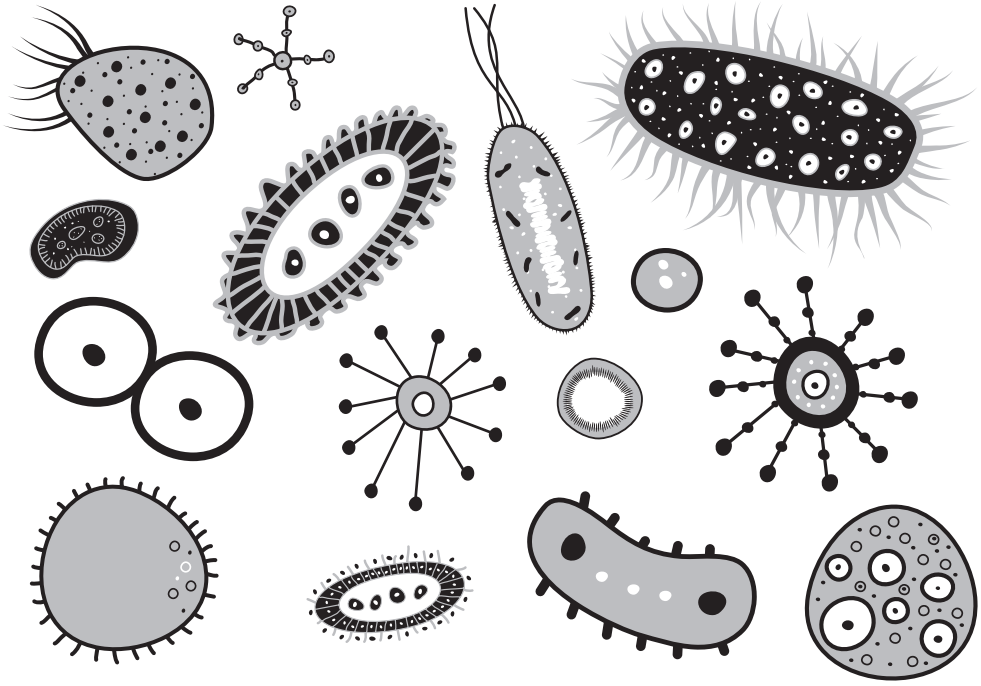
NASOPHARYNGEAL
COLONIZATION



with
RESPIRATORY
PATHOGENS

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Nasopharyngeal colonization with respiratory pathogens

Nasopharyngeale kolonisatie met respiratoire pathogenen

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof.dr. G.J. van der Zwaan, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op vrijdag 19 april 2013 des middags te 12:45 uur.

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"If we knew what it was we were doing, it would not be called research, would it?"
—Albert Einstein

Voor mijn lieve ouders;
voor Barbara, Daniël en Robin

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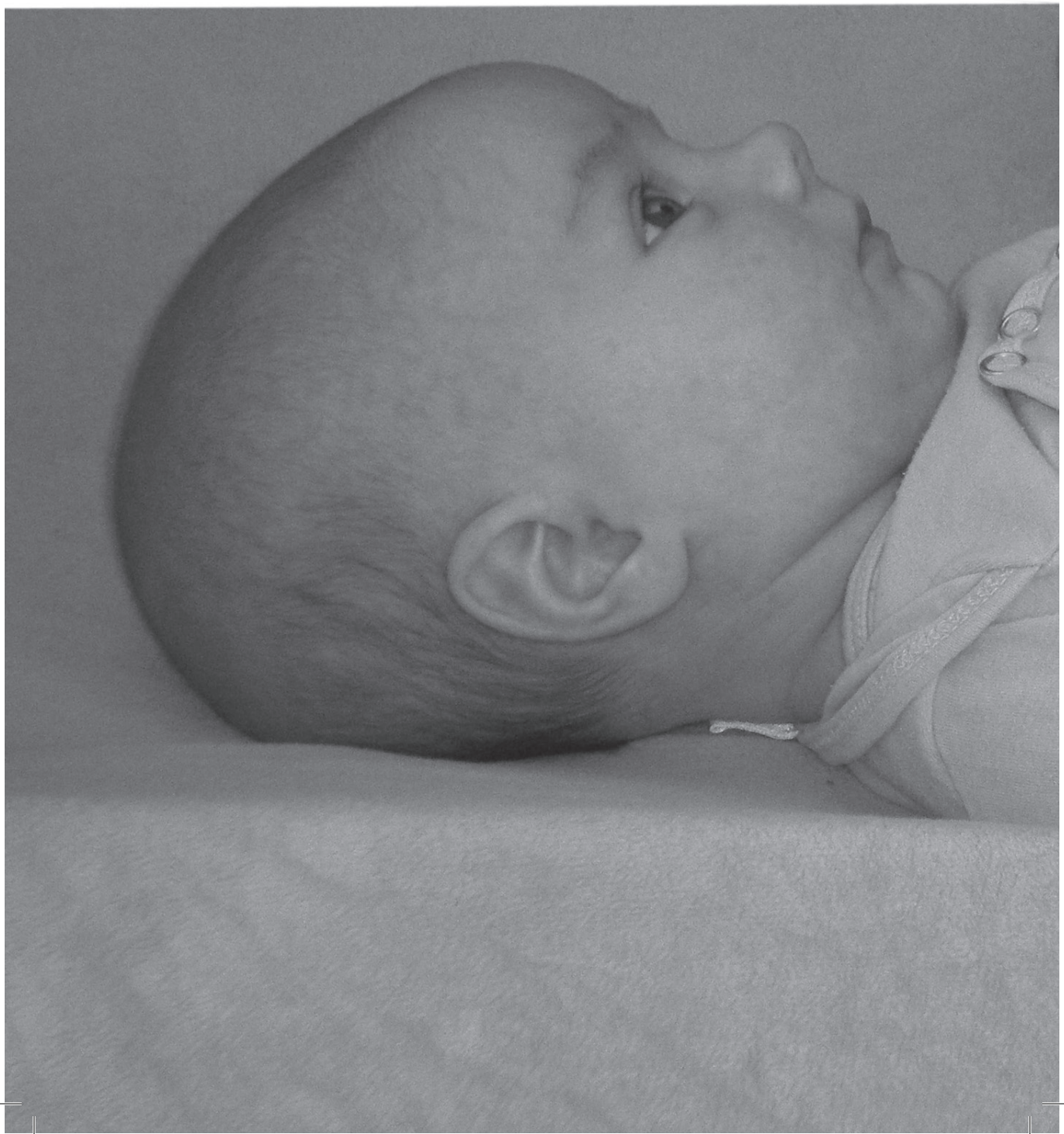

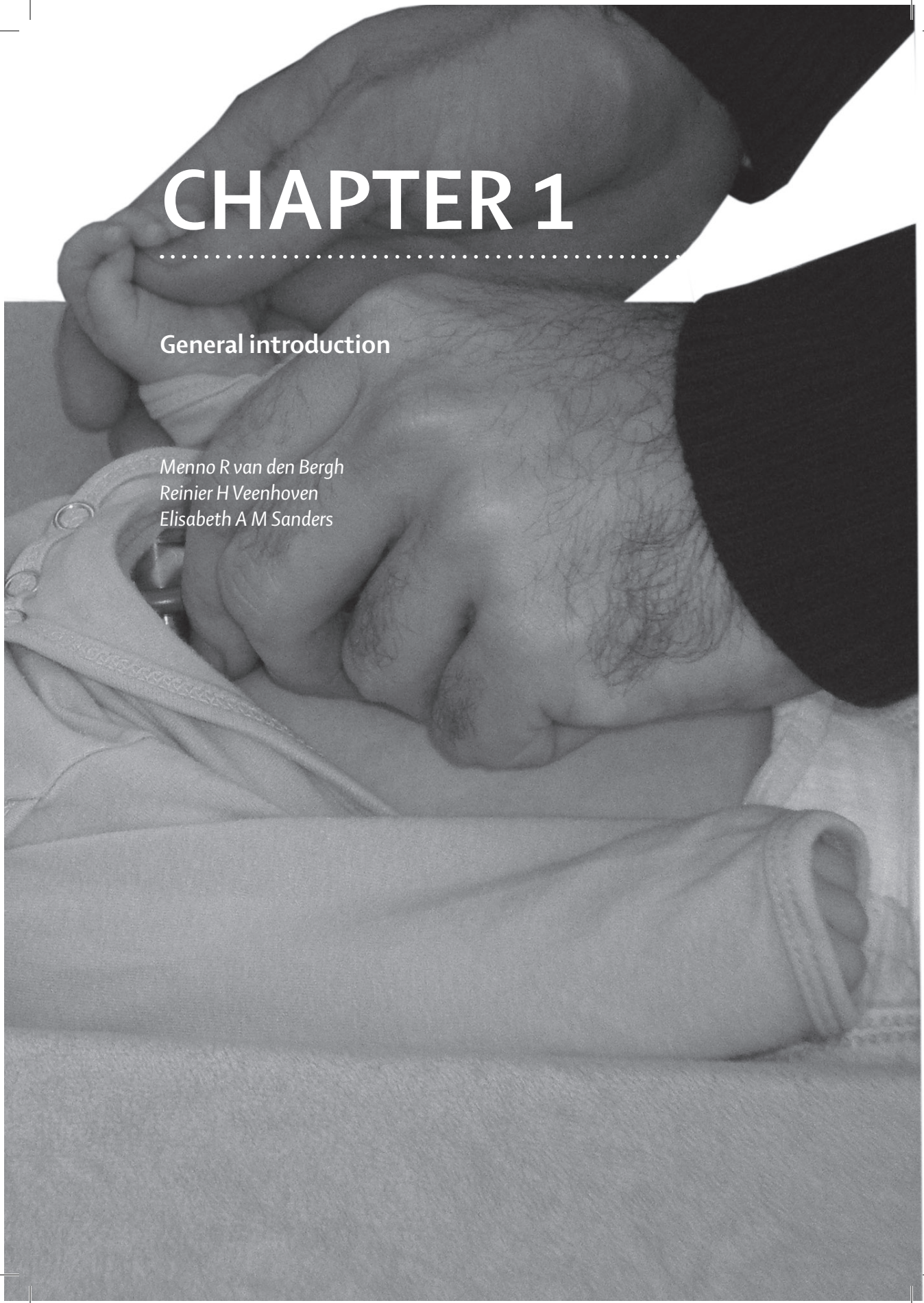

CHAPTER 1

General introduction

Menno R van den Bergh
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Infectious diseases are a major cause of morbidity and mortality worldwide. Pneumonia kills more children than any other illness—more than malaria, AIDS and measles combined, mainly in developing countries.[1,2] In 2010, bacterial pneumonia and invasive diseases caused nearly one in five deaths in children under five years of age worldwide, totaling 1.4 million.[3] Although rarely lethal in developed countries like the Netherlands, childhood pneumonia is still an important reason for hospitalization.[4] Pneumonia also causes substantial morbidity and mortality in the elderly.[5] Community-acquired pneumonia results in an estimated 350,000 to 620,000 hospitalizations per year in the United States alone.[6] The bacterium *Streptococcus pneumoniae* (pneumococcus) is the major pathogen believed to cause invasive diseases and pneumonia in both children and older adults. The incidence of respiratory and invasive pneumococcal disease (IPD, consisting of meningitis, sepsis and bacteremic pneumonia) typically follows a U-shaped curve, peaking at the extremes of life (Figure 1).

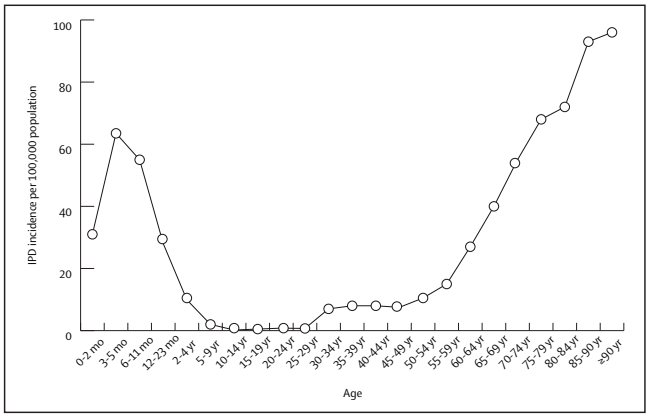

***Figure 1:* IPD incidence by age in the Netherlands, 2004-2006.** Adapted from Ref. 4

Apart from pneumonia, acute otitis media (AOM) is one of the most frequently-diagnosed childhood infection worldwide — far more common than pneumonia.[7] Its incidence peaks in children between 6 and 18 months of age. In developed countries, 80% of all children has suffered from AOM at least once by their third birthday,[8,9] and at seven years of age, 40% had six or more recurrences.[10] Although in most children the natural course is favorable, it is a leading cause of pediatric medical visits and antibiotic prescription. It is also the main reason for childhood surgery, such as ventilation tube placement or adenoidectomy.[11] In addition, complications and sequelae like intracranial infection and hearing loss due to chronic suppurative otitis cause substantial morbidity and even mortality in developing countries.[11] Altogether, these features mark AOM as a considerable burden of disease.[7,12]

Pneumonia and AOM specifically are polymicrobial diseases, meaning that a variety of pathogens, including respiratory viruses and bacteria, either separately or combined, can result in a symptomatic infection.[11,13] *Streptococcus pneumoniae* and nontypeable *Haemophilus influenzae* (NTHi) are recognized as the two leading bacterial pathogens causing these respiratory infections. Therefore, research in the last decades has focused on unravelling the pathogenesis of these infections and the continuous development of effective vaccines to target these microorganisms. Questions remain about vaccine effects, potential interactions between viral and bacterial pathogens, methodology, and prevalence of *S. pneumoniae* in subpopulations. This thesis aims to address each of these topics in-depth. However, some background to the current knowledge of this field of research is essential.

S. pneumoniae is a Gram-positive diplococcus categorized by the composition of its polysaccharide capsule.[14] To date, over 94 structurally and antigenically different serotypes have been described.[15] The polysaccharide capsule is a major virulence factor,[16] preventing entrapment in nasal mucus and inhibiting effective opsonophagocytosis.[17] Unencapsulated pneumococci are generally regarded to be avirulent. Capsular polysaccharides have therefore been the primary target in the development of pneumococcal vaccines.

H. influenzae is a Gram-negative, rod-shaped bacterium divided into encapsulated (serotypes labeled a to f) and unencapsulated or nontypeable strains (NTHi). Like *S. pneumoniae*, the polysaccharide capsule of encapsulated *H. influenzae* is a major virulence factor. For NTHi, several protein antigens have been proposed as potential vaccine candidates. One of these is protein D (PD), an outer membrane lipoprotein highly conserved among *Haemophilus* strains.[18] It is a well-described virulence factor and animal studies showed that anti-PD antibodies protect against NTHi disease.[19,20]

From colonization to disease

The human nasopharynx, a specific part of the upper respiratory tract, is the reservoir and natural habitat for *S. pneumoniae*, *H. influenzae*, and other bacterial pathogens. Transmission is thought to occur by airborne droplets or through direct contact with secretions of colonized individuals.[17] Upon acquisition, bacteria interact with each other and the host immune system before being cleared.[21]

S. pneumoniae

The continuous process of acquisition, colonization, and clearance is most extensively studied for *S. pneumoniae*. Acquisition and subsequent colonization with a new pneumococcal serotype usually proceeds asymptomatically. Clearance is mediated by two arms of the immune system: antibody- and T cell-mediated immunity.[22-24] *S. pneumoniae* colonization is

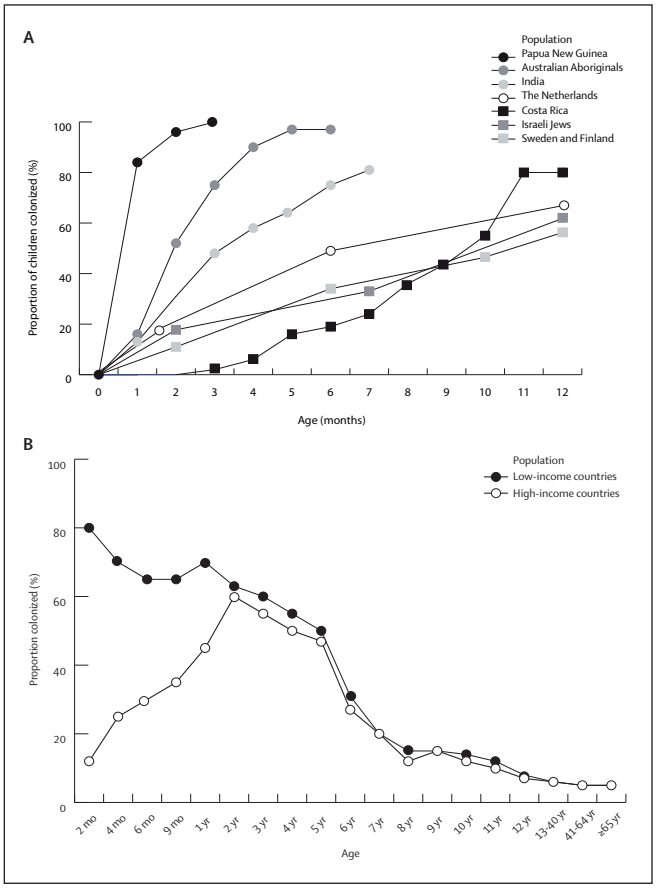

Figure 2: **Prevalence of nasopharyngeal pneumococcal colonization during infancy (A) and later in life (B)**. Adapted from Ref. 25, 26 (A) and 27 (B).

dependent on age (Figure 2) and ethnic background, but also influenced by environmental conditions and exposure.[21] Pneumococcal colonization rates are highest in infants and toddlers with estimates of up to 60-70% in industrialized countries and approaching 100% in high-risk populations (Fig. 2a).[25,26] From around three to five years of age, colonization rates gradually decrease to approximately 10-20% in adulthood (Fig. 2b). Only limited data are available for the elderly, but nasopharyngeal *S. pneumoniae* colonization as measured by conventional culture methods is reported to be low, ranging from virtually non-existent to 5%.[28-30] However, it is uncertain whether this reflects the true prevalence, since low-density colonization can be missed when using conventional culture only.[31]

Under certain conditions, *S. pneumoniae* can spread from the nasopharynx to the surrounding tissue, causing mucosal diseases like pneumonia or AOM via the Eustachian tube. More rarely, it causes life-threatening IPD by breaching the mucosal barrier and invading the blood stream (Figure 3).

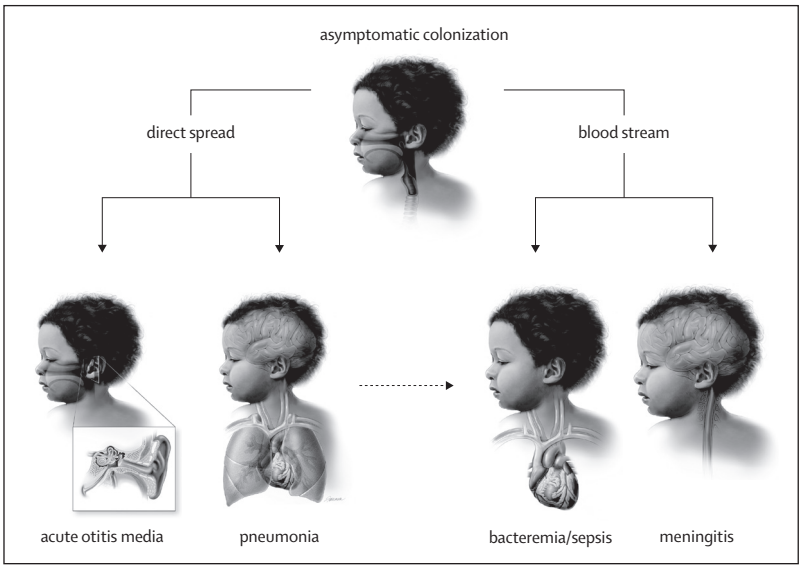

***Figure 3*: Nasopharyngeal acquisition: a prerequisite for pneumococcal disease.**
Courtesy of pneumoACTION of the International Vaccine Access Center (IVAC) at Johns Hopkins Bloomberg School of Public Health (available at: http://www.preventpneumo.org/data-tools/slides.cfm)

H. influenzae

Like *S. pneumoniae*, NTHi colonization rates are highest in infants and toddlers. Point estimates for the prevalence in the first two years of life range from 10 to 60%,[32,33] and essentially every child is colonized at some time.[34] High transmission and colonization rates occur among children in daycare centers.[35,36] In the pre-vaccine era, *H. influenzae* type b was a dreaded cause of meningitis, epiglottitis and pneumonia. Unencapsulated strains rarely cause invasive disease in healthy children and adults,[37] but are often involved in respiratory infections.[34,38] NTHi is the second most common bacterial pathogen involved in AOM and particularly associated with recurrent AOM episodes, presumably through biofilm formation.[39,40] The relationship between NTHi colonization and disease is, however, less characterized than for *S. pneumoniae*.

Microbial interactions

Although most research has focused historically on single pathogens, infectious diseases often occur in the context of pre-existing viral and bacterial infections. Probably the best-recognized and most-studied viral-bacterial interaction is that between influenza virus and *S. pneumoniae*.[41] The 1918 influenza pandemic caused an estimated 20 to 50 million deaths worldwide, and bacterial coinfection, notably *S. pneumoniae*, played a major role.[42] Strong and consistent evidence exists of clinically important interactions between influenza and secondary bacterial pathogens,[43,44] including the association between IPD and the 2009 H1N1 pandemic.[45]

Colonization with bacterial pathogens and progression toward inflammation with clinical symptoms are both likely promoted by viral infections. In vitro and animal studies demonstrated that viruses can enhance adherence of bacteria to respiratory epithelium.[46,47] In addition, increased pneumococcal colonization rates have been reported during a viral infection.[28,46,48,49] Interactions between viruses and bacteria may potentially be reflected in co-occurrence patterns in the nasopharyngeal niche—the point of origin for respiratory and invasive infections. Importantly, AOM is considered to result from the interplay between the microbial load of viruses and bacteria and the immune response.[11] Figure 4 illustrates mechanisms by which viruses affect bacterial colonization (Fig. 4a) and invasion (Fig. 4b).

Pneumococcal conjugate vaccination

A bacterial capsular polysaccharide is poorly immunogenic in children under two years of age. However, covalently binding (conjugation) of a polysaccharide to a carrier protein produces a T cell-dependent immune response. As a result, protein-polysaccharide conjugate vaccines induce production of functional antibodies and immunologic memory, even in early infancy.[50]

The first pneumococcal conjugate vaccine (PCV) was licensed for children in 2000 in the United States.[51-53] In this vaccine, polysaccharides of serotypes 4, 6B, 9V, 14, 18C, 19F, and 23F were each conjugated to cross-reactive material 197 (CRM197, a nontoxic diphtheria mutant) as the carrier protein (7vCRM; Prevenar™/Prevnar™, Pfizer, Inc.). Before introduction, these seven serotypes covered >80% of invasive isolates in US children under five years of age.[54] In the US, 7vCRM was implemented as a 3+1-dose schedule with the primary doses administered at 2, 4, and 6 months of age followed by a booster dose at 15-18 months of age.

