Antibody levels and functionality after reduced-dose schedules with the pneumococcal conjugate vaccine into the second year of life. In preparation.

Other publications

(12) van Gils EJM, Veenhoven RH, Hak E, Rodenburg GD, Bogaert D, IJzerman EP, Bruin JP, van Alphen L, Sanders EAM. Effect of reduced-dose schedules with 7-valent pneumococcal conjugate vaccine on nasopharyngeal pneumococcal carriage in children: a randomized controlled trial. *JAMA* 2009; 302 (2):159-167.

(13) van Gils EJM, Veenhoven RH, Hak E, Rodenburg GD, Keizers WCM, Bogaert D, Trzcinski K, Bruin JP, van Alphen L, van der Ende A, Sanders EAM. Pneumococcal conjugate vaccination and nasopharyngeal acquisition of serotype 19A strains. *JAMA* 2010; 304 (10):1099-1106.

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(15) van Wessel K, Rodenburg GD, Veenhoven RH, Spanjaard L, van der Ende A, Sanders EAM. *Nontypeable Haemophilus influenzae* invasive disease in the Netherlands; a retrospective surveillance study 2001-2009. *Clinical Infectious Diseases*, Accepted for publication.

(16) Rodenburg GD*, Fransen F*, Bogaert D, Schipper K, Claus H, Vogel U, Groenwold RHH, Hamstra HJ, Westerhuis BM, van de Beek D, van der Ley P, Sanders EAM, van der Ende A. Meningococcal endotoxin variants having impact on carriage and disease. Submitted.

(17) van Lier EA, van der Maas AT, Rodenburg GD, Sanders EAM, de Melker HE. Hospitalization due to varicella in the Netherlands. Submitted.