Efficacy against IPD, pneumonia and AOM

PCVs are proven to be highly efficacious in preventing vaccine serotype IPD in infants and toddlers, with efficacy estimates ranging from 77 to 100% (Table 1).[51-53,55,56] In addition, efficacy estimates of 20 to 40% for radiologically-confirmed pneumonia and around 6% for clinical (all-cause) pneumonia have been reported.[57-59] For pneumonia, vaccine efficacy estimates have been fairly comparable across studies, geographical areas and vaccine formulations (Table 1).

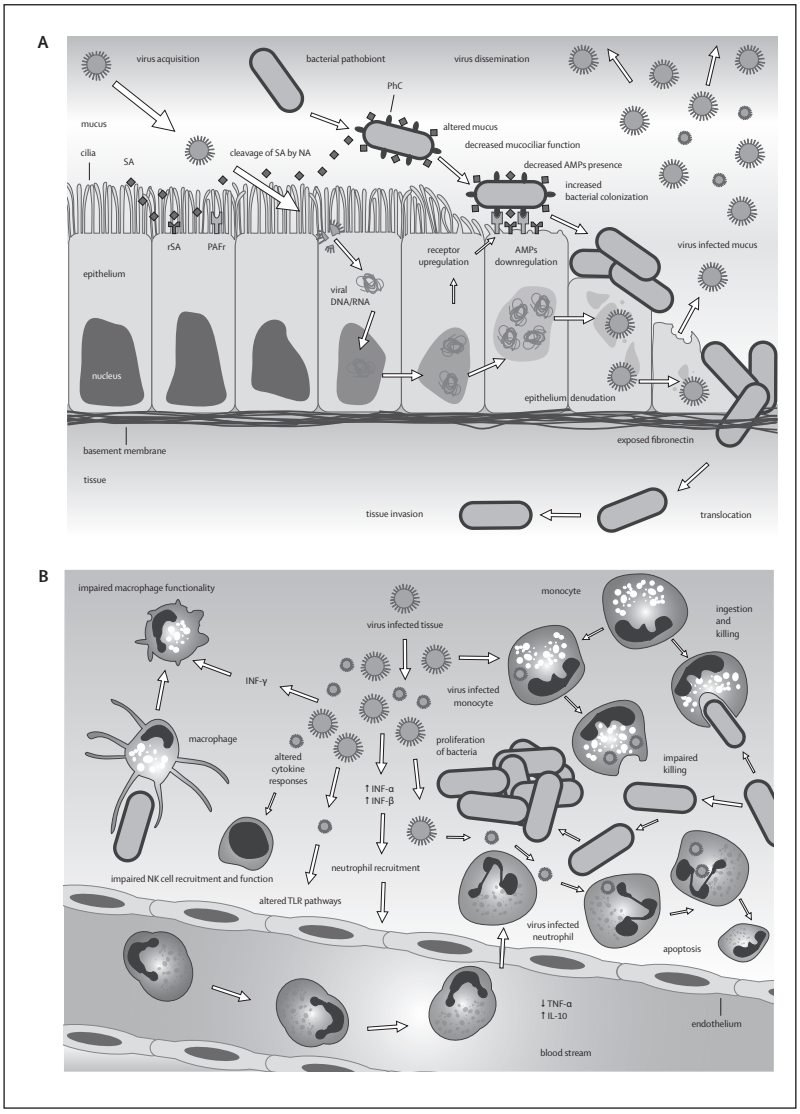

Figure 4: **Viral-bacterial interactions at the respiratory mucosal surface (A) and in relation to the host immune system (B).**
A Viruses may render the epithelium more susceptible to bacterial colonization by altering the mucosal surfaces. Ciliae may be damaged, leading to decreased mucociliar function of the respiratory epithelium. Additionally, due to viral-induced damage and loss of integrity of the epithelium layer, bacterial colonization may be enhanced and translocation may be increased. Virus-infected cells may decrease the expression of antimicrobial peptides. Viral neuraminidase (NA) activity is able to cleave sialic acids residues, thereby giving access to bacterial receptors that were covered by these residues. Finally, viruses may induce bacterial colonisation and replication both directly and indirectly, the latter by inducing upregulation of various receptors required for bacterial adherence. Abbreviations: PAFr, platelet activating factor receptor; SA, sialic acids; NA, neuraminidase; mRNA, messenger RNA.
B Viruses may also induce changes in immune function favourable to bacterial invasion: fewer natural killer (NK)-cells may be recruited into the tissue and their functionality may be suboptimal as a consequence of viral infection. Virus-induced INFN-α and IFN-β may impair recruitment and functionality of neutrophils, and subsequently induce apoptosis of neutrophils recruited to combat the viral invader. Furthermore, IFN-γ seems to negatively affect the activity of macrophages. Viral-infected monocytes appear less effective in ingesting and killing bacteria, predisposing to bacterial overgrowth and invasion. Viral infection seems to impair TLR-pathways, induce production of the anti-inflammatory cytokine IL-10, and decrease the concentration of the pro-inflammatory cytokine TNF-α. Abbreviations: IFN, interferon; TNF, tumor necrosis factor; TLR, toll like receptor; IL, interleukin; NK cell, natural killer cell.
Courtesy of A. Bosch and D. Bogaert

Population	California, USA[51]	American Indians[52]	Soweto, S-Africa[53]	The Gambia[55]	Philippines[58]	Finland[56]	Latin America[59]
Vaccine	7vCRM	7vCRM	9vCRM	9vCRM	PCV-11	PHiD-CV	PHiD-CV
No. of subjects	38,000	8,300	40,000	17,400	12,200	47,500	23,800
IPD							
Overall	89% (75 to 95)	46% (-6 to 73)	35% (-31 to 68)	45% (19 to 62)	NA	NA	NA
Vaccine serotypes	94% (80 to 98)	83% (22 to 96)	77% (19 to 93)	77% (49 to 91)	NA	100% (83 to 100)	NA
Pneumonia							
Radiologically-confirmed	26% (7 to 40)	NA	20% (3 to 35)	NA	23% (-1 to 41)	NA	23% (9 to 36)
All clinically diagnosed	6% (-2 to 11)	NA	7% (-1 to 14)	7% (1 to 12)	NA	NA	7% (2 to 12)

Table 1: **Pneumococcal conjugate vaccine efficacy against invasive pneumococcal disease (IPD) and pneumonia.** Data are vaccine efficacy estimates (95% confidence interval), unless otherwise stated. Abbreviations: 7vCRM, 7-valent pneumococcal CRM197-conjugate vaccine (Prevenar™, Pfizer, Inc.); 9vCRM, 9-valent pneumococcal CRM197-conjugate vaccine (unlicensed); PCV-11, 11-valent pneumococcal tetanus-diphtheria-conjugate vaccine (unlicensed); PHiD-CV, 10-valent pneumococcal nontypeable *H. influenzae* protein D-conjugate vaccine (Synflorix™, GlaxoSmithKline Vaccines); NA, not available.

Table 2 shows vaccine efficacy estimates against AOM for two PCVs in different trials.[51,61,62] In the Finnish Otitis Media trial (FinOM), 7vCRM reduced AOM caused by vaccine serotypes by 57%, but all-cause AOM was not significantly reduced (6%; 95% confidence interval (CI), -4 to 16%). This efficacy estimate for overall AOM was in line with results of the Northern California Kaiser Permanente trial (Table 2). In the Pneumococcal Otitis Efficacy Trial (POET), an experimental 11-valent vaccine was evaluated in which each polysaccharide from serotypes 1, 3, 4, 5, 6B, 7F, 9V, 14, 18C, 19F, and 23F was conjugated to NTHi-derived protein D (11Pn-PD, GlaxoSmithKline). 11Pn-PD was similarly efficacious in preventing vaccine serotype AOM as 7vCRM, but, in contrast, significantly reduced all-cause AOM by 34%.[62]

Two key findings offer an explanation for the divergent results of FinOM and POET. First, the decline in AOM caused by vaccine serotypes in FinOM was opposed by an increase in AOM caused by nonvaccine serotypes.[61] This kind of replacement was not observed in POET. Second, an increase in AOM caused by *H. influenzae* was found in FinOM, albeit not statistically significant.[61] In contrast, efficacy against NTHi-caused AOM was shown in POET (35%; 95% CI, 2 to 57%),[62] an effect attributed to the use of NTHi-derived PD as the carrier protein.[19] Nonetheless, differences in the trial results of POET and FinOM remained difficult to reconcile. For instance, the AOM incidence in POET was one-tenth of the incidence observed in Finnish children (1.24 versus 0.13 episodes per person year, respectively).[61,62] POET was however not designed or aiming to include every single AOM episode,[62] making it likely that ascertained cases were more severe than those in FinOM. While this has the potential to inflate efficacy estimates, differences in case definitions were later shown to not fully explain the observed differences.[63]

	Northern Kaiser Permanente trial	Finnish Otitis Media trial (FinOM)	Pneumococcal Otitis Efficacy Trial (POET)
Author	Black, et al.[51]	Eskola, et al.[61]	Prymula, et al.[62]
Vaccine	7vCRM	7vCRM	11Pn-PD
No. of subjects	38,000	1,662	4,968
Incidence (episodes/person year)	1.72	1.24	0.13
AOM			
Overall	6% (4 to 9)	6% (-4 to 16)	34% (21 to 44)
Vaccine serotypes	NA	57% (44 to 67)	58% (41 to 69)
Nonvaccine serotypes	NA	-33% (-80 to 1)	9% (-64 to 49)
Haemophilus influenzae	NA	-11% (-34 to 8)	35% (2 to 57)

Table 2: **Pneumococcal conjugate vaccine efficacy against acute otitis media (AOM).** Data are vaccine efficacy estimates (95% confidence interval), unless otherwise stated. Abbreviations: 7vCRM, 7-valent pneumococcal CRM197-conjugate vaccine (Prevenar,™ Pfizer, Inc.); 11Pn-PD, 11-valent pneumococcal nontypeable *H. influenzae* protein D-conjugate vaccine (unlicensed, GlaxoSmithKline Vaccines); NA, not available.

Efficacy against nasopharyngeal colonization

In general, PCVs reduce vaccine serotype colonization by 40 to 60%,[62,64-66] even after reduced-dose schedules.[26] This is similar to the decline in AOM caused by vaccine serotypes. Besides decreasing the crude presence of pneumococci (as determined by conventional culture methods), the density of colonizing vaccine serotypes may also be reduced.[67] In the nasopharyngeal niche, replacement by (or unmasking of) nonvaccine serotypes almost completely offsets the reduction of vaccine pneumococcal serotypes, leaving overall pneumococcal colonization rates nearly unchanged.[26,68-70] Although the prevalence of several nonvaccine serotypes increased, serotype 19A is the main replacing serotype following 7vCRM introduction, both in colonization and disease.[69,71-74] Despite other possible explanations for this increase of 19A, such as natural trends and antibiotic resistance,[75-77] vaccination with 7vCRM actually creates a "vacant niche" in the nasopharynx which is invaded by serotype 19A.[68] In addition, nonpneumococcal species seem to (temporarily) occupy the niche. For example, carriage of *Staphylococcus aureus* doubled shortly after the booster dose in healthy 7vCRM-vaccinated children compared to unvaccinated controls.[78] This is in line with a previous randomized study in otitis-prone children, showing increased involvement of *S. aureus* in AOM after pneumococcal vaccinations.[66]

Herd effects

Due to its effects on nasopharyngeal colonization, PCVs effectively prevent transmission of vaccine-targeted pneumococcal strains to others.[79] This has resulted in an unexpectedly large indirect (or herd) protection of the unvaccinated population. After introduction of 7vCRM in the US, substantial reductions in the incidence of IPD were observed in untargeted age groups as well, particularly in adults over 65 years.[80,81] In fact, it has been estimated that two thirds of the overall vaccine effectiveness against IPD could be attributed to herd effects.[82,83] Furthermore, widespread use of 7vCRM yielded greater than expected reductions in pneumonia and AOM than based on results of randomized trials.[17,84-87] The magnitude of these herd effects greatly accelerated the overall public health impact and cost-effectiveness of the

vaccine.[88] After using 7vCRM over a decade in the US, vaccine serotype IPD almost disappeared.[89,90] The same holds true for vaccine serotype AOM and colonization.
However, replacement by nonvaccine pneumococcal serotypes counteracts the great success of PCVs.[91] Whereas in colonization complete replacement was already observed in randomized studies evaluating experimental PCVs,[92] increases in nonvaccine serotypes only partially counterbalanced the reduction in vaccine serotype IPD after 7vCRM introduction in most countries.[91] These nonvaccine serotype strains tend to be less invasive than the vaccine serotypes.[93] Yet, as briefly mentioned, nonvaccine serotype 19A particularly emerged in colonization, AOM and IPD following universal infant vaccination with 7vCRM.[71,72,74]

Investigating the effects of PCVs on nasopharyngeal colonization in randomized trial settings is very useful for two main reasons. First, direct effects on vaccine and nonvaccine serotypes and, potentially, nonpneumococcal bacterial species can be readily detected with relatively small sample sizes.[26,78] The impact on colonization can thus be used as a functional marker to predict effects on disease endpoints.[87] Second, since colonization reduction is the mechanism through which herd immunity is conferred, impact on bacterial colonization is a predictor of herd effects. Therefore, the PneumoCarr Consortium, consisting of nine research institutes from different parts of the world (http://www.ktl.fi/roko/pneumocarr/), aimed to incorporate evaluation of the effects of new pneumococcal vaccine formulations on nasopharyngeal colonization as part of the registration process.[87]

Sampling techniques

A World Health Organization (WHO) working group recommended standardizing the methods used to assess nasopharyngeal colonization to facilitate comparisons of results between pneumococcal vaccination trials.[25] Because the preferred niche for *S. pneumoniae* (and NTHi) is the nasopharynx, a deep transnasal swab needs to be obtained reaching the posterior wall.[25] Nasopharyngeal sampling may, however, not always be well tolerated—especially when swabs are taken repeatedly. Various alternative sampling methods have been explored to detect respiratory pathogens.[94-99] In children with visible nasal secretions, Leach *et al.* showed that *S. pneumoniae* was detected at similar frequencies in cultures of samples obtained by simply wiping the child's nose with a paper tissue as in cultures of nasal swabs.[100] Nonetheless, direct culture of a paper tissue containing nasal secretions has not previously been compared to the current standards for detecting bacterial pathogens.

Current licensure criteria based on antibodies

It has become increasingly problematic to conduct efficacy trials evaluating clinical endpoints, especially IPD. IPD is relatively rare and, as a result, these studies generally require enrolment of tens of thousands of subjects (see Table 1). In addition, many countries have already introduced PCVs, which negates the possibility of using a non-PCV as a control. Consequently, licensure of new PCVs is at present based on serological criteria (Table 3). Specifically, noninferiority of the immune response of a new vaccine compared with a registered product (7vCRM) should be demonstrated in terms of the proportions of children reaching a predefined antibody concentration. Through pooling IPD efficacy data from three pivotal clinical trials,[51-53] a putative protective mean antibody concentration of 0.35 µg/mL was derived for anticapsular antibodies to the seven serotypes in 7vCRM one month after the third primary dose.[101] The WHO has recommended using this threshold as a benchmark for protection against IPD.[102] The antibody concentration of 0.35 µg/mL was measured using an enzyme-linked immunosorbant assay (ELISA) without pre-adsorption with serotype 22F. This level

Noninferiority criteria

- IgG antibody concentration (ELISA) in sera collected 4 weeks after a 3-dose primary series. A single threshold of 0.35 µg per mL is recommended, defined based on data obtained using ELISA without pre-adsorption with serotype 22F
- Direct clinical comparison of the registered vaccine with the new one is the preferred method evaluating new vaccine formulations
- Percentage of responders (those in whom antibody concentration exceeds the threshold) should be used as noninferiority criterium
- For serotypes present in a registered vaccine, the percentage of responders to each serotype in the new formulation or combination should be compared with the percentage of responders to the same serotype in the registered vaccine in the same population. Noninferiority for each serotype is desirable, but not an absolute requirement. Serotypes not contained in a registered formulation may be evaluated for noninferiority to the aggregate response to the serotypes in the registered vaccine.

Additional criteria

Functional antibodies

- Opsonophagocytic activity as measured by opsonophagocytic assay (OPA) after a three-dose priming series is required to demonstrate the functionality of antibodies. The method used to demonstrate OPA should be comparable to the reference assay.

Immunological memory

- Evidence of memory should be demonstrated. One possible method is to administer a booster dose of pneumococcal polysaccharide vaccine and to compare concentrations between age-matched unprimed and primed individuals; data from non-concurrent controls may be sufficient for the purposes of comparison.
- Avidity of antibodies is also a useful marker for immunological memory.

Table 3: **WHO criteria for licensure of new pneumococcal conjugate vaccines.**

was shown to be equivalent to 0.20 µg/mL when measured using the more specific third-generation 22F-ELISA.[103,104] In addition, the functionality of antibodies should be tested (Figure 5).

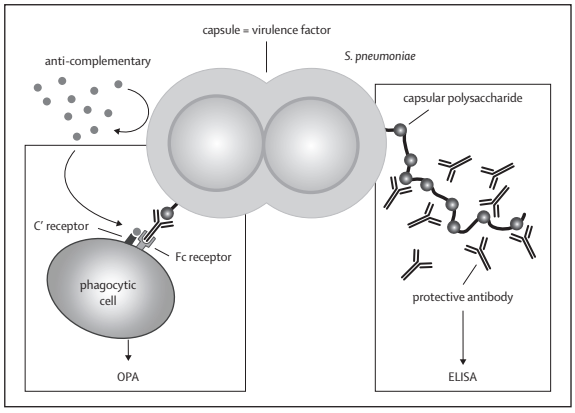

Figure 5: **Measuring anticapsular antibodies elicited by PCVs.** Abbreviations: ELISA, enzyme-linked immunosorbant assay; OPA, opsonophagocytic assay

In most immunization schedules, PCVs are concomitantly administered with other routinely used pediatric vaccines. Coadministration of various vaccines offers the advantage of preventing multiple infectious diseases while minimizing the number of vaccination visits needed. However, a limited number of carrier proteins are employed in protein-polysaccharide conjugate vaccines in clinical use, such as tetanus toxoid, diphtheria toxoid or its genetic mutant form CMR197. This has led to the problem of attenuated immune responses when vaccines using the same or similar carrier proteins are coadministered.[105,106]

Hyporesponsiveness has been reported even when vaccines with unrelated carriers are co-adminstered.[106,107] Conversely, enhancement of immune responses has also been described.[105,106] The precise effects of possible immune interactions on elicited immune responses (i.e., impairment or enhancement) when vaccines are coadministered are therefore difficult to predict.[105] A negative impact on immunogenicity may potentially result in diminished efficacy against one or several antigens of either of the coadministered vaccines. Consequently, as new vaccines are introduced into immunization programs, it is important to assess the immunogenicity (and safety profiles) of vaccines that are likely to be concomitantly administered.

In the Netherlands, 7vCRM was introduced as a 3+1-dose schedule in the national immunization program (NIP) for all infants born after March 31, 2006 and administered at 2, 3, and 4 months of age followed by a booster dose at 11-12 months of age.[108] At the time, this coincided with administration of a diphtheria-tetanus-acellular pertussis-inactivated polio and Hib combination vaccine (DTPa-IPV-Hib; Pediacel™, Sanofi Pasteur MSD).

PHiD-CV, a second-generation pneumococcal conjugate vaccine

The 10-valent pneumococcal nontypeable *Haemophilus influenzae* protein D-conjugate vaccine (PHiD-CV; Synflorix™, GlaxoSmithKline Vaccines) is based on the 11-valent vaccine in POET and the first licensed vaccine using NTHi-derived PD as a carrier protein. PD potentially serves a dual role: (1) as an effective alternative carrier that may avoid immunologic interference, and (2) as an antigen eliciting a protective response against NTHi.[62] Compared with the unlicensed 11-valent variant, serotype 3 was dropped and in PHiD-CV 8 out of 10 serotypes were conjugated PD. Serotype 18C and 19F were conjugated to tetanus toxoid and diphtheria toxoid, respectively. PHiD-CV was first licensed in Europe in 2009 on the basis of an immune response comparable to 7vCRM as well as on AOM efficacy data of the previous 11-valent vaccine.[62,109] Recently, data from a large-scale randomized trial in Finland provided confirmatory evidence that PHiD-CV is efficacious in preventing IPD.[109] PHiD-CV was evaluated when coadministered with various pediatric vaccines,[110,111] but no data were available with respect to coadministration with DTPa-IPV-Hib as used in the Netherlands.

In conjunction with the efficacy against NTHi-AOM, some evidence for *H. influenzae* colonization reduction was reported in POET, albeit maybe only temporary after the booster dose.[62,112] No consistent effect on NTHi colonization was observed for PHiD-CV.[32] It should be noted that NTHi (and *S. pneumoniae*) colonization rates in these former PD-conjugate vaccine studies were much lower than those in children at comparable ages in practically any other recent study.[32,62,111] In the Netherlands, nasopharyngeal bacterial colonization rates are high, including those for *H. influenzae*.[33,70] An effect on NTHi colonization and/or density would provide a biological mechanism by which a PD-conjugate vaccine confers protection against mucosal NTHi disease. Importantly, an impact of PHiD-CV on NTHi colonization would predict herd effects. Of note, PHiD-CV replaced 7vCRM in the Dutch NIP for all infants born after March 1, 2011.

Objectives and outline of this thesis

The main focus of this thesis was to investigate the impact of PHiD-CV and various aspects of nasopharyngeal colonization with respiratory pathogens.

The following research questions were formulated:

1. What is the immunogenicity of PHiD-CV and DTPa-IPV-Hib coadministration as compared to other combinations of pneumococcal conjugate and DTPa-combined vaccines? Is there evidence for immune interactions?
2. What are the effects of PHiD-CV immunization compared with 7vCRM on nasopharyngeal bacterial colonization, and in particular NTHi?
3. Are there specific associations among viral and bacterial pathogens present in the nasopharynx of healthy children?
4. Can alternative sampling methods reliably detect bacterial pathogens in children with an upper respiratory tract infection?
5. How prevalent is nasopharyngeal *S. pneumoniae* colonization in the elderly?

A randomized controlled trial was undertaken in 780 infants to evaluate the impact of PHiD-CV on immunogenicity, safety and nasopharyngeal bacterial colonization (Figure 6). The primary aim of this study was to demonstrate noninferiority of immune responses to PHiD-CV and DTPa-IPV-Hib when coadministered as a 3+1-dose schedule according to the Dutch NIP. We present the immunogenicity, safety and reactogenicity results of the primary series in **Chapter 2**, followed by the results of the booster phase of the study, including the long-term immune responses to pneumococcal serotypes, in **Chapter 3**. The study design allowed us to investigate the effects of PHiD-CV on nasopharyngeal bacterial colonization, particularly NTHi (using 7vCRM as a non-active control). In our study, we applied both conventional culture methods and quantitative polymerase chain reaction (PCR) assays to detect *S. pneumoniae* and *H. influenzae* (**Chapter 4**).

In **Chapter 5**, we describe the prevalence and co-occurrence of a wide range of respiratory viruses and four major bacterial pathogens in approximately 1000 nasopharyngeal samples obtained from healthy 6- to 24-month-old children who had participated in another pneumococcal vaccination trial in the Netherlands.[26] We also studied the accuracy of alternative sampling methods to detect bacterial pathogens in young children with an upper respiratory tract infection, focusing on culture of a paper tissue collected by simply wiping or blowing the nose. The results of this study in 66 children aged 0-4 years are described in **Chapter 6**. Finally, we explored the prevalence of *S. pneumoniae* in the nasopharynx of elderly, the other high-risk group for pneumococcal diseases. In **Chapter 7**, we present results of a cross-sectional study in 330 community-dwelling adults aged 65 years and older, using both conventional culture and molecular methods to detect *S. pneumoniae*. In **Chapter 8**, we follow with a summarizing discussion of the main findings, and make some recommendations for future research.

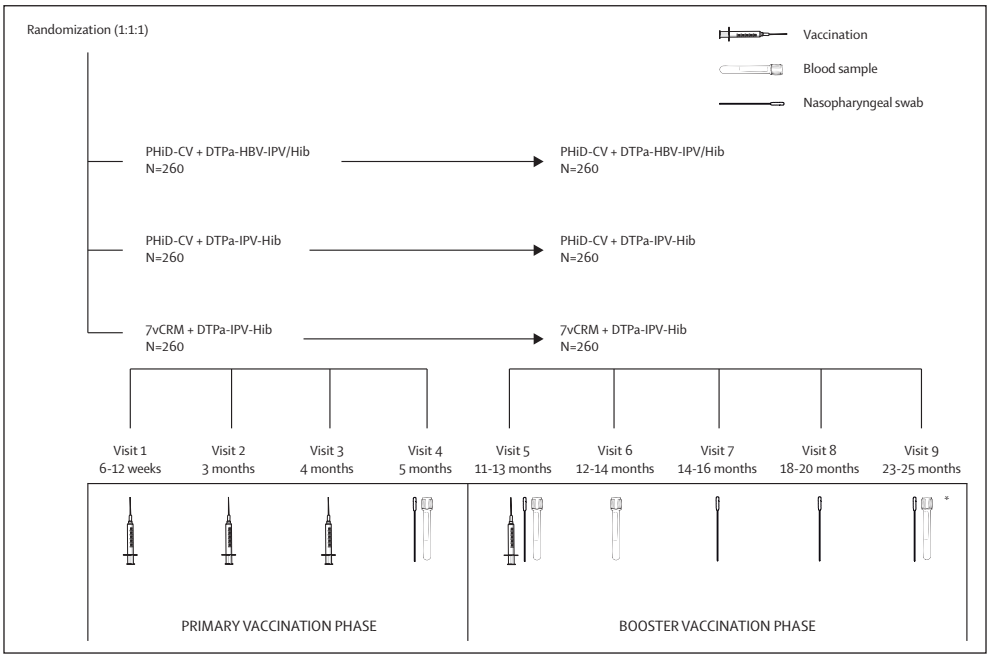

Figure 6: **Trial design.** Abbreviations: PHiD-CV, 10-valent pneumococcal nontypeable *Haemophilus influenzae* protein D-conjugate vaccine (Synflorix™, GlaxoSmithKline Vaccines); 7vCRM, 7-valent pneumococcal CRM197-conjugate vaccine (Prevenar™/Prevnar™, Pfizer, Inc.); DTPa-HBV-IPV/Hib (Infanrix hexa™, GlaxoSmithKline Vaccines); DTPa-IPV-Hib (Pediacel™, Sanofi Pasteur MSD). * Venipuncture at visit 9 (23-25 months of age) was optional.

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CHAPTER 2

Immunogenicity, safety, and reactogenicity of the 10-valent pneumococcal nontypeable *Haemophilus influenzae* protein D-conjugate vaccine (PHiD-CV) and DTPa-IPV-Hib when coadministered as a 3-dose primary vaccination schedule in the Netherlands: a randomized controlled trial

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CHAPTER 2

Immunogenicity, safety, and reactogenicity of the 10-valent pneumococcal nontypeable *Haemophilus influenzae* protein D-conjugate vaccine (PHiD-CV) and DTPa-IPV-Hib when coadministered as a 3-dose primary vaccination schedule in the Netherlands: a randomized controlled trial

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This trial is registered at www.ClinicalTrials.gov: NCT00652951

Abstract

Background Recent reviews have highlighted the unpredictability of immunologic interference when multivalent conjugate vaccines are coadministered with other pediatric vaccines.

Objective To evaluate immunogenicity, safety and reactogenicity of the 10-valent pneumococcal nontypeable *Haemophilus influenzae* protein D-conjugate vaccine (PHiD-CV; *Synflorix*™, GlaxoSmithKline Biologicals) and DTPa-IPV-Hib (*Pediacel*™, Sanofi Pasteur MSD) when coadministered as a three-dose primary vaccination course.

Material and methods Single-blind, single-center, randomized controlled trial in the Netherlands. Healthy infants were randomly assigned (1:1:1) to receive either (1) PHiD-CV + DTPa-HBV-IPV/Hib (*Infanrix hexa*™, GlaxoSmithKline Biologicals), (2) PHiD-CV + DTPa-IPV-Hib, or (3) 7vCRM (*Prevenar*™/*Prevnar*™, Pfizer, Inc.) + DTPa-IPV-Hib at 2, 3 and 4 months of age. Blood samples were collected one month post-dose 3. Diary cards were used to record safety and reactogenicity.

Results Antibody concentrations elicited by PHiD-CV coadministrated with DTPa-IPV-Hib were noninferior to those following DTPa-HBV-IPV/Hib coadministration for 9 out of 10 vaccine pneumococcal serotypes and protein D. For serotype 18C (conjugated to tetanus toxoid), the antibody concentration was higher with DTPa-HBV-IPV/Hib coadministration (1.73 µg/mL vs. 1.07 µg/mL). The percentages of infants with antibody concentrations ≥ 0.2 µg/mL (68.9–100% in the PHiD-CV + DTPa-HB-IPV/Hib group) vs. 64.9–100% in the PHiD-CV + DTPa-IPV-Hib group) and with measurable opsonophagocytic activity (56.1–100% in the PHiD-CV + DTPa-HBV-IPV/Hib group vs. 61.1–100% in the PHiD-CV + DTPa-IPV-Hib group) were comparable for all serotypes in both PHiD-CV groups. Group differences in antibody responses to the DTPa-IPV-Hib antigens remained within the predefined limit for noninferiority. Safety and reactogenicity profiles were comparable across groups.

Conclusions PHiD-CV and DTPa-IPV-Hib were immunogenic and well-tolerated when coadministered as a 3-dose primary vaccination course.

Funding GlaxoSmithKline Biologicals.

Introduction

Recent reviews have highlighted the unpredictability and complexity of immune response interference when multivalent conjugated vaccines are coadministered with other pediatric vaccines.[1–3] Since licensure in 2000, the 7-valent pneumococcal conjugate vaccine (7vCRM; Prevenar™/Prevnar™, Pfizer, Inc.), in which each of the pneumococcal capsular polysaccharides (4, 6B, 9V, 14, 18C, 19F and 23F) is conjugated to a non-toxic diphtheria toxoid mutant (CRM197), has been implemented in many national immunization programs. In the Netherlands, pneumococcal conjugate vaccination was introduced into the national immunization program (NIP) without a catch-up campaign using 7vCRM for all infants born after March 31, 2006 as a three-dose primary vaccination schedule at 2, 3 and 4 months followed by a booster dose at 11 months of age. This schedule currently coincides with the administration of DTPa-IPV-Hib (Pediacel™, Sanofi Pasteur MSD).

The recently licensed 10-valent pneumococcal nontypeable *Haemophilus influenzae* protein D conjugate vaccine (PHiD-CV; Synflorix™, GlaxoSmithKline Biologicals) differs in several aspects from 7vCRM. First, it contains three additional pneumococcal capsular polysaccharides (1, 5 and 7F). Second, different carrier proteins are used in PHiD-CV. Serotype 18C is conjugated to tetanus toxoid and serotype 19F to diphtheria toxoid, whereas each of the eight remaining pneumococcal serotypes are conjugated to protein D, a highly conserved 42 kD cell-surface lipoprotein of nontypeable *Haemophilus influenzae* (NTHi). Antibodies against protein D are thought to underlie the efficacy of the precursor vaccine of PHiD-CV against acute otitis media caused by NTHi.[4–6] The use of a novel carrier such as protein D might reduce the risk of potential carrier-mediated immune suppression, as described to occur with some conjugates that use tetanus toxoid or CRM197 as the carrier protein.[1–3, 7, 8]

PHiD-CV was shown to be immunogenic and well-tolerated when coadministered with various pediatric vaccines, including DTPa-HBV-IPV/Hib (Infanrix hexa™, GlaxoSmithKline Biologicals)[9], but no data are available regarding coadministration with DTPa-IPV-Hib. The aim of this study was to demonstrate noninferiority of PHiD-CV when coadministered with DTPa-IPV-Hib compared to coadministration with DTPa-HBV-IPV/Hib in terms of antibody responses one month after the third primary dose to each of the 10 vaccine pneumococcal serotypes and protein D. Noninferiority of DTPa-IPV-Hib when coadministered with PHiD-CV compared to coadministration with 7vCRM in terms of antibody responses to the DTPa-IPV-Hib antigens was also assessed, as well as safety and reactogenicity following (co)administration of the different vaccines.

Material and methods

Study design

This single-center randomized controlled trial (NCT00652951) was performed in the Netherlands and consisted of two consecutive study phases: a primary and a booster phase. Results from the primary study phase are reported here. Infants were randomly assigned (1:1:1) to receive either (1) PHiD-CV coadministered with DTPa-HBV-IPV/Hib, (2) PHiD-CV coadministered with DTPa-IPV-Hib or (3) 7vCRM coadministered with DTPa-IPV-Hib at 2, 3 and 4 months of age. Treatment allocation was performed at the investigator site using a central randomization system. The study was single-blind, i.e. study staff members were aware of the treatment assignment, but parents and laboratory technicians were not. Vaccines were prepared for injection out of the sight of the parents; however, the appearance of the syringes and vials used was distinct.

An Independent Ethics Committee (Centrale Commissie Mensgebonden Onderzoek, available at: http://www.ccmo-online.nl) approved the study protocol. The study was conducted in accordance with the Declaration of Helsinki and Good Clinical Practice guidelines. Written informed consent was obtained from each infant's parent(s) or guardian(s) before enrolment.

Study subjects

The study area covered a birth cohort of approximately 35 000 children per year in the western and central part of the Netherlands. Parents of newborn infants eligible for routine infant vaccination according to the NIP, excluding those requiring hepatitis B vaccination (20% of the birth cohort), were provided written information about the study and offered the possibility to participate. After parent(s) or guardian(s) granted informed consent, a study physician determined eligibility of the infant by obtaining a medical history and performing physical examination prior to enrolment. Healthy infants born after a gestation period of at least 36 weeks and aged 6 to 12 weeks at the time of the first primary vaccination were eligible. Usual exclusion criteria were applied such as immunosuppression of any cause, serious chronic illness, a history of seizures or progressive neurological disease, major congenital defects, administration of immunoglobulin or blood products since birth, history of disease caused by pathogens targeted by DTPa-HBV-IPV/Hib or prior vaccination against pathogens targeted by the study vaccines. Infants were visited at home for all study procedures. Participants did not receive any financial compensation.

Vaccines and vaccinations

The composition of PHiD-CV (Synflorix™, GlaxoSmithKline Biologicals, Rixensart, Belgium), 7vCRM (Prevenar™/Prevnar™, Pfizer, Inc., New York, USA), DTPa-HBV-IPV/Hib (Infanrix hexa™, GlaxoSmithKline Biologicals, Rixensart, Belgium) and DTPa-IPV-Hib (Pediacel™, Sanofi Pasteur MSD, Lyon, France) were as described previously.[10–12] One vaccine lot was used for each study vaccine except for the Hib vaccine component of DTPa-HBV-IPV/Hib, for which two lots were used. All vaccines were administered intramuscularly; PHiD-CV or 7vCRM in the right and DTPa-HBV-IPV/Hib or DTPa-IPV-Hib in the left anterolateral thigh.

Blood sampling and serological methods

Venous blood samples of approximately 5.0 mL were collected one month after the third primary dose. The Ethics Committee allowed only a single attempt to draw blood per infant. The aliquots of serum were stored at -20˚C to -80˚C and sent to GlaxoSmithKline Biologicals for assessment of immune responses. Pneumococcal serotype-specific IgG antibodies to the 10 vaccine pneumococcal serotypes were measured by an enzyme-linked immunosorbant assay (ELISA) including adsorption with pneumococcal serotype 22F polysaccharide (GSK's 22F-ELISA) to increase assay specificity, as described previously.[13, 14] It has been established that an antibody concentration of 0.20 µg/mL as determined by GSK's 22F-ELISA is equivalent to the 0.35 µg/mL threshold recommended by the World Health Organization (WHO) for an ELISA without 22F pre-adsorption.[15, 16] Serotype-specific opsonophagocytic activity (OPA) was determined using a modification of the HL-60 cell WHO reference method, as described previously.[17] IgG antibodies to the H. influenzae protein D were determined using an in-house ELISA and expressed in ELISA units (EL.U) per milliliter. The analyses of immune responses to diphtheria and tetanus toxoids, Hib polysaccharide PRP, pertussis antigens and hepatitis B surface antigen were performed using standard ELISA.

For anti-PRP antibodies, the percentages of subjects reaching an antibody concentration of at least 0.15 µg/mL and 1.0 µg/mL were evaluated. Seropositivity was used to describe immune responses to the acellular pertussis components PT, FHA and PRN (i.e., antibody concentration ≥ 5 EL.U/mL).[18–20] The presence of antibodies against poliovirus types 1, 2 and 3 was determined by a virus microneutralization test adopted from the WHO Guidelines and expressed in terms of the 50% inhibitory dose (ID50).[21, 22]

Evaluation of safety and reactogenicity

Assessment of reactogenicity was performed as described previously.[23] Following each administration of study vaccines, study staff observed the infant for 30 minutes for immediate post-vaccination reactions. Parents filled out a diary card during the 4 days following vaccination. Local (pain, redness and swelling at the injection site) and general symptoms (fever, irritability, drowsiness and loss of appetite) were actively solicited. Temperature was measured rectally using a standard digital thermometer. Any other (i.e., unsolicited) adverse event occurring within one month after vaccination was also recorded. All solicited local symptoms were defined to be causally related to vaccination. Study physicians performed causality assessment regarding all solicited general symptoms and unsolicited events. Serious adverse events were reported at any time throughout the course of the study.

Statistical analyses

The primary objective was defined to be reached if the upper limit of the 2-sided 95% confidence interval (CI) of the geometric mean antibody concentration (GMC) ratio between groups (PHiD-CV + DTPa-HBV-IPV/Hib group over PHiD-CV + DTPa-IPV-Hib group) was lower than 2 for antibodies against each of the 10 vaccine pneumococcal serotypes and protein D. The secondary objective was reached if the upper limit of the 2-sided 95% CI of the geometric mean antibody concentration/titer (GMC/GMT) ratio between groups (7vCRM + DTPa-IPV-Hib group over PHiD-CV + DTPa-IPV-Hib group) was lower than 2 for the DTPa-IPV-Hib antigens. This noninferiority margin was chosen since lot-to-lot consistency was shown to be within a 2-fold range for PHiD-CV.[10]

The immunogenicity analyses were performed on the according-to-protocol (ATP) cohort, defined as subjects who (1) met all eligibility criteria, (2) complied with all procedures and intervals defined in the protocol and (3) for whom post-immunization data for at least one study vaccine antigen was available. Assuming identical antibody responses between groups, 180 evaluable subjects per group would allow at least 97% overall power to meet the primary objective. Estimating that in up to 30% of infants an insufficient blood volume would be available for all serological tests, a target sample of 260 subjects per group was calculated to be required for the study. If more than 5% of the subjects needed to be excluded from the ATP cohort, a complementary analysis based on the total vaccinated cohort would be performed. Antibody GMCs/GMTs and seropositivity/seroprotection rates with 95% CIs were computed for each serotype/antigen.

The safety and reactogenicity analyses were conducted on the total vaccinated cohort. For each symptom the percentage of doses followed by the symptom with exact 95% CI was calculated per group. All comparisons other than those for the primary and secondary objectives were exploratory in nature and statistical significance was based on the observation of nonoverlapping 95% CIs.

Results

All infants (n=780) were enrolled between 1 April 2008 and 30 January 2009 and all completed the primary study phase (Figure 1). The baseline characteristics of the infants in the ATP cohort (n=579) were comparable between the three treatment groups. The mean age at the time of the first primary vaccination was 7.5 weeks (standard deviation 1.26). The overall percentage of male participants was 50.9% and most infants (99.1%) were White-Caucasian.

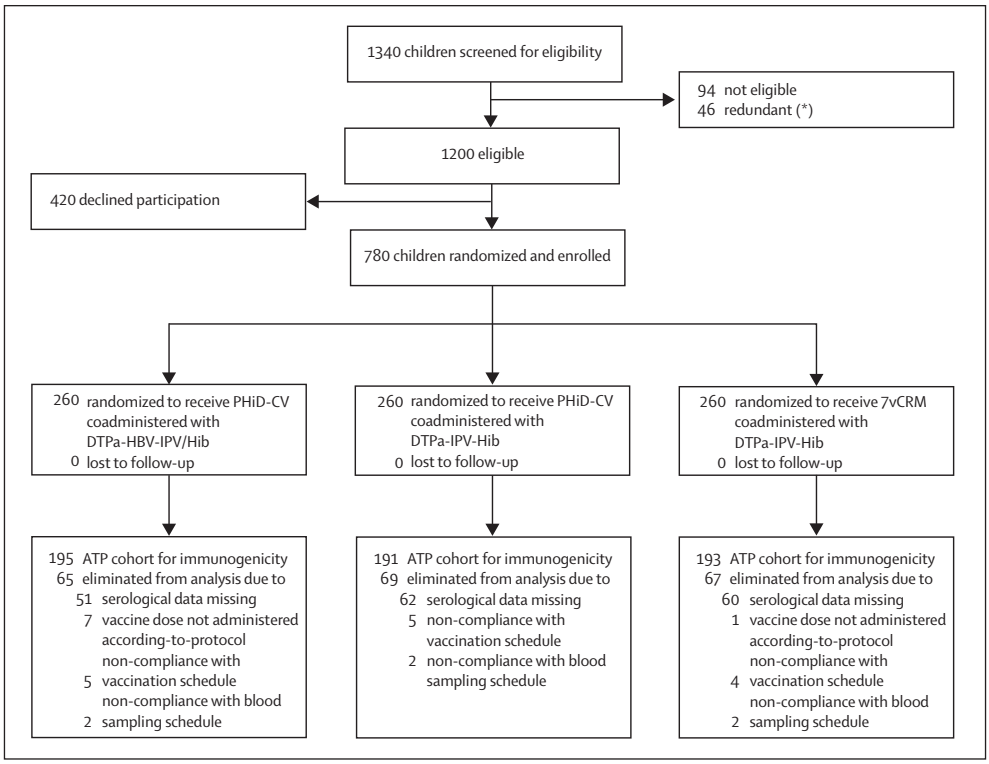

Figure 1: **Trial profile.** * Parents of children were considered "redundant" if they had received study information and indicated to be interested in participating in the study after the enrolment target (n=780) had already been achieved; subsequently, the informed consent process was cancelled.

Evaluation of immunogenicity

Immune responses to pneumococcal conjugate vaccine antigens

When comparing the two groups vaccinated with PHiD-CV coadministered with either DTPa-HBV-IPV/Hib or DTPa-IPV-Hib, antibody responses one month after the third primary dose met the criteria for noninferiority for 9 out of 10 serotypes and protein D (Table 1). For serotype 18C, the predefined limit for noninferiority between the two PHiD-CV groups was marginally exceeded. The antibody GMC was in the same range for both PHiD-CV groups for 9 out of 10 vaccine pneumococcal serotypes. The antibody GMC against 18C was higher

Antigen	PHiD-CV DTPa-HBV-IPV/Hib N=195 GMC (95% CI)	PHiD-CV DTPa-IPV-Hib N=189 GMC (95% CI)	7vCRM DTPa-IPV-Hib N=192 GMC (95% CI)	[PHiD-CV + DTPa-HBV-IPV/Hib] over [PHiD-CV + DTPa-IPV-Hib] GMR (95% CI)
1	1.17 (1.02 - 1.33)	1.31 (1.16 - 1.48)	0.03 (0.03 - 0.03)	0.89 (0.74 - 1.07)
4	1.61 (1.41 - 1.84)	1.59 (1.38 - 1.83)	2.44 (2.19 - 2.73)	1.01 (0.84 - 1.23)
5	2.11 (1.88 - 2.37)	2.16 (1.92 - 2.43)	0.03 (0.03 - 0.03)	0.98 (0.83 - 1.15)
6B	0.33 (0.26 - 0.40)	0.35 (0.28 - 0.43)	0.41 (0.34 - 0.51)	0.93 (0.69 - 1.26)
7F	1.70 (1.52 - 1.90)	1.77 (1.57 - 1.99)	0.04 (0.03 - 0.04)	0.96 (0.82 - 1.13)
9V	1.40 (1.20 - 1.63)	1.47 (1.29 - 1.68)	2.14 (1.91 - 2.40)	0.95 (0.78 - 1.16)
14	3.38 (2.99 - 3.81)	3.33 (2.93 - 3.78)	3.64 (3.24 - 4.10)	1.01 (0.85 - 1.21)
18C	1.73 (1.45 - 2.05)	1.07 (0.92 - 1.25)	2.10 (1.83 - 2.40)	1.61 (1.28 - 2.03)
19F	2.07 (1.73 - 2.48)	1.96 (1.64 - 2.34)	3.04 (2.71 - 3.42)	1.06 (0.82 - 1.36)
23F	0.50 (0.41 - 0.60)	0.54 (0.44 - 0.66)	1.24 (1.04 - 1.47)	0.92 (0.70 - 1.23)
Protein D	1580.0 (1409.5 - 1771.1)	1743.0 (1560.2 - 1947.2)	69.7 (63.0 - 77.1)	0.91 (0.77 - 1.06)
6A	0.10 (0.08-0.12)	0.10 (0.09-0.12)	0.08 (0.06-0.09)	NA
19A	0.10 (0.09-0.13)	0.09 (0.08-0.11)	0.08 (0.07-0.10)	NA

Table 1: **Pneumococcal serotype-specific and anti-protein D antibody GMCs and ratios of GMCs (GMR) one month after the third primary dose (ATP cohort for immunogenicity).** N = maximum number of infants with available results; actual number of infants included in the analysis varies slightly per serotype, depending on serum availability for testing; NA = not applicable.

when PHiD-CV was coadministered with DTPa-HBV-IPV/Hib (Table 1). The percentage of infants with antibody concentrations ≥ 0.20 µg/mL was in the same range for both PHiD-CV groups for all vaccine pneumococcal serotypes, including 18C (Table 2). In addition, OPA GMTs and the percentage of infants with opsonophagocytic titers ≥ 8 were in the same range for both PHiD-CV groups for each vaccine pneumococcal serotype (Table 3).
When comparing the two groups vaccinated with either PHiD-CV or 7vCRM coadministered with DTPa-IPV-Hib, antibody GMCs against the seven serotypes common to both pneumococcal conjugate vaccines were higher in the 7vCRM + DTPa-IPV-Hib group for serotypes 4, 9V, 18C, 19F and 23F (Table 1). Regarding the proportion of infants with antibody concentrations ≥ 0.20 µg/mL, a higher percentage in the 7vCRM + DTPa-IPV-Hib group reached this threshold for serotypes 19F and 23F (Table 2). OPA GMTs and the percentage of infants with measurable opsonophagocytic titers were higher for serotypes 18C and 23F in the 7vCRM + DTPa-IPV-Hib group, whereas the OPA GMT for serotype 19F was higher in the PHiD-CV + DTPa-IPV-Hib group (Table 3).

Immune responses to the coadministered DTPa-combined vaccine antigens

When comparing the two groups vaccinated with DTPa-IPV-Hib coadministered with either 7vCRM or PHiD-CV, antibody responses to the DTPa-IPV-Hib antigens remained within the predefined limit for noninferiority (Table 4). Antibody GMCs (Table 4) and seroprotection rates (Table 5) for antibodies against diphtheria toxoid and seropositivity rates for pertussis antigens were in the same range for both DTPa-IPV-Hib groups. Similarly, GMTs of antibo-

Antigen	PHiD-CV DTPa-HBV-IPV/Hib	PHiD-CV DTPa-IPV-Hib	7vCRM DTPa-IPV-Hib
	N=194 %≥ 0.20 µg/mL (95% CI)	N=189 %≥ 0.20 µg/mL (95% CI)	N=192 %≥ 0.20 µg/mL (95% CI)
1	96.1 (92.2–98.4)	98.9 (96.0–99.9)	2.8 (0.9–6.4)
4	97.9 (94.8–99.4)	97.3 (93.8–99.1)	99.0 (96.3–99.9)
5	100 (98.0–100)	98.9 (96.1–99.9)	0.0 (0.0–2.1)
6B	68.9 (61.5–75.7)	64.9 (57.4–72.0)	68.9 (61.6–75.6)
7F	99.0 (96.3–99.9)	99.5 (97.1–100)	6.0 (3.0–10.5)
9V	95.1 (91.0–97.8)	97.3 (93.8–99.1)	98.4 (95.4–99.7)
14	99.5 (97.1–100)	100 (98.0–100)	100 (98.1–100)
18C	94.3 (90.1–97.1)	94.2 (89.8–97.1)	97.9 (94.7–99.4)
19F	95.2 (91.2–97.8)	95.1 (90.9–97.7)	100 (98.1–100)
23F	74.9 (67.8–81.0)	76.0 (69.0–82.1)	92.9 (88.2–96.2)
6A	31.4 (24.7-38.6)	29.0 (22.4-36.3)	22.4 (16.6-29.1)
19A	31.1 (24.4-38.4)	28.2 (21.7-35.5)	22.2 (16.4-29.0)

Table 2: **Percentage of infants with antipneumococcal serotype-specific 22F-ELISA antibody concentration ≥ 0.20 µg/mL one month after the third primary dose (ATP cohort for immunogenicity).** N = maximum number of infants with available results; actual number of infants included in the analysis varies slightly per serotype, depending on serum availability for testing.

dies and the percentage of infants who developed seroprotective immune responses against the poliovirus antigens were comparable between these groups (Table 4 and 5). The antibody GMC against tetanus toxoid was higher when DTPa-IPV-Hib was coadminstered with PHiD-CV, but the seroprotection rates were comparable. For the Hib polysaccharide, GMCs of antibodies against PRP were higher in the PHiD-CV + DTPa-IPV-Hib group (Table 4) with a trend for a higher percentage of infants reaching seroprotective antibody levels of 0.15 µg/mL than in the 7vCRM + DTPa-IPV-Hib group (Table 5). Likewise, a higher percentage of infants with anti-PRP levels ≥ 1.0 µg/mL was observed in the group with PHiD-CV coadministration (Table 5). Remarkably, at least 60.2% of infants that did not receive a hepatitis B vaccine dose (i.e., subjects in both DTPa-IPV-Hib groups) showed antibody levels that are assumed to offer seroprotection against hepatitis B (Table 5), although the anti-HBs antibody GMC levels were very low (Table 4). When comparing the two groups vaccinated with either DTPa-HBV-IPV/Hib or DTPa-IPV-Hib coadministered with PHiD-CV, antibody GMCs against diphtheria, tetanus toxoid and pertussis antigens FHA and PRN, as well as the GMT for antibodies against poliovirus type 1 were higher in the group receiving DTPa-HBV-IPV/Hib (Table 4). However, seroprotection and seropositivity rates were comparable for all antigens common to both vaccines except for poliovirus type 1 (higher in the PHiD-CV + DTPa-HBV-IPV/Hib group). A complementary analysis of immunogenicity on the total vaccinated cohort yielded similar results (data not shown).

	PHiD-CV DTPa-HBV-IPV/Hib N=139		PHiD-CV DTPa-IPV-Hib N=135		7vCRM DTPa-IPV-Hib N=132	
Antigen	GMT (95% CI)	% ≥ 8 (95% CI)	GMT (95% CI)	% ≥ 8 (95% CI)	GMT (95% CI)	% ≥ 8 (95% CI)
1	20.7 (15.6-27.6)	56.1 (47.2 - 64.7)	20.8 (15.8-27.4)	61.1 (52.0 - 69.7)	4.7 (4.1-5.2)	6.4 (2.8 - 12.2)
4	592.9 (475.9-738.7)	96.2 (91.4 - 98.8)	600.4 (492.2-732.3)	98.5 (94.6 - 99.8)	838.4 (718.7-978.2)	100 (97.2 - 100)
5	54.8 (44.0-68.4)	89.1 (82.7 - 93.8)	60.1 (48.2-74.8)	91.0 (84.9 - 95.3)	4.2 (3.9-4.6)	1.5 (0.2 - 5.4)
6B	261.3 (176.0-388.0)	80.6 (72.7 - 87.0)	296.4 (198.0-443.7)	80.0 (72.1 - 86.5)	633.0 (419.0-956.4)	85.4 (77.9 - 91.1)
7F	2063.3 (1691.7-2516.6)	99.2 (95.8 - 100)	2136.1 (1707.9-2671.5)	98.4 (94.4 - 99.8)	18.4 (12.1-28.0)	33.3 (24.8 - 42.8)
9V	863.6 (687.6-1084.7)	98.5 (94.6 - 99.8)	1277.7 (1053.3-1550.1)	100 (97.2 - 100)	1194.0 (1009.5-1412.1)	100 (97.1 - 100)
14	990.4 (820.6-1195.5)	100 (97.3 - 100)	1086.4 (899.6-1311.9)	100 (97.3 - 100)	1373.3 (1040.4-1812.7)	96.9 (92.1 - 99.1)
18C	122.6 (89.4-168.2)	82.7 (75.2 - 88.7)	84.2 (60.9-116.5)	78.3 (70.2 - 85.1)	213.6 (163.6-278.8)	93.0 (87.2 - 96.8)
19F	133.0 (98.1-180.2)	87.6 (80.9 - 92.6)	143.8 (108.6-190.3)	91.0 (84.8 - 95.3)	39.2 (30.6-50.4)	81.5 (73.8 - 87.8)
23F	847.4 (626.5-1146.3)	93.5 (88.1 - 97.0)	1089.0 (800.2-1482.0)	93.2 (87.5 - 96.9)	3703.4 (3119.4-4396.8)	100 (97.2 - 100)
6A	23.2 (16.1-33.6)	44.5 (35.7-53.6)	25.4 (17.1-37.8)	44.2 (35.1-53.5)	33.0 (21.4-50.8)	47.1 (38.0-56.4)
19A	9.0 (6.9-11.7)	25.9 (18.8-34.2)	8.0 (6.3-10.2)	24.0 (16.9-32.3)	4.9 (4.4-5.4)	13.1 (7.8-20.1)

Table 3: **Antipneumococcal serotype-specific opsonophagocytic activity (OPA) titers and percentage of infants with OPA titer ≥ 8 one month after the third primary dose (ATP cohort for immunogenicity).** N = maximum number of infants with available results; actual number of infants included in the analysis varies slightly per serotype, depending on serum availability for testing.

Antigen	PHiD-CV DTPa-HBV-IPV/Hib N=189 GMC (95% CI)	PHiD-CV DTPa-IPV-Hib N=180 GMC (95% CI)	7vCRM DTPa-IPV-Hib N=189 GMC (95% CI)	[7vCRM + DTPa-IPV-Hib] over [PHiD-CV + DTPa-IPV-Hib] GMR (95% CI)
Antidiptheria (IU/mL)	1.475 (1.307-1.664)	1.078 (0.939-1.237)	1.077 (0.949-1.222)	1.00 (0.83-1.20)
Antitetanus (IU/mL)	2.873 (2.622-3.147)	1.702 (1.528-1.897)	0.934 (0.837-1.043)	0.55 (0.47-0.64)
Anti-PT (EL.U/mL)	42.7 (39.1-46.6)	36.4 (33.4-39.8)	40.1 (37.0-43.6)	1.10 (0.98-1.24)
Anti-FHA (EL.U/mL)	145.6 (130.4-162.5)	100.8 (89.6-113.5)	100.5 (89.9-112.4)	1.00 (0.85-1.17)
Anti-PRN (EL.U/mL)	97.6 (86.8-109.7)	40.1 (34.8-46.1)	45.1 (39.3-51.7)	1.13 (0.92-1.37)
Anti-poliovirus type 1	27.2 (21.7-34.1)	16.0 (13.0-19.7)	18.1 (14.8-22.1)	1.13 (0.85-1.51)
Anti-poliovirus type 2	37.1 (29.1-47.4)	29.0 (23.0-36.6)	23.2 (18.5-29.1)	0.80 (0.58-1.10)
Anti-poliovirus type 3	47.3 (35.8-62.4)	34.2 (27.0-43.4)	26.7 (21.5-33.0)	0.78 (0.57-1.07)
Anti-PRP (µg/mL)	2.139 (1.766-2.590)	4.796 (3.829-6.007)	2.219 (1.724-2.857)	0.46 (0.33-0.65)
Anti-HBs (mIU/mL)	356.9 (279.4-455.8)	14.0 (11.6-16.9)	11.5 (9.8-13.5)	NA

Table 4: **GMC/GMTs for antibodies against the coadministered vaccine antigens and ratios of GMCs/GMTs (GMR) one month after the third primary dose (ATP cohort for immunogenicity).** N = maximum number of infants with available results; actual number of infants included in the analysis varies slightly, depending on serum availability for testing.

Antigen	PHiD-CV DTPa-HBV-IPV/Hib N=189 % (95% CI)	PHiD-CV DTPa-IPV-Hib N=180 % (95% CI)	7vCRM DTPa-IPV-Hib N=189 % (95% CI)
Antidiphtheria (≥ 0.1 IU/mL)[38]	100 (98.0-100)	99.4 (96.9-100)	99.5 (97.1-100)
Antitetanus (≥ 0.1 IU/mL)[39]	100 (98.0-100)	100 (98.0-100)	100 (98.1-100)
Anti-PT (≥ 5 EL.U/mL)[18-20]	100 (98.0-100)	100 (98.0-100)	100 (98.1-100)
Anti-FHA (≥ 5 EL.U/mL)[18-20]	100 (98.0-100)	100 (97.9-100)	100 (98.0-100)
Anti-PRN (≥ 5 EL.U/mL)[18-20]	100 (98.0-100)	98.3 (95.2-99.7)	98.4 (95.4-99.7)
Anti-poliovirus type 1 (≥ 8)[21,22]	84.5 (77.8-89.8)	70.0 (62.0-77.2)	75.2 (67.4-81.9)
Anti-poliovirus type 2 (≥ 8)[21,22]	82.7 (75.8-88.3)	82. 6 (75.5-88.3)	75.8 (68.2-82.5)
Anti-poliovirus type 3 (≥ 8)[21,22]	84.0 (77.3-89.4)	84.5 (77.6-89.9)	81.9 (74.7-87.7)
Anti-PRP			
≥ 0.15 µg/mL[40]	97.4 (93.9-99.1)	97.2 (93.6-99.1)	89.9 (84.7-93.8)
≥ 1.0 µg/mL	72.5 (65.5-78.7)	84.4 (78.2-89.3)	70.7 (63.7-77.1)
Anti-HBs (≥ 10 mIU/mL)[41,42]	99.1 (95.1-100)	67.9 (58.4-76.4)	60.2 (50.5-69.3)

Table 5: **Seroprotection/seropositivity rates for the coadministered vaccine antigens one month after the third primary dose (ATP cohort for immunogenicity).** N = maximum number of infants with available results; actual number of infants included in the analysis varies slightly, depending on serum availability. % = percentage of infants; Cut-off values of the assays for antibody concentrations considered to be associated with seroprotection or seropositivity are shown in parentheses.

Symptom	PHiD-CV DTPa-HBV-IPV/Hib		PHiD-CV DTPa-IPV-Hib		7vCRM DTPa-IPV-Hib	
	PHiD-CV N=779 % (95% CI)	DTPa-HBV-IPV/Hib N=778 % (95% CI)	PHiD-CV N=779 % (95% CI)	DTPa-IPV-Hib N=779 % (95% CI)	7vCRM N=780 % (95% CI)	DTPa-IPV-Hib N=780 % (95% CI)
Pain						
Any	47.9 (44.3-51.5)	44.9 (41.3-48.4)	43.0 (39.5-46.6)	40.6 (37.1-44.1)	35.5 (32.2-39.0)	35.9 (32.5-39.4)
Grade 3	5.4 (3.9-7.2)	5.8 (4.2-7.7)	4.7 (3.4-6.5)	5.0 (3.6-6.8)	4.4 (3.0-6.0)	4.1 (2.8-5.7)
Redness						
Any	41.3 (37.9-44.9)	37.8 (34.4-41.3)	43.8 (40.3-47.3)	37.2 (33.8-40.7)	40.1 (36.7-43.7)	36.2 (32.8-39.6)
> 30 mm	0.9 (0.4-1.8)	1.2 (0.5-2.2)	2.1 (1.2-3.3)	3.2 (2.1-4.7)	1.2 (0.5-2.2)	2.1 (1.2-3.3)
Swelling						
Any	45.6 (42.0-49.1)	44.1 (40.6-47.7)	45.6 (42.0-49.1)	38.9 (35.5-42.4)	35.6 (32.3-39.1)	35.5 (32.2-39.0)
> 30 mm	1.8 (1.0-3.0)	2.4 (1.5-3.8)	2.3 (1.4-3.6)	3.2 (2.1-4.7)	1.8 (1.0-3.0)	3.2 (2.1-4.7)

Table 6: **Overall per dose incidence [% (95% CI)] of local symptoms at injection site after primary vaccinated (Total Vaccinated Cohort).** N indicates number of documented doses; %, percentage of doses followed by at least one specified symptom; 95% CI, exact 95% confidence interval; grade 3 pain, cried when limb was moved/sponeously painful

Evaluation of safety and reactogenicity

Serious adverse events

Overall, 32 out of 780 infants (4.1%) reported at least one serious adverse event during the primary study phase: 17 infants in the PHiD-CV + DTPa-HBV-IPV/Hib group, 5 infants in the PHiD-CV + DTPa-IPV-Hib group and 10 infants in the 7vCRM + DTPa-IPV-Hib group. None of them was fatal. One reported serious adverse event in the PHiD-CV + DTPa-HBV-IPV/Hib group was considered by the investigator to be related to vaccination. This event, described by the investigator as an "apparent life-threatening event", started 7 hours after administration of the second dose of the study vaccines. Symptoms lasted approximately one minute and included hypotonia and hyporesponsiveness. During hospitalization, blood tests and electrocardiography showed no abnormalities and no other episodes were observed. The infant was considered fully recovered within 24 hours. There was no recurrence after administration of the third primary dose of the study vaccines.

Local solicited adverse events

The overall per dose incidence of solicited local symptoms within 4 days following vaccination is shown in Table 6. Local symptoms at the PHiD-CV and DTPa-IPV-Hib injection sites did not seem to change when different vaccines were coadministered. Pain and swelling were reported less frequently at the 7vCRM injection site compared to PHiD-CV, whereas redness was reported at comparable rates across groups. The incidence of local symptoms was within the same range at the DTPa-HBV-IPV/Hib and DTPa-IPV-Hib injection sites in the groups with PHiD-CV coadministration, whereas pain and swelling at the DTPa-IPV-Hib injection site were reported less frequently in the group with 7vCRM coadministration.

Symptom	PHiD-CV DTPa-HBV-IPV/Hib N=779 % (95% CI)	PHiD-CV DTPa-IPV-Hib N=779 % (95% CI)	7vCRM DTPa-IPV-Hib N=780 % (95% CI)
Drowsiness			
Any	59.9 (56.4–63.4)	54.7 (51.1–58.2)	54.5 (50.9–58.0)
Grade 3	2.1 (1.2–3.3)	1.9 (1.1–3.2)	1.0 (0.4–2.0)
Irritability			
Any	65.3 (61.9–68.7)	63.9 (60.4–67.3)	61.9 (58.4–65.3)
Grade 3	6.5 (4.9–8.5)	5.8 (4.2–7.7)	2.3 (1.4–3.6)
Loss of appetite			
Any	29.9 (26.7–33.3)	29.0 (25.8–32.3)	29.6 (26.4–33.0)
Grade 3	0.5 (0.1–1.3)	0.5 (0.1–1.3)	0.1 (0.0–0.7)
Temperature (rectal)			
≥ 38.0°C	30.8 (27.6–34.2)	22.2 (19.3–25.3)	17.7 (15.1–20.6)
> 38.5°C	9.0 (7.1–11.2)	3.2 (2.1–4.7)	2.3 (1.4–3.6)
> 39.0°C	2.4 (1.5–3.8)	0.5 (0.1–1.3)	0.5 (0.1–1.3)
> 39.5°C	0.4 (0.1–1.1)	0.3 (0.0–0.9)	0.1 (0.0–0.7)
> 40.0°C	0.0 (0.0–0.5)	0.0 (0.0–0.5)	0.0 (0.0–0.5)

Table 7: **Overall per dose incidence [% (95% CI)] of general symptoms after primary vaccination (Total Vaccinated Cohort).** N indicates number of documented doses; %, percentage of doses followed by at least one specified symptom; 95% CI, exact 95% confidence interval; grade 3 drowsiness, preventing normal everyday activity; grade 3 irritability, crying inconsolably; grade 3 loss of appetite, not eating at all.

General solicited adverse events

The overall per dose incidence of solicited general symptoms within 4 days following vaccination was comparable across groups for all solicited general symptoms, except for fever (Table 7). The prevalence of fever (i.e., rectal temperature ≥ 38.0°C) was higher in the PHiD-CV + DTPa-HBV-IPV/Hib group than in the two other groups. No cases of rectal temperature > 40.0°C were reported. Irritability was the most frequently reported general symptom in each group.

Unsolicited adverse events

The unsolicited symptoms occurring within one month following each vaccine dose were those usually seen in a pediatric population and were reported with similar frequencies for all groups. The most frequently reported event was upper respiratory tract infection. Across groups, 2.9% to 4.5% of vaccine doses were followed by an adverse event with grade 3 intensity and 2.4% to 3.2% by an adverse event considered to be related to vaccination. Unsolicited adverse events with grade 3 intensity considered to be related to vaccination were reported twice in the PHiD-CV + DTPa-HBV-IPV/Hib group (apparent life-threatening event [same event as mentioned in the Serious adverse events section] and injection site rash) and twice (in the same infant) in the PHiD-CV + DTPa-IPV-Hib group (vomiting and enteritis). All events resolved < 72 hours.

Discussion

This article presents an evaluation of immunogenicity, safety and reactogenicity of PHiD-CV and DTPa-IPV-Hib when coadministered as a three-dose primary vaccination course. Antibody responses to the PHiD-CV antigens were noninferior between groups with different coadministered DTPa-combined vaccines for 9 out of 10 vaccine pneumococcal serotypes and protein D. The predefined limit for noninferiority was only marginally exceeded for serotype 18C. However, this difference is expected to be of limited clinical relevance, considering that for all vaccine pneumococcal serotypes the proportion of infants with antibody concentrations ≥ 0.20 µg/mL as well as the proportion of infants with measurable opsonophagocytic activity were comparable between both groups primed with PHiD-CV. Functional opsonophagocytic antibodies are considered to reflect the primary host defense mechanism against pneumococcal disease[24, 25] and results from opsonophagocytic assays were observed to correlate better with clinical protection against both inva sive and mucosal disease than antibody levels.[26, 27] Compared to other European PHiD-CV trials, the GMCs for antibodies against serotype 18C in the present study are at the lower end of the 18C responses previously reported and are comparable with studies evaluating a two-dose priming schedule or prophylactic use of paracetamol in a three-dose priming schedule.[28, 29] However, prophylactic use of paracetamol was limited in our study and did not differ between groups (1.7% in the PHiD-CV + DTPa-HBV-IPV/Hib group, 2.2% in the PHiD-CV + DTPa-IPV-Hib group). A recently published study evaluating antibody responses to a booster dose of 7vCRM after a two- or three-dose priming schedule in the Netherlands showed that the antibody GMCs pre- and post-booster were lowest for serotype 18C.[30] It is currently unknown why the antibody responses against serotype 18C among all vaccine pneumococcal serotypes seem to be rather low in the Netherlands and the clinical relevance of this observation remains unclear.

The higher immune response to pneumococcal serotypes common to both pneumococcal conjugate vaccines in 7vCRM primed infants is in line with previous studies comparing PHiD-CV and 7vCRM, although the impact of this difference on vaccine efficacy against invasive pneumococcal disease may be considered to be low.[9, 10, 31–33] The higher antitetanus antibody GMCs observed in both PHiD-CV groups compared to the 7vCRM group are in line with previous findings[12] and consistent with enhancement of the antitetanus response by the tetanus toxoid used as the carrier protein for serotype 18C in PHiD-CV. The antitetanus antibody GMC was also higher when PHiD-CV was coadministered with DTPa-HBV-IPV/Hib compared to coadministration with DTPa-IPV-Hib. This difference possibly reflects the overall higher content of tetanus in DTPa-HBV-IPV/Hib and might, in turn, account for the higher antibody GMC for 18C in the PHiD-CV + DTPa-HBV-IPV/Hib group compared to the PHiD-CV + DTPa-IPV-Hib group. It has been well documented that tetanus and diphtheria carrier proteins in conjugate vaccines can influence the response of coadministered vaccines containing tetanus and diphtheria.[1–3, 34]

For the Hib polysaccharide PRP, which is also conjugated to tetanus toxoid, higher antibody GMCs were observed in the group in which DTPa-IPV-Hib was coadministered with PHiD-CV compared to coadministration with 7vCRM. This may suggest enhancement of the anti-PRP response by the tetanus toxoid used as the carrier in PHiD-CV. However, the anti-PRP antibody GMC was lower in the PHiD-CV + DTPa-HBV-IPV/

Hib group than in the PHiD-CV + DTPa-IPV-Hib group, even though the percentage of infants reaching the 0.15 µg/mL cut-off was comparable between these groups. The higher anti-PRP antibody response in the group in which PHiD-CV was coadministered with DTPa-IPV-Hib could possibly be related to differences in formulation of the DTPa-combined vaccines.

The clinical relevance of the observed differences in antibody levels against several DTPa-IPV-Hib antigens across groups can be considered to be limited, given the fact that the titers for these antigens (i.e., diphtheria, tetanus and PRP) are well above the protective thresholds.

Compared to previous findings,[12] lower than expected antibody GMTs and seroprotection rates against the poliovirus antigens were observed in all three groups. The clinical relevance of these findings is also unknown, but should be limited considering that all infants receive a booster dose at 11–12 months of age.

Another unexpected finding in this study was the relatively high percentage of infants reaching seroprotective anti-HBs antibody levels among DTPa-IPV-Hib vaccinees, despite very low antibody concentrations. Maternal antibodies were not measured, but are unlikely to explain these findings. First, the seroprevalence of hepatitis B in the Dutch population is low, justifying a "risk group only" approach for hepatitis B vaccination for a long time.[35] Second, it is questionable whether such antibodies would remain at protective levels until the post-primary blood sampling time point at the age of 5 months. The ELISA used is the same assay GlaxoSmithKline Biologicals has always used to assess anti-HBs antibodies.[36] With these considerations taken into account, our observation remains as yet unexplained.

It should be noted that all comparisons other than those for the primary and secondary objectives were exploratory. These exploratory comparisons should therefore be interpreted with caution and should only be considered indicative of possible differences between groups. Another caveat to consider is the period in which this study was conducted, i.e. 2 to 3 years after implementation of 7vCRM in the Dutch NIP. With regard to nasopharyngeal pneumococcal carriage, it was recently shown that three years after nationwide implementation of 7vCRM, circulation of the seven vaccine pneumococcal serotypes had virtually disappeared in the Netherlands.[44] For meningococcal C conjugate vaccine, waning immunity has been described following reduction in meningococcal carriage, subsequent interruption of transmission and, consequently, absence of natural boosting.[37, 38] Such a phenomenon might also apply to pneumococcal conjugate vaccines and should be taken into account when interpreting the results of our study. Finally, it should be noted that the immunogenicity of the pneumococcal conjugate vaccines was evaluated after a three-dose priming schedule. However, many European countries are adopting or already have adopted a two-dose priming schedule. Comparisons between PHiD-CV and 7vCRM following such a two-dose dose priming might be different from our findings for some serotypes, especially with regard to the functional opsonophagocytic activity testing.[31]

In conclusion, the results of this study show that PHiD-CV and DTPa-IPV-Hib are immunogenic and well-tolerated when coadministered as a three-dose primary immunization course in the Netherlands.

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CHAPTER 3

Immunogenicity, safety, and reactogenicity of a booster dose of the 10-valent pneumococcal nontypeable *Haemophilus influenzae* protein D-conjugate vaccine (PHiD-CV) coadministered with DTPa-IPV-Hib in Dutch children

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Abstract

Background Immune responses and safety profiles may be affected when vaccines are coadministered. We evaluated the immunogenicity, safety and reactogenicity of a booster dose of the 10-valent pneumococcal nontypeable *Haemophilus influenzae* protein D-conjugate (PHiD-CV; *Synflorix*™, GlaxoSmithKline Vaccines) and DTPa-IPV-Hib (*Pediacel*™, Sanofi Pasteur MSD) when coadministered.

Methods This booster assessment was part of a randomized controlled trial in the Netherlands. Of 780 enrolled healthy infants, 774 toddlers participated in the booster phase and received (1:1:1) either (1) PHiD-CV + DTPa-HBV-IPV/Hib (*Infanrix hexa*™, GlaxoSmithKline Vaccines), (2) PHiD-CV + DTPa-IPV-Hib, or (3) 7-valent pneumococcal conjugate vaccine (7vCRM; *Prevenar*™/*Prevnar*™, Pfizer, Inc.) + DTPa-IPV-Hib at 2, 3, 4 and 11-13 months of age. Blood samples were taken post-primary, pre-booster, one month and 12 months post-booster.

Results Antipneumococcal antibody responses one month post-booster were comparable between both PHiD-CV groups, except for serotype 18C (conjugated to tetanus toxoid). Anti-18C antibody GMCs were higher when coadministered with DTPa-HBV-IPV/Hib. For each vaccine serotype the percentages of children with antibody concentration ≥ 0.20 µg/mL were within the same ranges between PHiD-CV groups (94-100%), as were the percentages of subjects with with OPA titer ≥ 8 (91-100%). When comparing both DTPa-IPV-Hib groups, post-booster antidiphtheria antibody GMCs were higher when coadministered with 7vCRM, while antitetanus and anti-PRP antibody GMCs were higher with PHiD-CV coadministration. Regardless, antibody levels to these antigens were well above protective thresholds. Safety and reactogenicity profiles were comparable between groups.

Conclusion Coadministration of a booster dose of PHiD-CV and DTPa-IPV-Hib was immunogenic and well-tolerated.

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Introduction

Coadministration of protein-polysaccharide conjugate vaccines with other pediatric vaccines offers the advantage to prevent multiple infectious diseases in children while limiting the number of vaccination visits, thereby upholding compliance to the immunization schedule. However, immune responses to vaccine antigens sharing similar or unrelated carrier proteins (e.g., diphtheria toxoid, tetanus toxoid and cross-reactive material 197 (CRM197, a diphtheria mutant)) could be affected when vaccines are coadministered.[1] Several mechanisms for immune interactions (that is, enhancement or interference) have been proposed,[2] but they have also been shown to be complex and unpredictable.[3]

The 10-valent pneumococcal conjugate vaccine (PHiD-CV; Synflorix™, GlaxoSmithKline Vaccines) is the first licensed vaccine using nontypeable *Haemophilus influenzae*-derived protein D as a carrier protein for 8 of 10 pneumococcal serotypes (18C and 19F are conjugated to tetanus toxoid and diphtheria toxoid, respectively). PHiD-CV was shown immunogenic and well-tolerated when coadministered with various pediatric vaccines, including hexavalent DTPa-HBV-IPV/Hib (Infanrix hexa™, GlaxoSmithKline Vaccines).[4] We have previously reported that coadministration of PHiD-CV and DTPa-IPV-Hib (Pediacel™, Sanofi Pasteur MSD) was well-tolerated and immunogenic after the primary series.[5] However, differences in antibody levels between vaccine groups were observed for pneumococcal serotype 18C, *Haemophilus influenzae* type b (Hib) polysaccharide polyribosyl-ribitol-phosphate (PRP), tetanus and diphtheria.

Here, we report the results of a booster dose of the same vaccines used for priming when administered at 11-13 months of age. In addition, we measured serotype-specific antipneumococcal immune responses 12 months after the booster dose as persistence of antibodies is an important aspect of long-term protection against encapsulated bacteria, next to herd immunity and immunologic memory.[6]

Material and methods

Study design and subjects

This study was a continuation of a randomized controlled trial (NCT00652951) designed to assess noninferiority of PHiD-CV when coadministered with DTPa-IPV-Hib compared with coadministration with DTPa-HBV-IPV/Hib in terms of antibody responses one month post-primary to each of the 10 vaccine pneumococcal serotypes and protein D.[5] Exploratory immunogenicity analysis was planned for the pre-, one month post-booster and 12 months post-booster timepoints, as well as analysis of the safety and reactogenicity after (co)administration of the different vaccines. At enrolment, infants were randomly assigned (1:1:1) to receive either (1) PHiD-CV + DTPa-HBV-IPV/Hib, (2) PHiD-CV + DTPa-IPV-Hib, or (3) 7-valent pneumococcal conjugate vaccine (7vCRM; Prevenar™/Prevnar™, Pfizer, Inc.) + DTPa-IPV-Hib at 2, 3, 4 and 11-13 months of age. An Independent Ethics Committee (Centrale Commissie Mensgebonden Onderzoek (CCMO), available at: http://www.ccmo-online.nl) approved the study protocol. This study was undertaken in accordance with Good Clinical Practice guidelines, which incorporate provisions of the Declaration of Helsinki. Results of the primary immunization were previously published.[5] Here we report the results of the booster phase of this study.

Vaccines and vaccinations

The composition of PHiD-CV, containing polysaccharides from serotypes 1, 4, 5, 6B, 7F, 9V, 14, 18C, 19F, and 23F (Synflorix™, GlaxoSmithKline Vaccines, Rixensart, Belgium); 7vCRM, containing polysaccharides from serotypes 4, 6B, 9V, 14, 18C, 19F, and 23F each conjugated to CRM917 (Prevenar™/Prevnar™, Pfizer, Inc., New York, USA), DTPa-HBV-IPV/Hib (Infanrix hexa™, GlaxoSmithKline Vaccines, Rixensart, Belgium), and DTPa-IPV-Hib (Pediacel™, Sanofi Pasteur MSD, Lyon, France) were as described previously.[7-9] All vaccines were administered intramuscularly; PHiD-CV or 7vCRM in the right and DTPa-HBV-IPV/Hib or DTPa-IPV-Hib in the left anterolateral thigh.

Blood sampling and serologic methods

Venous blood samples were collected one month after the third primary dose (results previously published),[5] pre-booster, one month and 12 months post-booster. Serotype-specific IgG antibodies to the 10 vaccine pneumococcal serotypes and cross-reactive serotypes 6A and 19A were measured using an ELISA, which includes pre-adsorption with pneumococcal serotype 22F polysaccharide (GSK's 22F-ELISA),[10,11] with a threshold of 0.2 µg/mL. Percentages of vaccine recipients reaching a pneumococcal antibody concentration of 1.0 µg/mL one month post-booster were also calculated. Serotype-specific opsonophagocytic activity (OPA) was determined using a modification of the HL-60 cell WHO reference method, with a dilution of 1:8 as a cut-off.[12] IgG antibodies to the non-lipidated *H. influenzae*-derived protein D were measured by an in-house ELISA. Standard methods were used for the analyses of immune responses to DTPa-HBV-IPV/Hib antigens.[5] Of note, further testing is being performed to confirm the assay status for FHA, polioviruses and hepatitis B and corroborate current findings.

Evaluation of safety and reactogenicity

Assessment of safety and reactogenicity was perfomed on the total vaccinated cohort, as previously described in detail.[5] In short, serious adverse events were reported at any time throughout follow-up. Solicited symptoms and unsolicited adverse events were recorded occurring within 4 and 31 days after the booster vaccination, respectively. Local reactions are known to occur more frequent and potentially be more severe following DTPa booster vaccinations.[13] Parents were therefore instructed to contact the study staff in case of a large swelling reaction following the booster dose, defined as any local swelling >50 mm, any increase in circumference of the limb >30 mm, or any diffuse swelling.

Statistical analyses

The sample size of the randomized controlled trial (N=780) was based on the primary objective.[5] All other endpoints were secondary objectives. The immunogenicity analysis was performed on the according-to-protocol (ATP) booster cohort. This cohort was defined as participants who (1) met all eligibility criteria, (2) complied with all procedures and intervals defined in the protocol, and (3) from whom postimmunization data at at least one time point and for at least one study vaccine antigen were available. Standard seroprotection and seropositivity rates were calculated with their exact 95% CIs for all evaluated antigens. Safety and reactogenicity analyses were performed on the total vaccinated cohort. All comparisons reported here were descriptive and statistical significance was based on the observation of nonoverlapping 95% CIs. They should therefore be interpreted with caution and should only be considered indicative of possible differences between groups. The statistical analyses were performed using the Statistical Analysis System (SAS) Drug and Development (SDD), web portal version 3.5 with SAS version 9.2. Adobe® Illustrator CS4 was used to graph data.

Results

Participants were enrolled between April 1, 2008 and January 30, 2009. Follow-up ended on December 1, 2010. Of the 780 infants, 772 (99%) completed follow-up (Figure 1). In total, 723 (93%) children were included in the ATP cohort for booster immunogenicity. Baseline characteristics of participating children were comparable between the vaccine groups.[14]

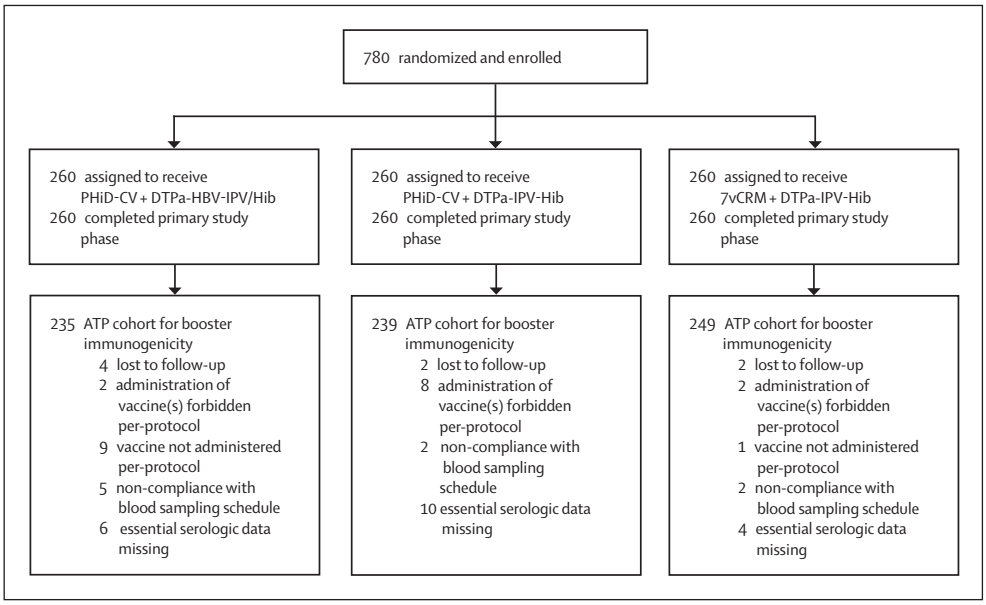


Figure 1: **Trial profile.** ATP, according-to-protocol.

Evaluation of immunogenicity

Immune response against pneumococcal conjugate vaccine antigens

Post-booster antibody GMCs were comparable (i.e., overlapping 95% CIs) between PHiD-CV groups for all vaccine pneumococcal serotypes, except for 18C (Table 1, Figure 2). The anti-18C antibody GMC was higher when PHiD-CV was coadministered with DTPa-HBV-IPV/Hib.

Regardless of the coadministered DTPa-combined vaccine, robust booster responses were measured in both PHiD-CV groups, ranging from 4-fold (serotype 14) to 11-fold (serotypes 1 and 18C) increases in antibody GMCs (Table 1) and 5-fold (serotype 7F) to 101-fold (serotype 4) increases in OPA GMTs (Table 2). After the booster dose, the percentage of children with protective levels was within the same range between PHiD-CV groups (94-100% antibody concentration ≥ 0.20 µg/mL, 69-100% ≥ 1.0 µg/mL, and 91-100% OPA ≥ 8) for all vaccine pneumococcal serotypes (Table 1).

With respect to cross-reactive serotypes 6A and 19A, antibody GMCs (Table 1) and OPA GMTs (Table 2) were generally lower than those for the vaccine pneumococcal serotypes. Still, antibody levels ≥ 0.20 µg/mL and OPA ≥ 8 were reached by a considerable proportion of children for serotype 6A (72-80% and 75-88%, respectively), regardless of the coadministered vaccine.

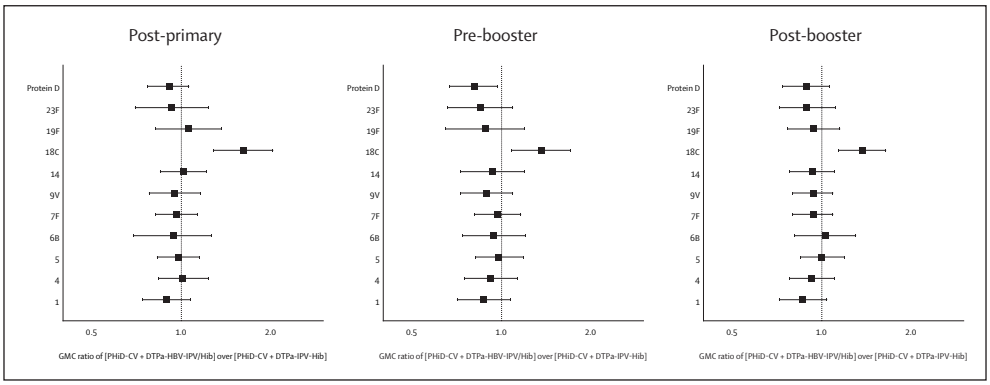


Figure 2: **Ratios of antipneumococcal and anti-protein D antibody GMCs between PHiD-CV groups.**[a] GMC ratios with error bars indicating 95% confidence intervals (CI). A ratio of 1 (dashed line) indicates no difference in antibody GMC between groups. ATP cohort for booster immunogenicity. [a] Ratio of PHiD-CV coadministered with DTPa-HBV-IPV/Hib over PHiD-CV coadministered with DTPa-IPV-Hib.

Kinetics of the immune responses (ELISA and OPA) are visualized in Figure 3. For the PHiD-CV groups, serotype-specific antibody GMCs and OPA GMTs were comparable at each sampling moment, apart from serotype 18C. When comparing PHiD-CV with 7vCRM, a steeper decline in antibody GMCs and OPA GMTs to serotypes 6A and 6B, 9V, 19A and 19F, and 23F was observed in the 7vCRM group from post-primary to pre-booster. However, after the booster dose, antibody GMCs to serotypes 6A, 6B and 23F were higher in the 7vCRM group. At 24 months of age, approximately 12 months post-booster, antibody GMCs and OPA GMTs had declined to near pre-booster levels for all pneumococcal serotypes, except for 19A (Figure 3). For most serotypes, the majority of vaccinees still had antipneumococcal antibody concentrations ≥0.2 µg/mL at 24 months of age, but the proportion of children with opsonophagocytic antibody levels ≥ 8 dropped below 50% for some serotypes. In 7vCRM recipients, although GMCs remained low (Table 2, Figure 3) OPA GMTs for serotype 7F increased with age (Table 3, Figure 3), with 94% of 24-month-old children reaching opsonophagocytic titers ≥ 8.

Immune response against coadministered DTPa-combined vaccine antigens

When comparing both DTPa-IPV-Hib groups, post-booster antidiphtheria antibody GMCs were higher when coadministered with 7vCRM, while antitetanus and anti-PRP antibody GMCs were higher with PHiD-CV coadministration (Figure 4). Booster responses were robust, resulting in adequate seroprotection/-positivity rates for all antigens (Table 3). When comparing both DTPa-IPV-Hib groups, post-booster GMCs for antibodies against diphtheria toxoid were higher in the 7vCRM + DTPa-IPV-Hib group, while anti-tetanus and anti-PRP antibody GMCs were higher in the PHiD-CV + DTPa-IPV-Hib (Table 3).

	PHiD-CV + DTPa-HBV-IPV/Hib group					PHiD-CV + DTPa-IPV-Hib group					7vCRM + DTPa-IPV-Hib group				
	Pre-booster N=183		Post-booster N=190			Pre-booster N=185		Post-booster N=198			Pre-booster N=199		Post-booster N=202		
Serotype	GMC (95% CI)	%≥0.2 (95% CI)	GMC (95% CI)	%≥0.2 (95% CI)	% ≥1.0 (95% CI)	GMC (95% CI)	%≥0.2 (95% CI)	GMC (95% CI)	%≥0.2 (95% CI)	% ≥1.0 (95% CI)	GMC (95% CI)	%≥0.2 (95% CI)	GMC (95% CI)	%≥0.2 (95% CI)	% ≥1.0 (95% CI)
1	0.20 (0.17-0.22)	47 (40-55)	2.16 (1.89-2.46)	100 (98-100)	80 (74-86)	0.22 (0.19-0.26)	50 (42-57)	2.50 (2.19-2.86)	100 (97-100)	84 (78-89)	0.03 (0.03-0.04)	3 (1-7)	0.03 (0.03-0.04)	4 (1-7)	3 (1-6)
4	0.39 (0.34-0.46)	78 (71-84)	3.04 (2.70-3.42)	100 (98-100)	95 (90-97)	0.43 (0.37-0.50)	81 (75-87)	3.29 (2.89-3.73)	100 (98-100)	96 (92-98)	0.38 (0.34-0.43)	76 (70-82)	4.01 (3.53-4.56)	100 (98-100)	98 (94-99)
5	0.41 (0.36-0.47)	79 (72-84)	3.27 (2.90-3.70)	100 (97-100)	91 (86-95)	0.42 (0.37-0.48)	84 (78-89)	3.27 (2.90-3.68)	100 (98-100)	91 (86-95)	0.03 (0.03-0.04)	2 (0-4)	0.04 (0.03-0.04)	2 (1-5)	0 (0-2)
6B	0.30 (0.25-0.36)	70 (59-74)	1.45(1.23-1.71)	95 (90-97)	75 (68-81)	0.36 (0.29-0.44)	71 (63-77)	1.41 (1.19-1.67)	94 (89-97)	69 (62-76)	0.13 (0.11-0.16)	32 (26-39)	2.52 (2.15-2.96)	97 (93-99)	87 (81-91)
7F	0.60 (0.54-0.68)	94 (89-97)	3.79 (3.40-4.23)	100 (98-100)	98 (95-99)	0.62 (0.55-0.71)	91 (85-94)	4.06 (3.63-4.54)	100 (98-100)	99 (96-100)	0.03 (0.03-0.03)	3 (1-6)	0.03 (0.03-0.04)	4 (2-8)	2 (1-5)
9V	0.68 (0.60-0.78)	93 (89-97)	3.96 (3.58-4.39)	100 (98-100)	100 (97-100)	0.76 (0.66-0.89)	93 (89-97)	4.23 (3.78-4.74)	100 (98-100)	97 (94-99)	0.55 (0.49-0.62)	90 (85-94)	6.05 (5.38-6.80)	100 (98-100)	99 (97-100)
14	1.06 (0.90-1.26)	93 (88 -96)	4.59 (4.06-5.19)	100 (98-100)	97 (93-99)	1.14 (0.95-1.36)	94 (89-97)	4.95 (4.38-5.60)	100 (98-100)	98 (95-99)	1.55 (1.35-1.79)	96 (92-98)	7.31 (6.37-8.39)	99 (96-100)	97 (94-99)
18C	0.58 (0.49-0.68)	85 (79-90)	6.36 (5.56-7.27)	100 (97-100)	99 (96-100)	0.43 (0.36-0.50)	79 (72-85)	4.63(4.09-5.25)	100 (97-100)	99 (96-100)	0.40 (0.35-0.45)	78 (71-84)	5.08 (4.47-5.78)	100 (97-100)	98 (94-99)
19F	0.79 (0.64-0.99)	87 (81-91)	5.45 (4.72-6.30)	98 (95-100)	96 (92-98)	0.90 (0.73-1.11)	89 (83-93)	5.80 (5.04-6.68)	99 (96-100)	96 (92-98)	0.31 (0.25-0.37)	55 (48-62)	2.40 (2.14-2.70)	100 (98-100)	88 (83-92)
23F	0.33 (0.27-0.39)	68 (61-75)	2.32 (1.98-2.71)	97 (93-99)	87 (81-92)	0.38 (0.32-0.46)	77 (70-83)	2.60 (2.24-3.02)	98 (95-99)	87 (81-91)	0.26 (0.22-0.30)	55 (48-62)	5.32 (4.49-6.31)	98 (94-99)	97 (93-99)
6A	0.10 (0.09-0.12)	28 (21-35)	0.45 (0.36-0.55)	72 (65-79)	32 (26-39)	0.11 (0.09-0.13)	30 (24-37)	0.49 (0.40-0.61)	72 (66-79)	34 (27-41)	0.06 (0.05-0.07)	15 (10-20)	0.71 (0.58-0.88)	80 (73-85)	50 (43-57)
19A	0.12 (0.10-0.15)	34 (27-41)	0.50 (0.39-0.63)	70 (62-76)	41 (34-48)	0.12 (0.09-0.14)	34 (27-42)	0.52 (0.41-0.66)	71 (64-79)	41 (34-48)	0.05 (0.05-0.07)	15 (10-20)	0.21 (0.17-0.25)	49 (41-56)	12 (8-17)

Table 1: **Serotype-specific antibody GMCs and proportion of children with IgG ≥ 0.2 µg/mL and ≥ 1.0 µg/mL (ATP cohort for booster immunogenicity).** N indicates the maximum number of children with available results; actual number included in the analysis varies slightly per antigen, depending on serum availability for testing;

	PHiD-CV + DTPa-HBV-IPV/Hib group				PHiD-CV + DTPa-IPV-Hib group				7vCRM + DTPa-IPV-Hib group			
	Pre-booster N=149		Post-booster N=156		Pre-booster N=152		Post-booster N=154		Pre-booster N=168		Post-booster N=164	
Serotype	GMT (95% CI)	%≥8 (95% CI)	GMT (95% CI)	%≥8 (95% CI)	GMT (95% CI)	%≥8 (95% CI)	GMT (95% CI)	%≥8 (95% CI)	GMT (95% CI)	%≥8 (95% CI)	GMT (95% CI)	%≥8 (95% CI)
1	5.5 (4.7-6.4)	11 (7-18)	208.8 (160.6-271.7)	92 (87-96)	5.6 (4.7-6.7)	12 (7-18)	221.4 (165.1-297.0)	91 (85-95)	5.0 (4.4-5.8)	7 (4-12)	4.6 (4.2-5.1)	5 (2-9)
4	11.3 (8.6-14.9)	34 (26-42)	1046.8 (865.8-1266.0)	99 (97-100)	15.3 (11.3-20.6)	41 (33-49)	1121.6 (909.4-1383.3)	99 (95-100)	12.3 (9.3-16.2)	37 (29-45)	2335.8 (1946.3-2803.3)	100 (98-100)
5	7.2 (6.1-8.5)	30 (23-38)	149.3 (119.2-187.0)	97 (93-99)	7.0 (6.0-8.1)	31 (23-39)	132.4 (103.8-168.9)	93 (88-97)	4.3 (4.0-4.6)	2 (1-6)	4.1 (4.0-4.3)	1 (0-4)
6B	52.0 (34.8-77.7)	62 (53-70)	681.4 (514.6-902.1)	94 (86-97)	96.7 (63.1-148.0)	68 (60-76)	763.3 (581.9-1001.3)	95 (90-98)	27.4 (18.6-40.3)	46 (38-54)	1807.5 (1408.0-2320.3)	97 (93-99)
7F	818.9 (642.2-1044)	97 (93-99)	3936.5 (3413.0-4540.0)	100 (98-100)	754.3 (598.4-950.8)	97 (92-99)	3976.0 (3390.2-4663.1)	100 (98-100)	138.1 (91.2-209.2)	69 (61-77)	129.1 (85.9-193.9)	68 (60-75)
9V	267.2 (212.7-335.8)	94 (89-98)	2512.9 (2213.0-2853.0)	100 (98-100)	338.7 (264.8-433.2)	96 (91-99)	2257.6 (1974.3-2581.5)	100 (98-100)	149.5 (112.4-198.9)	86 (80-91)	3889.5 (3260.1-4640.2)	99 (97-100)
14	117.3 (82.9-165.9)	79 (71-85)	1534.2 (1291.0-1823.0)	99 (96-100)	142.5 (101.3-200.4)	81 (74-87)	1896.3 (1591.3-2259.7)	99 (96-100)	156.0 (114.5-212.5)	84 (77-89)	1867.9 (1540.5-2265.1)	98 (95-100)
18C	8.2 (6.2-10.8)	22 (15-29)	720.7 (597.5-869.4)	99 (96-100)	6.8 (5.3-8.5)	16 (11-23)	385.9 (303.5-490.8)	95 (90-98)	7.0 (5.7-8.7)	19 (13-26)	660.4 (520.9-837.3)	99 (96-100)
19F	13.8 (10.6-18.1)	44 (36-52)	435.7 (336.3-564.5)	94 (88-97)	15.4 (11.8-20.2)	48 (40-57)	475.4 (377.8-598.1)	98 (94-100)	7.8 (6.2-9.9	20 (14-27)	123.2 (95.9-158.2)	92 (86-95)
23F	314.1 (198.6-496.7)	76 (68-82)	2895.4 (2276.0-3683.0)	98 (94-100)	350.4 (229.8-534.2)	80 (73-87)	2895.3 (2419.3-3465.0)	99 (96-100)	338.9 (219.9-522.3)	75 (68-82)	12418.7 (10172.0-15162.0)	100 (98-100)
6A	23.3 (15.7-34.6)	41 (32-50)	143.3 (99.1-207.3)	75 (67-81)	30.7 (20.8-45.2)	47 (38-55)	197.1 (138.0-281.3)	80 (72-86)	15.5 (11.1-21.6)	32 (25-40)	493.3 (357.1-681.6)	88 (82-93)
19A	5.7 (4.6-7.0)	10 (5-15)	34.9 (24.8-49.2)	60 (51-68)	5.5 (4.6-6.5)	10 (5-15)	24.6 (17.7-34.3)	53 (44-61)	5.5 (4.6-6.5)	8 (4-13)	7.7 (6.2-9.5)	25 (18-32)

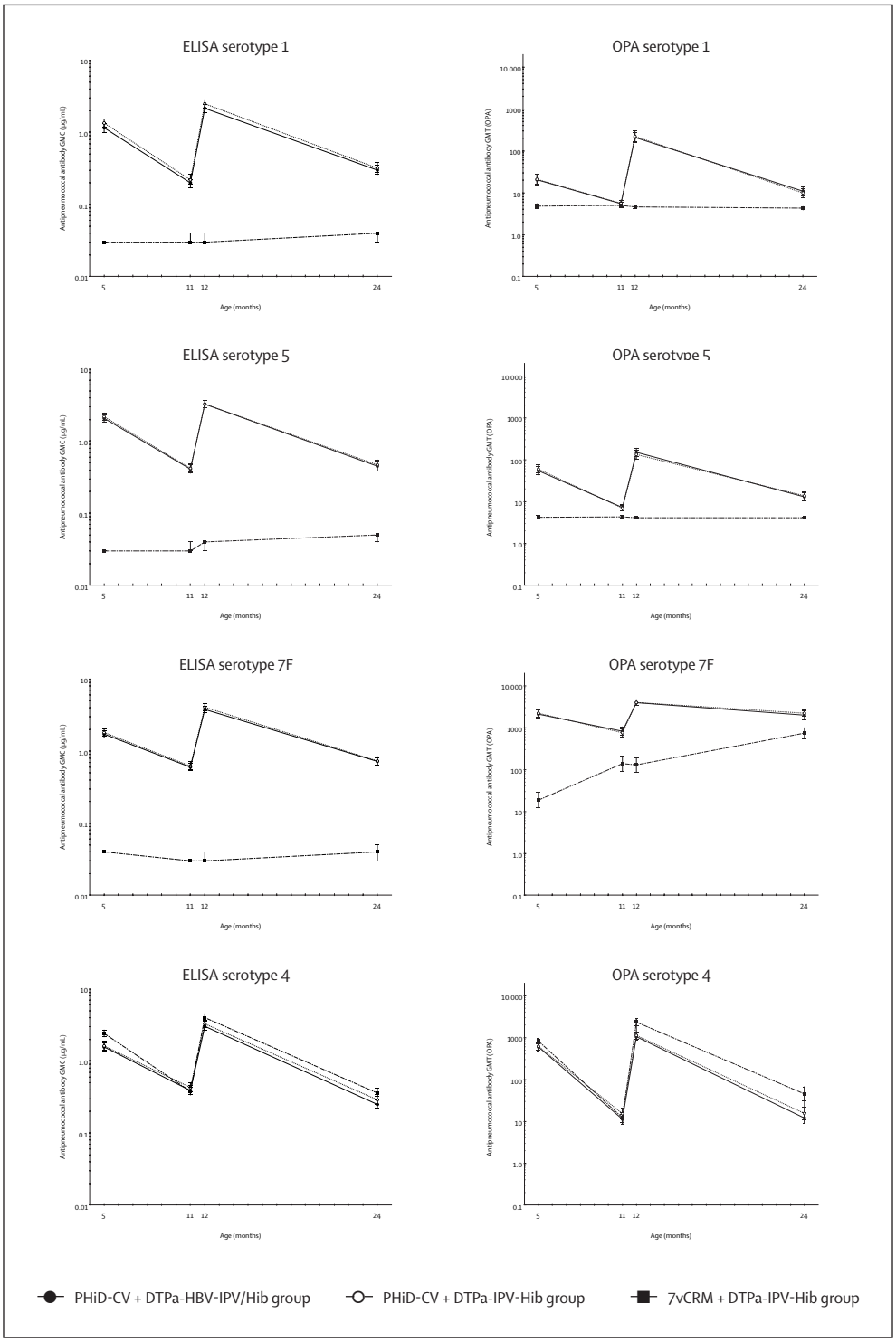
***Table 2*: Serotype-specific opsonophagocytic activity and proportion of children with titers ≥ 8 (ATP cohort for booster immunogenicity).** N indicates the maximum number of children with available results; actual number included in the analysis varies slightly per antigen, depending on serum availability for testing;

		PHiD-CV + DTPa-HBV-IPV/Hib group		PHiD-CV + DTPa-IPV-Hib group		7vCRM + DTPa-IPV-Hib group	
Antigen		Pre-booster	Post-booster	Pre-booster	Post-booster	Pre-booster	Post-booster
Diphtheria	N	175	186	179	194	192	197
	GMC [IU/mL] (95% CI)	0.235 (0.205-0.270)	4.061 (3.601-4.580)	0.232 (0.203-0.264)	3.226 (2.866-3.632)	0.266 (0.235-0.301)	4.882 (4.425-5.386)
	% ≥0.1 IU/mL (95% CI)	85 (78-90)	100 (98-100)	84 (78-89)	100 (98-100)	89 (83-93)	100 (98-100)
Tetanus	N	175	186	180	194	193	197
	GMC (IU/mL)	0.728 (0.656-0.809)	8.628 (7.867-9.462)	0.536 (0.477-0.603)	5.989 (6.568)	0.232 (0.202-0.267)	3.248 (2.859-3.690)
	% ≥0.1 IU/mL	99 (97-100)	100 (98-100)	97 (94-100)	100 (98-100)	80 (73-85)	100 (98-100)
PT	N	174	186	176	194	176	194
	GMC [EL.U/mL] (95% CI)	7.4 (6.6-8.4)	53.5 (48.3-59.2)	6.0 (5.4-6.7)	47.4 (42.9-52.5)	6.0 (5.4-6.7)	47.4 (42.9-52.5)
	% ≥5 EL.U/mL	71 (64-78)	100 (98-100)	67 (59-73)	100 (97-100)	67 (59-73)	100 (97-100)
FHA	N	175	184	179	192	191	194
	GMC [EL.U/mL] (95% CI)	34.0 (29.4-39.4)	343.5 (308.0-383.1)	35.3 (30.8-40.6)	135.7 (121.2-151.9)	34.7 (30.1-39.9)	140.0 (123.2-159.1)
	% ≥5 EL.U/mL	98 (94-99)	100 (98-100)	99 (97-100)	100 (98-100)	99 (96-100)	100 (98-100)
PRN	N	175	186	179	193	192	197
	GMC [EL.U/mL] (95% CI)	14.1 (12.3-16.2)	281.7 (247.2-321.0)	6.8 (5.9-7.9)	97.8 (85.4-112.0)	8.6 (7.4-10.0)	106.4 (91.5-123.8)
	% ≥5 EL.U/mL	87 (82-92)	100 (98.0-100)	60 (52-67)	100 (98-100)	68 (61-75)	99 (96-100)

***Table 3*: Antibody GMCs/GMTs and seroprotection/-positivity rates to the coadministered vaccine antigens (ATP cohort for booster immunogenicity).** N indicates the number of children with available results; NA, not available. For FHA, poliovirus types 1, 2 and 3, hepatitis B, data are preliminary and may be adopted pending verification of the assay results.

		PHiD-CV + DTPa-HBV-IPV/Hib group		PHiD-CV + DTPa-IPV-Hib group		7vCRM + DTPa-IPV-Hib group	
Antigen		Pre-booster	Post-booster	Pre-booster	Post-booster	Pre-booster	Post-booster
Poliovirus type 1	N	156	166	166	172	182	170
	GMT (95% CI)	8.3 (7.0-10.0)	370.7 (289.8-474.2)	9.0 (7.5-10.7)	311.9 (240.4-404.6)	7.0 (6.1-8.0)	221.2 (171.5-285.3)
	% ≥8	41 (33-49)	98 (94-99)	42 (35-50)	95 (90-98)	34 (27-41)	93 (88-96)
Poliovirus type 2	N	156	167	164	173	182	169
	GMT (95% CI)	13.4 (10.8-16.6)	710.8 (588-859.3)	10.6 (8.8-12.7)	338.8 (272.7-420.8)	10.4 (8.9-12.2)	481.4 (391.0-592.7)
	% ≥8	54 (46-62)	100 (98-100)	50 (42-58)	97 (93-99)	56 (48-63)	98 (95-100)
Poliovirus type 3	N	156	167	166	172	180	170
	GMT (95% CI)	11.3 (9.3-13.9)	631.5 (495.7-804.4)	9.0 (7.5-10.7)	311.9 (240.4-404.6)	8.7 (7.4-10.3)	348.3 (263.6-460.2)
	% ≥8	50 (42-58)	98 (94-99)	42 (35-50)	95 (90-98)	43 (35-50)	95 (91-98)
PRP	N	174	184	179	192	193	197
	GMC [µg/mL] (95% CI)	0.475 (0.386-0.585)	19.331 (16.144-23.147)	0.855 (0.682-1.072)	39.383 (32.617-47.551)	0.371 (0.298-0.461)	23.676 (18.944-29.591)
	% ≥0.15 µg/mL (95% CI)	79 (73-85)	100 (98-100)	84 (78-89)	100 (98-100)	64 (57-71)	99 (96-100)
	% ≥1.0 µg/mL (95% CI)	27 (21-34)	99 (96-100)	47 (39-55)	99 (96-100)	99 (96-100)	96 (92-98)
Hepatitis B	N	121	125	124	123	135	135
	GMC [mIU/mL] (95% CI)	142.1 (113.4-178.1)	1981.0 (1552.0-2528.7)	NA	NA	NA	NA
	% ≥10 mIU/mL (95% CI)	98 (94-100)	100 (97-100)	NA	NA	NA	NA

Table 3: **Continued.**


ELISA serotype 1
OPA serotype 1
ELISA serotype 5
OPA serotype 5
ELISA serotype 7F
OPA serotype 7F
ELISA serotype 4
OPA serotype 4
Age (months)
PHiD-CV + DTPa-HBV-IPV/Hib group
PHiD-CV + DTPa-IPV-Hib group
7vCRM + DTPa-IPV-Hib group

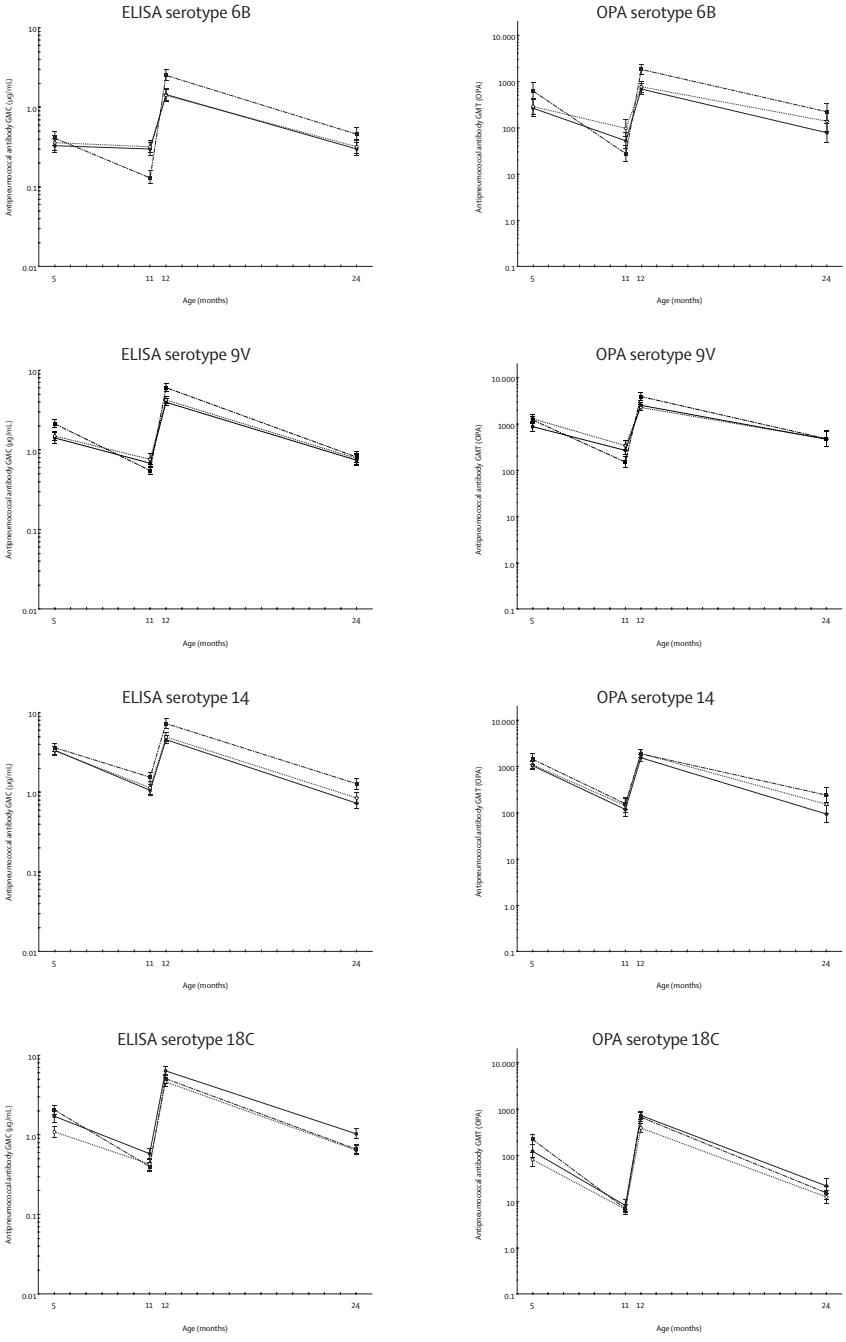


Figure 3: **Kinetics of serotype-specific antipneumococcal antibody GMCs and OPA GMTs** (ATP cohort for booster immunogenicity). Antibody GMCs and OPA GMTs for vaccine pneumococcal serotypes and cross-reative serotypes during follow-up. Error bars indicate 95% confidence intervals.

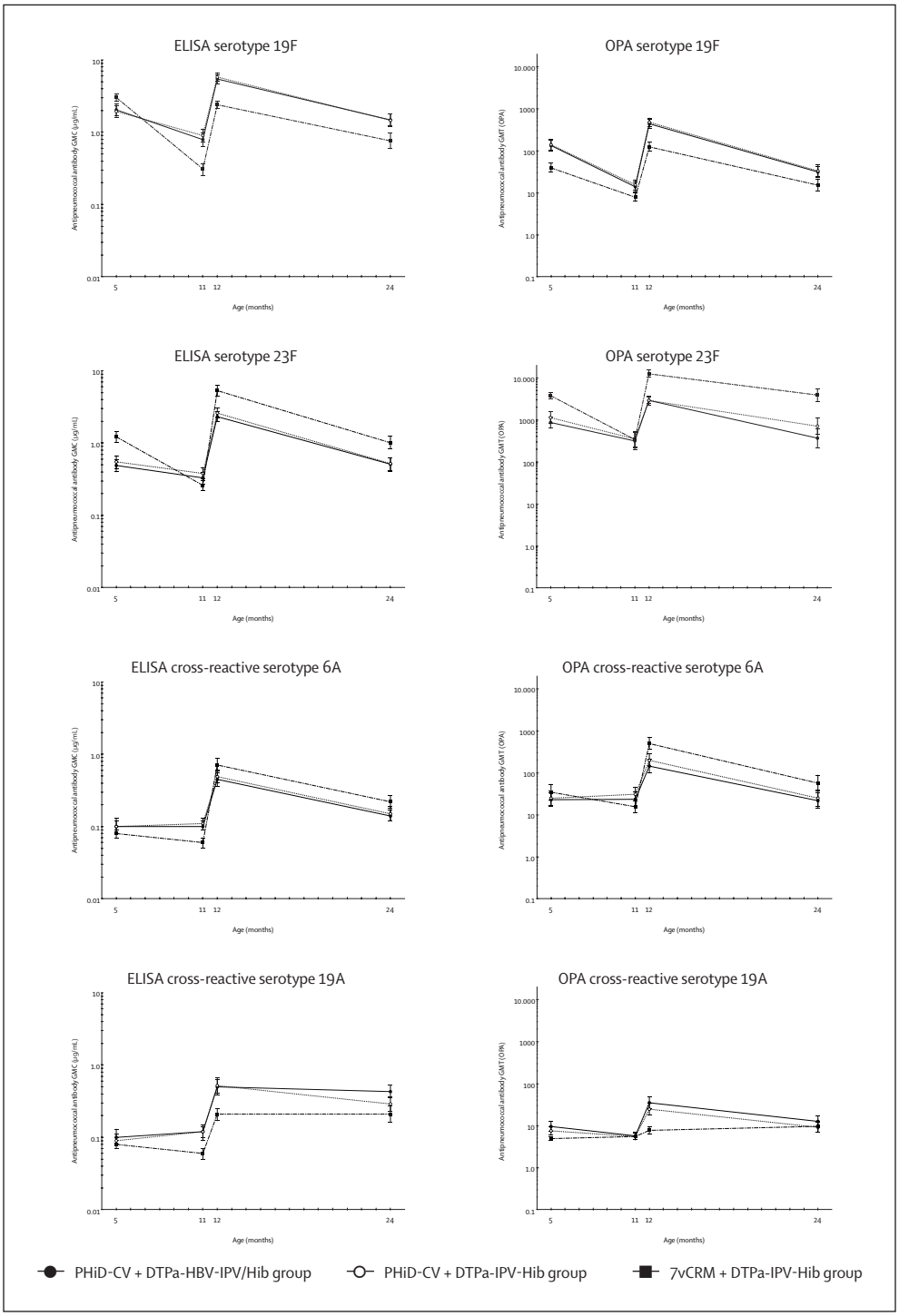


Figure 3: **Continued.**

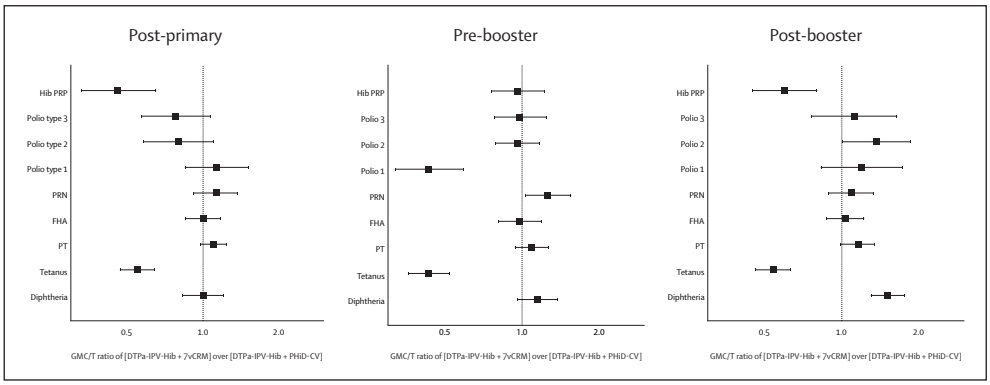


Figure 4: **Ratios of antibody GMC/GMTs between DTPa-IPV-Hib groups.** [a] GMC/GMT ratios with error bars indicating 95% confidence intervals (CI). A ratio of 1 (dashed line) indicates no difference in antibody GMC/GMTs between groups. ATP cohort for booster immunogenicity. [a] Ratio of DTPa-IPV-Hib coadministered with 7vCRM over DTPa-IPV-Hib coadministered with PHiD-CV.

Evaluation of safety and reactogenicity

Serious adverse events were reported in 96 of 780 subjects (12%) during the entire study period, equally distributed across groups. During the booster study phase, no serious adverse event was considered to be causally related to vaccination. The incidence of solicited local symptoms (Supplementary Figure 1), general symptoms (Supplementary Figure 2) and unsolicited events (data not shown) were generally comparable across groups. Fifteen large swelling reactions were reported: 4 at the PHiD-CV injection site, 2 at the 7vCRM injection site, 4 at the DTPa-HBV-IPV/Hib injections site and 5 at the DTPa-IPV-Hib injection site. All reported large swelling reactions were local or diffuse, did not involve the adjacent joint, none was accompanied by functional impairment and all resolved without sequelae.

Discussion

In this randomized controlled trial, we have shown that coadministration of PHiD-CV and DTPa-IPV-Hib as a 3+1-dose schedule at 2, 3, 4 and 11-13 months of age is immunogenic and well-tolerated. The combination of coadministered vaccines affected the antibody levels to pneumococcal vaccine serotype 18C and the coadministered vaccine components Hib-PRP, tetanus and diphtheria, mainly pointing toward enhancement of immune responses.

In our study, anti-18C antibodies were higher at each time point when PHiD-CV was coadministered with DTPa-HBV-IPV/Hib compared with DTPa-IPV-Hib coadministration. The total tetanus toxoid content is higher in DTPa-HBV-IPV/Hib (10 Lf + 30 µg as carrier) than in DTPa-IPV-Hib (5 Lf + 10 µg as carrier) and this may explain the higher elicited antibodies to both the carrier protein as well as the polysaccharide components (18C, PRP) conjugated to it. In line with this observation, higher antitetanus and anti-PRP antibody GMCs are found when DTPa-IPV-Hib is coadministered with PHiD-CV compared to 7vCRM coadministration. All findings are consistent with immunologic enhancement by sharing the same carrier protein (tetanus toxoid) and conjugation of serotype 18C to tetanus toxoid in PHiD-CV. While we and others observed enhancement of the immune response to both tetanus,[9] PRP and serotype 18C,[5] Ruiz-Palacios, et al. instead reported a lower anti-18C booster response when

PHiD-CV was coadministered with a tetravalent meningococcal conjugate vaccine using tetanus toxoid as carrier protein,[15] possibly related to competition for T helper cells.[2,16] Indeed, both enhancement and interference has been described to occur when vaccines using tetanus toxoid as carrier protein are coadministered,[1-3,17] highlighting the unpredictability of the impact on immune responses when vaccines sharing the same carrier protein are concomitantly administered.

Post-booster antidiphtheria antibody GMCs were higher when DTPa-IPV-Hib was coadministered with 7vCRM than with PHiD-CV coadministration. Thist may be due to the CRM197 used as carrier protein in 7vCRM,[1] although post-primary and pre-booster no differences between groups were observed. It should be stressed that the elicited immune responses to pneumococcal serotype 18C, tetanus, PRP and diphtheria were well above the (putative) protective thresholds, irrespective of the coadministered vaccine.
Post-primary anti-poliovirus antibody GMTs and seroprotection rates[5] and post-booster GMTs were lower than expected compared to previous findings,[9] regardless of the administered vaccine. Interestingly, lower polio responses have been observed previously in the Netherlands.[18] The clinical relevance is at present unknown, as post-booster seroprotection rates were largely unaffected (92-100%) and because polio has been eliminated in most parts of the world, including Europe.[19] To verify our results, sera will be retested.
In line with post-primary results, a substantial proportion of DTPa-IPV-Hib vaccinated children reached protective anti-HBs levels after the booster dose. Additional testing will be performed to corroborate current findings.

Next to herd immunity and immunologic memory, a key aspect of sustaining protection in the population against encapsulated bacteria is maintenance of antibody levels above the (putative) protective threshold.[20,21] In the UK, the importance of long-term antibody persistence was emphasized as vaccine failures were reported for *Haemophilus influenzae* type b and group C meningococci, even in the context of proven herd protection.[21] However, circulating antibodies elicited by protein-polysaccharide conjugate vaccines are known to wane over time.[6] Apart from the necessicity of a booster dose, these vaccine failures emphasized the importance of long-term circulating antibody persistence. Long-term antibody responses have been reported for related experimental pneumococcal conjugate vaccines, but no such data are published with respect to PHiD-CV or 7vCRM.[22,23] In this study, we have investigated the persistence of antipneumococcal antibodies one year after the booster dose. We found that within a year after the booster dose, antibody levels had declined to approximate pre-booster levels. The clinical impact of these observations is unknown, as long-term protection against encapsulated bacteria also depends on immunologic memory, next to herd immunirty. Both the incidence of invasive pneumococcal disease[24] and nasopharyngeal pneumococcal colonization rates decline after the second year of life.[25] This occurs independent from the development of serotype-specific anticapsular antibodies and is likely due to maturation of the innate immunity and development of acquired immunity to non-capsular pneumococcal antigens.[26] Furthermore, children up to five years of age are protected against vaccine serotype disease owing to the profound effect of herd immunity.[27] In the Netherlands, already three years after 7vCRM introduction (without a catch-up campaign), vaccine pneumococcal serotypes virtually disappeared from the nasopharynx of young children and their parents.[28] However, it should be noted that, similar to the experience with Hib and group C meningococci, pneumococcal seroepidemiology might change in the absence of natural boosting. We did not observe a substantial decline in antibodies to the cross-reactive serotype 19A between 12 and

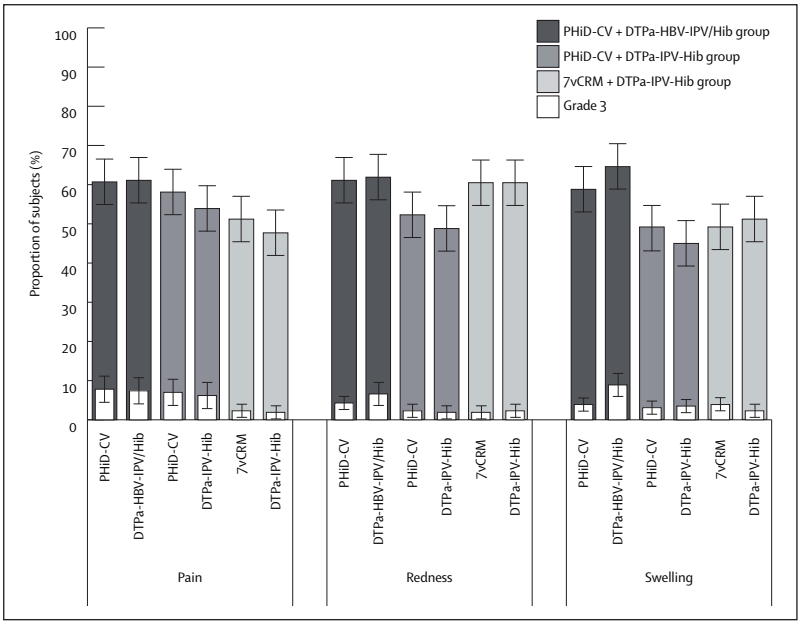
24 months of age. This might be due to continuous natural boosting of the immune system, since serotype 19A was found to be the most prevalent colonizing strain during the study period.[14] This reasoning is in line with previously reported long-term increases in antibodies GMCs to vaccine pneumococcal serotypes in the pre-pneumococcal conjugate vaccine era (that is, with circulating vaccine serotypes),[23] and particularly in populations with high pneumococcal colonization and disease rates.[22,29]

A limitation of our study was that all comparisons reported here (i.e., secondary objectives) were descriptive. In addition, immunogenicity studies only provide indirect evidence for protection against disease. Furthermore, bridging of immunogenicity data from one study to the next harbors the danger of drifting away from the primary efficacy trials in which the protective thresholds were established.[30]

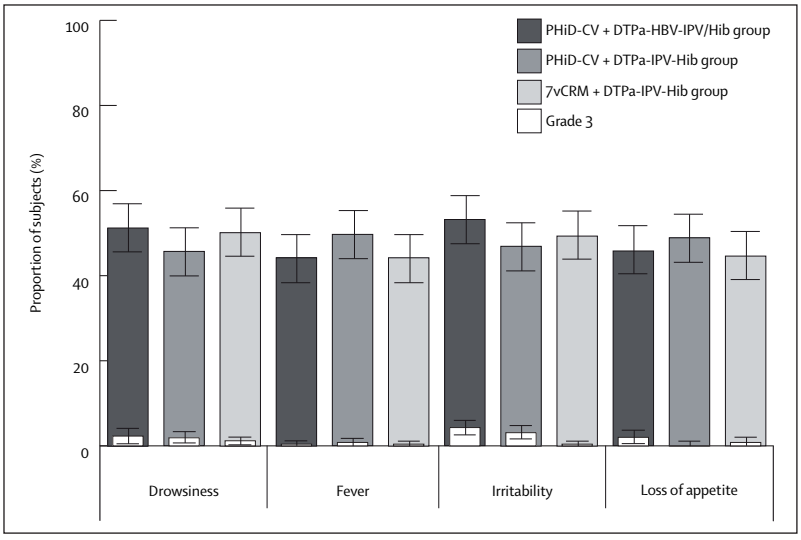
Strengths of this study include the randomized controlled longitudinal trial setting, virtual absence of loss to follow-up, evaluation of the immunogenicity of different combination of vaccines, including assessment of functional antipneumococcal antibodies at several time points during a period when protection against disease is most warranted.[24]

In conclusion, we have shown that PHiD-CV and DTPa-IPV-Hib are immunogenic and well-tolerated when coadministered as a 3+1-dose schedule at 2, 3, 4 and 11-12 months of age in the Netherlands. Nevertheless, some differences in antibody concentrations were found between vaccine combinations, highlighting the importance of both serological and disease surveillance.

Supplementary material



Supplementary Figure 1: **Incidence of solicited local symptoms reported during the 4-day follow-up period after booster vaccination** (Total Vaccinated Cohort). Error bars indicate 95% confidence intervals. Grade 3 intensity for pain: crying when the limb was moved or if it was spontaneously painful; redness and swelling: diameter at the injection site of >30 mm.



Supplementary Figure 2: **Incidence of solicited general symptoms reported during the 4-day follow-up period after booster vaccination** (Total Vaccinated Cohort) Error bars indicate 95% confidence intervals. Grade 3 intensity for fever: rectal temperature >40°C; irritability: crying inconsolably or preventing normal daily activity; loss of appetite: not eating at all; other symptoms: preventing normal daily activity.

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CHAPTER 4

Effects of the 10-valent pneumococcal nontypeable *Haemophilus influenzae* protein D-conjugate vaccine (PHiD-CV) on nasopharyngeal bacterial colonization in young children: a randomized controlled trial

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This trial is registered at www.ClinicalTrials.gov: NCT00652951

Abstract

Background This study evaluated the effects of the 10-valent pneumococcal nontypeable *Haemophilus influenzae* protein D-conjugate vaccine (PHiD-CV) on nasopharyngeal bacterial colonization compared with the 7-valent pneumococcal conjugate vaccine (7vCRM) in young children.

Methods A randomized controlled trial in the Netherlands, initiated 2 years post-7vCRM introduction, was conducted between April 1, 2008 and December 1, 2010. Infants (n=780) received either PHiD-CV or 7vCRM (2:1) at 2, 3, 4 and 11-13 months of age. Nasopharyngeal samples taken at 5, 11, 14, 18 and 24 months of age were cultured to detect *Haemophilus influenzae* (Hi), *Streptococcus pneumoniae* (Spn), *Moraxella catarrhalis* (Mc) and *Staphylococcus aureus* (Sa). PCR assays quantified Hi and Spn and confirmed Hi as nontypeable (NTHi). Primary outcome measure was vaccine efficacy (VE) against NTHi colonization.

Results In both groups, NTHi colonization increased with age from 33% in 5-month-old to 65% in 24-month-old children. 3 months post-booster, VE against colonization was 0.5% (95% CI, -21.8% to 18.4%) and VE against acquisition 10.9% (95% CI, -31.3% to 38.9%). At each sampling moment, no differences between groups in either NTHi prevalence or Hi density were detected. Spn (range, 39%–57%), Mc (range, 63%¬–69%) and Sa (range, 9%–30%) colonization patterns were similar between groups.

Conclusion PHiD-CV had no differential effect on nasopharyngeal NTHi colonization or Hi density in healthy Dutch children up to two years of age, implying that herd effects for NTHi are not to be expected. Other bacterial colonization patterns were also similar.

Funding GlaxoSmithKline Biologicals.

Introduction

Pneumococcal conjugate vaccination is highly effective in preventing serotype-specific invasive pneumococcal disease, such as meningitis, sepsis and invasive pneumonia.[1] In addition, *Streptococcus pneumoniae* is frequently involved in common mucosal bacterial infections in childhood, like pneumonia and acute otitis media (AOM).[1,2] Unencapsulated or nontypeable *Haemophilus influenzae* (NTHi) is the second most common bacterial respiratory pathogen.[2,3] This otopathogen is particularly associated with recurrent otitis and its sequelae, presumably through biofilm formation.[3–5]

The human nasopharynx is the reservoir for both pneumococcus and NTHi, and the source of transmission between individuals.[6] The great success of polysaccharide-protein conjugate vaccines, targeting encapsulated bacteria like pneumococcus, group C meningococcus and *H. influenzae* type b, is based not only on inducing protective antibodies in immunized individuals, but also on reducing nasopharyngeal colonization and preventing transmission of the vaccine-targeted bacteria.[7–9] The consequent indirect (herd) effects following pneumococcal conjugate vaccination resulted in protection of the unimmunized population, which had a major impact on the overall effectiveness.[10] Assessment of nasopharyngeal colonization of vaccine-targeted bacteria in immunized individuals is therefore important for understanding the mechanism and to predict effects after widespread implementation.[11]

An experimental polysaccharide-protein conjugate vaccine, in which each of 11 pneumococcal capsular saccharides was conjugated to NTHi-derived protein D (PD) as a carrier protein (11Pn-PD, GlaxoSmithKline Vaccines), was found to not only protect against vaccine serotype-specific pneumococcal AOM, but also to prevent 35% of NTHi AOM episodes.[12] The licensed 10-valent pneumococcal nontypeable *H. influenzae* protein D-conjugate vaccine (PHiD-CV, GlaxoSmithKline Vaccines) is based on its 11-valent predecessor and uses PD as a carrier for 8 out of 10 pneumococcal serotypes.[13] Data regarding the effects of these PD-conjugate vaccines on nasopharyngeal bacterial colonization are limited.[12,14,15] Therefore, irrefutable evidence that PD-conjugate vaccines affect NTHi colonization and thus prevent NTHi transmission is still lacking. Biological effects of the carrier protein may differ from those of conjugated polysaccharides and may not affect nasopharyngeal colonization.

Additionally, pneumococcal conjugate vaccination shifts colonization from vaccine to nonvaccine pneumococcal serotypes,[16] and may affect colonization with non-pneumococcal species, such as Staphylococcus aureus or NTHi.[17, 18] In a randomized controlled trial, we compared nasopharyngeal bacterial colonization in children immunized with PHiD-CV with children receiving the 7-valent pneumococcal conjugate vaccine, in which 7 serotypes are covalently bound to the carrier protein CRM197, a non-toxic diphtheria mutant (7vCRM, Pfizer, Inc.). This trial was initiated approximately two years after introduction of 7vCRM in the Dutch national immunization program (NIP).

Material and methods

Study design and population

We conducted this trial between April 1, 2008 and December 1, 2010. The study area covered the western and central parts of the Netherlands.[19] Healthy infants born after a gestation period of at least 36 weeks and aged 6 to 12 weeks at the time of the first primary vaccination were eligible. Exclusion criteria have been detailed previously.[19] Infants were visited at home for all study procedures. Participants received no financial compensation. An Independent

Ethics Committee (Centrale Commissie Mensgebonden Onderzoek (CCMO), available at: http://www.ccmo-online.nl) approved the study protocol (NCT00652951). The study was undertaken in accordance with the European guidelines for Good Clinical Practice, which incorporate provisions of the Declaration of Helsinki. Written informed consent was obtained from each infant's parent(s)/guardian(s) before enrolment. 7vCRM was introduced in the Dutch NIP for all infants born after March 31, 2006 with no catch-up campaign. PHiD-CV replaced 7vCRM in the Dutch NIP for all infants born after March 1, 2011.

Randomization and masking

Infants were randomly assigned (1:1:1) to receive either (1) PHiD-CV + DTPa-HBV-IPV/Hib, (2) PHiD-CV + DTPa-IPV-Hib, or (3) 7vCRM + DTPa-IPV-Hib at 2, 3, 4 and 11-13 months of age, resulting in a 2:1 ratio for immunization with either PHiD-CV or 7vCRM. A randomization list used to number the vaccines was generated using a standard SAS (Statistical Analysis System) program (version 9.2) with a block size of 6 (blocking scheme of 2:2:2). Each participant was assigned to a group via a web-based central randomization system that, on receipt of the infant's birth date and scheduled date for the first home visit, determined the vaccine number to be used. Parents and study site staff were aware of the treatment assignment, but outcome assessors were not.

Procedures

Vaccines were administered intramuscularly in the left and right anterolateral thigh. The composition of PHiD-CV (Synflorix™; GlaxoSmithKline Vaccines), 7vCRM (Prevenar™/Prevnar™; Pfizer, Inc.), DTPa-HBV-IPV/Hib (Infanrix hexa™; GlaxoSmithKline Vaccines) and DTPa-IPV-Hib (Pediacel™, Sanofi Pasteur MSD) has been previously described.[19]

Nasopharyngeal samples were obtained transnasally using a flexible, sterile swab with a flocked nylon tip (Eswab 482CE; Copan, Brescia, Italy) by trained study personnel according to World Health Organization procedures[20] at 5, 11, 14, 18 and 24 months of age. With each nasopharyngeal swab, a questionnaire addressing risk factors for bacterial colonization was completed. After sampling, swabs were immediately inoculated in 1 mL of modified liquid Amies transport medium and transferred at room temperature to the Regional Laboratory of Public Health (Haarlem, the Netherlands). Samples were plated as soon as possible (< 8 hours), but at the latest within 24 hours after sampling. Swabs were used to inoculate the plates. Identification of *H. influenzae*, *S. pneumoniae*, *M. catarrhalis* and *S. aureus* was based on colony morphology and conventional methods of determination, as previously described.[9, 17, 21] To differentiate *H. influenzae* from *H. haemolyticus*, a closely related but non-pathogenic species,[22] all *H. influenzae* isolates underwent polymerase chain reaction (PCR), targeting the glycosyltransferase (*lgtC*) and outer membrane protein P6 (*P6*) genes.

One *S. pneumoniae* colony per plate was subcultured and pneumococcal isolates were serotyped by Quellung reaction. Likewise, one *H. influenzae* colony per plate was subcultured and typed by slide agglutination serotyping. After plating, the remaining medium was stored at -70°C until being transferred to GlaxoSmithKline Vaccines' laboratories.

Here, the presence of *H. influenzae* and *S. pneumoniae* was quantified in all available, properly stored original samples using two real-time quantitative PCR (qPCR) assays, targeting the *lgtC* gene and autolysin (*lytA*) gene, respectively. DNA was extracted using the NucliSENS™ easyMAG™ (BioMérieux), and DNA fragments amplified using the 7900HT Real-Time PCR System (Applied Biosystems). Bacterial densities were expressed as genome equivalents per milliliter (GE/mL). A serotype-specific PCR for 19A assay was also developed.

Statistical analysis

This trial was primarily designed to assess immunogenicity.[19] The study was adequately powered to assess effects of PHiD-CV on NTHi colonization using 7vCRM as a non-active control. Given an estimated 55% prevalence for *H. influenzae* in the 7vCRM group based on previous experience,[21] a sample size of 520 subjects in the PHiD-CV group and 260 subjects in the 7vCRM group would allow demonstration of a positive vaccine efficacy of 19.8% with 80% power at a one-sided alpha level of 2.5% (calculated with PASS2005). Statistical analysis was performed on the total vaccinated cohort, meaning that all available data from all participants allocated to a treatment group were analyzed according to the assigned intervention. The percentage of subjects with a positive culture for the considered bacterium (or serotype) was calculated per group at each sampling moment and across visits, as was the frequency of acquisition of new bacteria (or serotypes). Acquisition was defined as a subject whose nasopharyngeal swab became positive for a bacterium (or serotype) after a previously negative swab for that bacterium (or serotype).[15] In case of missing data, the occurrence of bacteria (or serotypes) was not considered as a 'new acquisition'. Vaccine efficacy of PHiD-CV against bacterial colonization (VEcol) and acquisition (VEacq) was estimated as [(1 – relative risk) x 100] and calculated with 95% confidence intervals (CI) at each time point and across visits. Statistical significance of vaccine efficacy was based on a lower limit of the 95% CI greater than 0. No adjustment for multiple comparisons was made and conclusions on statistical significance should therefore be interpreted with caution. The statistical analyses were performed using SAS version 9.2 and SDD (SAS Drug and Development) web portal version 3.5.

Results

A total of 780 children were enrolled and allocated to one of three groups (Figure 1). Recruitment started on April 1, 2008 and was completed on January 30, 2009. Follow-up ended on December 1, 2010. Characteristics of the children did not differ between groups (Table 1). A total of 3863 (99% of planned) nasopharyngeal swabs were collected.

Nasopharyngeal NTHi colonization, acquisition and density

Based on conventional culture methods, 65% of cultured swabs were positive for *H. influenzae*. After differentiation from *H. haemolyticus*, 59% of all samples were confirmed *H. influenzae*. Of these, 92% were NTHi, while the remainder was encapsulated. NTHi colonization increased with age from approximately 33% at 5 months to 65% at 24 months (Table 2). Prevalence rates were similar in both groups at each sampling moment (Table 2), as were the frequencies of acquisition (Supplementary Table 1). At 3 months post-booster, VEcol was 0.5% (95% CI, -21.8% to 18.4) and VEacq was 10.9% (95% CI, -31.3% to 38.9%).

The presence of *H. influenzae* detected by qPCR was 44% in 5-month-olds in both groups and increased with age to 84% (95% CI, 80% to 87%) and 80% (95% CI, 75% to 85%) in 24-month-olds vaccinated with PHiD-CV and 7vCRM, respectively. Of all culture-positive samples (confirmed as true *H. influenzae* by PCR), 96% was found to be also positive by qPCR, while qPCR detected *H. influenzae* in 30% of culture-negative samples (Supplementary Table 2). *H. influenzae* density increased with age, reaching a plateau in the second year of life. Densities were comparable at each sampling moment, irrespective of the administered vaccine (Figure 2).

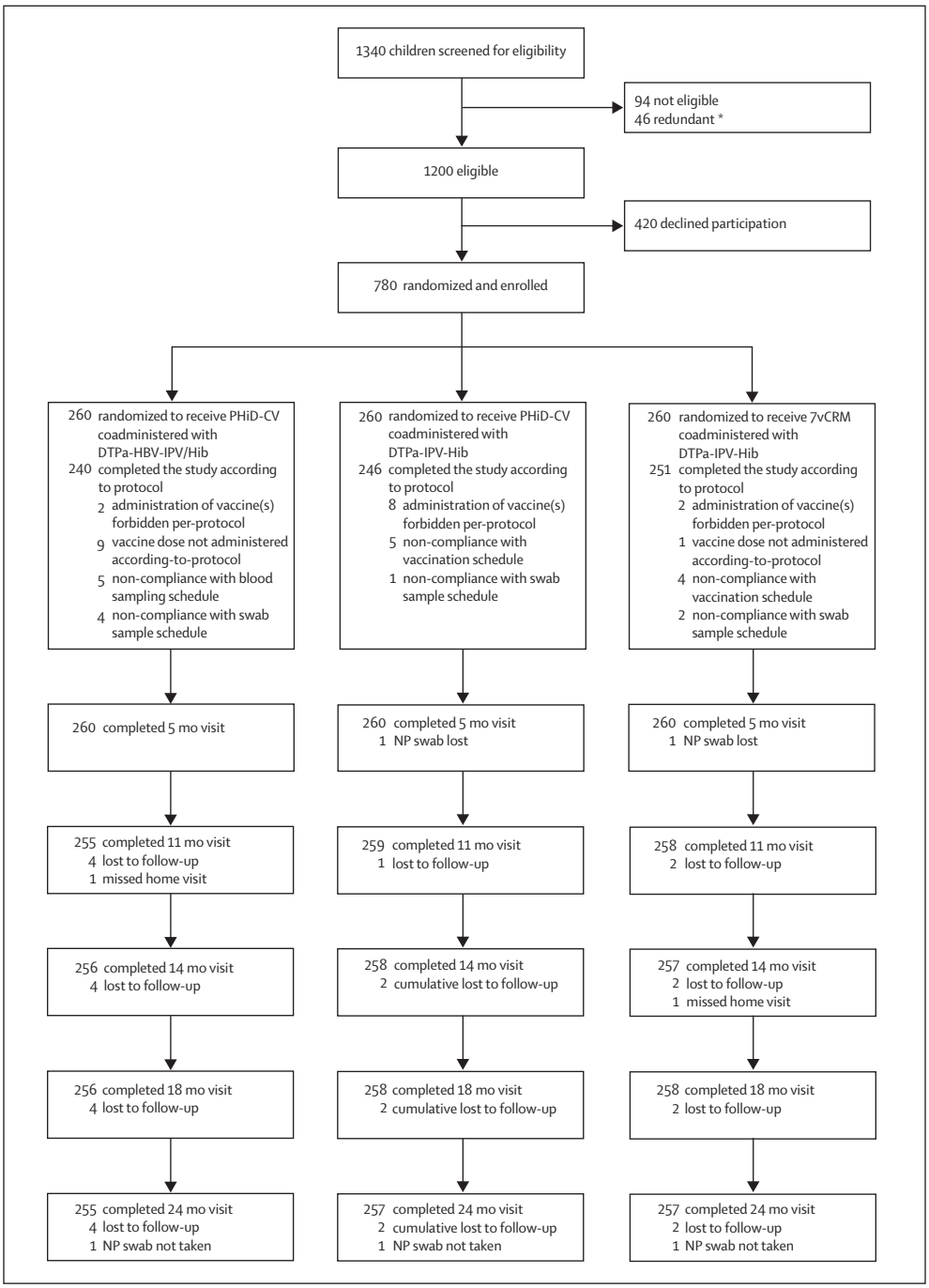

Figure 1: **Trial profile** * Parents of children interested in participating in the study were 'redundant' when they were still in the information process after target enrolment had already been achieved. Consequently, the informed consent procedure was cancelled. [a] Coadministered with DTPa-HBV-IPV/Hib (GlaxoSmithKline Vaccines). [b] Coadministered with DTPa-IPV-Hib (Sanofi Pasteur). Abbreviations: NP, nasopharyngeal; mo, month.

	PHiD-CV DTPa-HBV-IPV/Hib group	PHiD-CV DTPa-IPV-Hib group	7vCRM DTPa-IPV-Hib group
Participants	260	260	260
Sex (male)	142 (55%)	130 (50%)	124 (48%)
Feeding from birth[a]			
breastfed (partially) > 3 mo	125 (49%)	131 (51%)	157 (61%)
breastfed (partially) > 6 mo	78 (31%)	78 (30%)	99 (39%)
Presence of siblings[b]	147 (57%)	131 (50%)	116 (45%)
Day care attendance[c]			
At 11-13 mo[d]	172 (67%)	171 (66%)	172 (67%)
At 23-25 mo[e]	189 (74%)	186 (72%)	180 (70%)
Use of oral or intravenous antibiotics one month before sampling			
At 11-13 mo[d]	26 (10%)	21 (8%)	25 (10%)
At 23-25 mo[e]	15 (6%)	11(4%)	18 (7%)
Passive tobacco smoke exposure indoors			
At 11-13 mo[d]	16 (6%)	17 (7%)	13 (5%)
At 23-25 mo[e]	16 (6%)	19 (7%)	13 (5%)
Age at time of vaccination [mean (SD)]			
Dose 1 (wk)	7.4 (1.2)	7.6 (1.3)	7.6 (1.3)
Dose 2 (wk)	12.0 (1.4)	12.1 (1.5)	12.1 (1.4)
Dose 3 (wk)	16.4 (1.6)	16.6 (1.6)	16.6 (1.6)
Booster dose (mo)	11.0 (0.2)	11.1 (0.3)	11.0 (0.2)
Age at time of nasopharyngeal sampling [mean (SD)]			
Post-primary	4.4 (0.5)	4.5 (0.5)	4.5 (0.5)
Pre-booster	11.0 (0.1)	11.0 (0.2)	11.0 (0.1)
3 mo post-booster	14.1 (0.4)	14.1 (0.4)	14.1 (0.3)
7 mo post-booster	18.1 (0.3)	18.1 (0.3)	18.1 (0.3)
12 mo post-booster	23.2 (0.4)	23.2 (0.4)	23.1 (0.4)

***Table 1*: Characteristics of participating children.** Data are n (%) unless otherwise stated. [a] Information was asked at 11 months of age. [b] Data represent presence of siblings (yes/no) at 5 months of age. [c] Defined as at least 4 continuous hours per week with at least one child under 5 years of age from a different family. [d] For PHiD-CV + DTPa-HBV-IPV/Hib group, n=256; PHiD-CV + DTPa-IPV-Hib group, n=259; 7vCRM + DTPa-IPV-Hib group, n=257. e For PHiD-CV + DTPa-HBV-IPV/Hib group, n=256; PHiD-CV + DTPa-IPV-Hib group, n=258; 7vCRM + DTPa-IPV-Hib group, n=258. Abbreviations: mo, months; wk, weeks; SD, standard deviation.

	PHiD-CV group			7vCRM group			Vaccine efficacy	
	n	N	% (95% CI)	n	N	% (95% CI)	% (95% CI)	P-value[c]
Any *H. influenzae*[a]								
Age 5 mo	185	517	35.8 (31.6 to 40.1)	85	258	32.9 (27.2 to 39.0)	-8.6 (-42.1 to 16.4)	0.5747
Age 11–13 mo	307	512	60.0 (55.6 to 64.2)	144	256	56.3 (49.9 to 62.4)	-6.6 (-30.9¬ to 12.8)	0.5626
Age 14–16 mo	319	514	62.1 (57.7 to 66.3)	165	257	64.2 (58.0 to 70.1)	3.3 (-17.4 to 20.1)	0.7564
Age 18–20 mo	354	510	69.4 (65.2 to 73.4)	162	256	63.3 (57.1 to 69.2)	-9.7 (-32.9 to 9.2)	0.3535
Age 23–25 mo	384	509	75.4 (71.5 to 79.1)	177	254	69.7 (63.6 to 75.3)	-8.3 (-30.1 to 9.6)	0.4079
Across all visits	486	520	93.5 (91.0 to 95.4)	238	260	91.5 (87.5 to 94.6)	-2.1 (-19.7¬ to 12.7)	0.8265
Nontypeable *H. influenzae*[a]								
Age 5 mo	177	517	34.2 (30.1 to 38.5)	83	258	32.2 (26.5 to 38.2)	-6.4 (-39.8 to 18.5)	0.6923
Age 11–13 mo	296	512	57.8 (53.4 to 62.1)	138	255	54.1 (47.8 to 60.4)	-6.8 (-31.7 to 13.0)	0.5578
Age 14–16 mo	302	513	58.9 (54.5 to 63.2)	152	257	59.1 (52.9 to 65.2)	0.5 (-21.8 to 18.4)	0.9980
Age 18–20 mo	314	509	61.7 (57.3 to 65.9)	141	254	55.5 (49.2 to 61.7)	-11.1 (-36.5 to 9.2)	0.3214
Age 23–25 mo	335	505	66.3 (62.0 to 70.5)	165	254	65.0 (58.7 to 70.8)	-2.1 (-23.8 to 15.5)	0.8667
Across all visits	480	520	92.3 (89.7 to 94.4)	234	260	90.0 (85.7 to 93.4)	-2.6 (-20.4 to 12.5)	0.7843

***Table 2*: Nasopharyngeal *H. influenzae* colonization (Total Vaccinated Cohort).** For each visit, N indicates the number of subjects with swabs cultured and n (%) the number (percentage) of swabs positive for the specified bacterium. For across all visits, N indicates the number of subjects with swabs cultured after at least one visit and n (%) the number (percentage) of swabs positive for the pecified bacterium after at least one visit. Vaccine efficacy of PHiD-CV compared to 7vCRM was estimated as ([1 – relative risk] x 100). [a] Data include only results from samples confirmed by PCR as positive for *H. influenzae* after discrimination from *H. haemolyticus*. Samples with invalid test results were excluded from the analysis. c Two-sided conditional exact test. Abbreviations: mo, months; CI, confidence interval.

Nasopharyngeal pneumococcal colonization, acquisition and density

Based on conventional culture, 50% of all swabs were positive for *S. pneumoniae*. Overall pneumococcal colonization rates ranged in both groups from around 39% at 5 months of age to 57% in the second year of life (Table 3). Prevalence of the 7 serotypes included in both vaccines was comparable between groups and declined from approximately 8% in 5-month-old to 3% in 24-month-old children. Serotypes 1, 5 and 7F were rarely detected (n=17 (0.4%) of all samples). Acquisition of pneumococci was within the same range in both groups (Supplementary Table 3). Throughout follow-up, 19A was the predominant colonizing serotype, ranging similarly in both groups from 6% to 11% (Table 3), followed by 11A (2% to 6%) and 16F (2% to 4%; Supplementary Table 4).

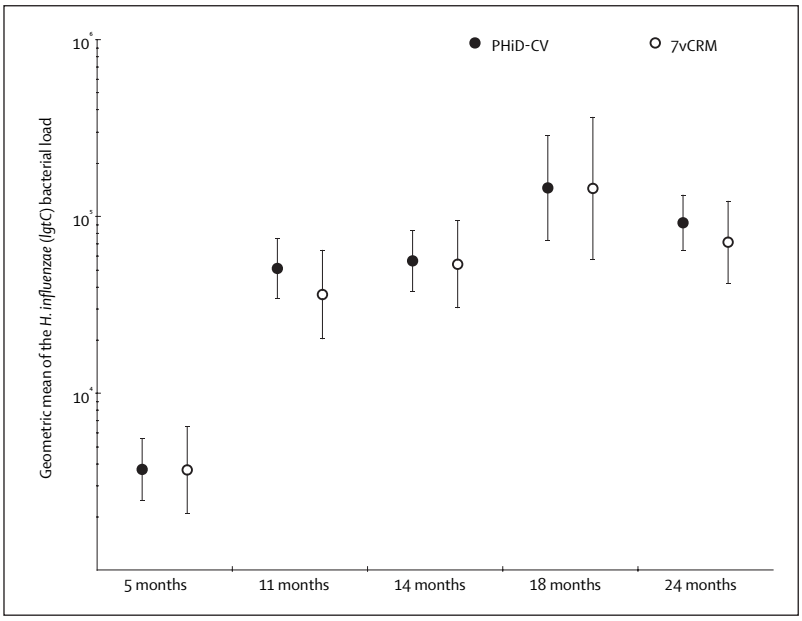

Figure 2: **Density of *H. influenzae* in nasopharyngeal samples of children (Total Vaccinated Cohort).** Density of *H. influenzae* was measured in original swab media by qPCR targeting the glycosyltransferase (*lgtC*) gene with values of ≥ 200 genomic equivalents per millilitre (GE/mL) defined as positive for the presence of *H. influenzae*. Point estimates of the geometric means of GE/mL are shown with their 95% confidence intervals (error bars).

Using qPCR, the detection of pneumococci ranged from 57% (95% CI, 52 to 61%) and 52% (95% CI, 46% to 58%) at 5 months to 80% (95% CI, 76% to 84%) and 77% (95% CI, 66 to 85%) in the second year of life in children immunized with PHiD-CV and 7vCRM, respectively. Of all culture-positive samples, 99% were found to be also positive by qPCR, while qPCR detected pneumococci in 43% of culture-negative samples (Supplementary Table 2). Of the tested samples across all visits, serotype-specific PCR increased the detection of 19A to an average of 20% and 19% in PHiD-CV and 7vCRM vaccinees, respectively (Supplementary Table 2). Detection of 19A using PCR remained constant during follow-up. In both groups alike, *S. pneumoniae* density increased with age, reaching a plateau in the second year of life (Figure 3).

Nasopharyngeal colonization with other bacterial species

M. catarrhalis colonization rates ranged from 63% to 69% during follow-up, with no differences between groups (data not shown). Nasopharyngeal colonization with *S. aureus* decreased with age from around 30% at 5 months to 11% at 24 months of age (data not shown). Colonization rates remained within the same ranges between groups, although a trend for a difference was found at 11-13 months (15% [7vCRM] vs 10% [PHiD-CV]).

	PHiD-CV group			7vCRM group		
	n	N	% (95% CI)	n	N	% (95% CI)
Any *S. pneumoniae*[a]						
Age 5 mo	211	519	40.7 (36.4 to 45.0)	100	259	38.6 (32.6 to 44.8)
Age 11–13 mo	249	514	48.4 (44.0 to 52.9)	119	258	46.1 (39.9 to 52.4)
Age 14–16 mo	252	514	49.0 (44.6 to 53.4)	126	257	49.0 (42.8¬ to 55.3)
Age 18–20 mo	286	514	55.6 (51.2 to 60.0)	148	258	57.4 (51.1 to 63.5)
Age 23–25 mo	293	512	57.2 (52.8 to 61.6)	131	257	51.0 (44.7 to 57.2)
Across all visits	477	520	91.7 (89.0 to 94.0)	233	260	89.6 (85.3 to 93.0)
Serotypes common to both vaccines[b]						
Age 5 mo	42	519	8.1 (5.9 to 10.8)	18	259	6.9 (4.2 to 10.8)
Age 11–13 mo	36	514	7.0 (5.0 to 9.6)	15	257	5.8 (3.3 to 9.4)
Age 14–16 mo	27	514	5.3 (3.5 to 7.6)	12	257	4.7 (2.4¬ to 8.0)
Age 18–20 mo	26	514	5.1 (3.3 to 7.3)	9	258	3.5 (1.6 to 6.5)
Age 23–25 mo	14	512	2.7 (1.5 to 4.5)	7	257	2.7 (1.1 to 5.5)
Across all visits	90	520	17.3 (14.2 to 20.8)	43	260	16.5 (12.2 to 21.6)
Serotype 19A						
Age 5 mo	41	519	7.9 (5.7 to 10.6)	17	259	6.6 (3.9 to 10.3)
Age 11–13 mo	33	514	6.4 (4.5 to 8.9)	19	257	7.4 (4.5 to 11.3)
Age 14–16 mo	42	514	8.2 (6.0 to 10.9)	22	257	8.6 (5.4 to 12.7)
Age 18–20 mo	56	514	10.9 (8.3 to 13.9)	28	258	10.9 (7.3 to 15.3)
Age 23–25 mo	31	512	6.1 (4.2 to 8.5)	20	257	7.8 (4.8 to 11.8)
Across all visits	146	520	28.1 (24.3 to 32.2)	80	260	30.8 (25.2 to 36.8)

***Table 3*: Nasopharyngeal *S. pneumoniae* colonization (Total Vaccinated Cohort).** For each visit, N indicates the number of subjects with swabs cultured and n (%) the number (percentage) of swabs positive for the specified bacterium. For across all visits, N indicates the number of subjects with swabs cultured after at least one visit and n (%) the number (percentage) of swabs positive for the specified bacterium after at least one visit. [a] Data include any *S. pneumoniae* as identified by conventional culture methods, excluding nontypeable strains. [b] Pneumococcal serotypes common to both vaccines are serotypes 4, 6B, 9V, 14, 18C, 19F, and 23F. Abbreviations: mo, months; CI, confidence interval.

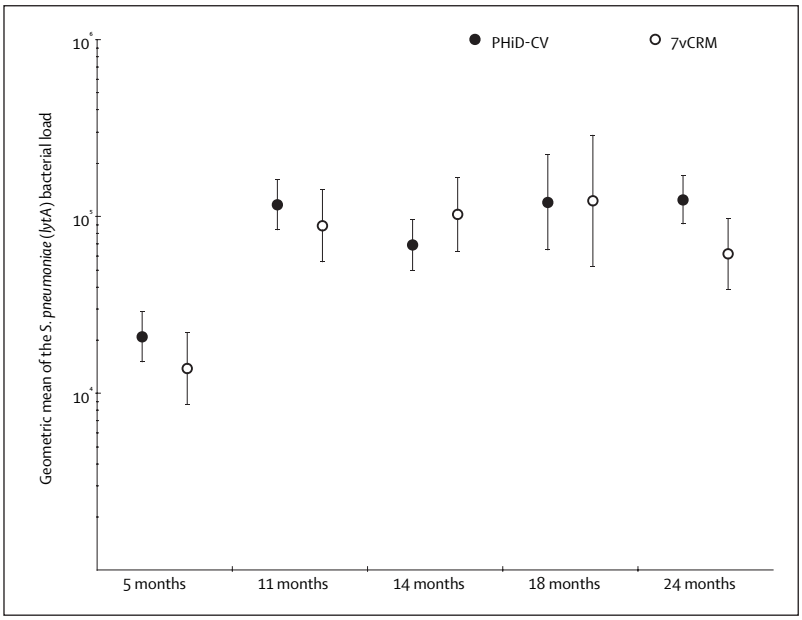

Figure 3: **Density of *S. pneumoniae* in nasopharyngeal samples of children (Total Vaccinated Cohort).** Density of *S. pneumoniae* was measured in original swab media by qPCR targeting the autolysin (lytA) gene with values of ≥ 900 genomic equivalents per millilitre (GE/mL) defined as positive for the presence of *S. pneumoniae*. Point estimates of the geometric means of GE/mL are shown with their 95% confidence intervals (error bars).

Discussion

It is well described that nasopharyngeal colonization precedes disease.[6] The effect of pneumococcal conjugate vaccination, in particular on prevention of mucosal diseases like AOM and pneumonia, is presumed to be associated with the reduction of nasopharyngeal colonization and density of vaccine pneumococcal serotypes.[23] As mentioned, subsequent group protection has contributed greatly to the success of pneumococcal conjugate vaccination. In our study, we found no differential effect of PHiD-CV immunization on NTHi colonization, acquisition or density of *H. influenzae* compared to 7vCRM in healthy Dutch children up to two years of age. Therefore, we expect no indirect effects of PHiD-CV on NTHi through prevention of transmission.

The unlicensed 11Pn-PD vaccine reduced 35% of NTHi-caused AOM episodes in the Pneumococcal Otitis Efficacy Trial (POET) with a trend of reduced NTHi colonization.[12,14] However, the group difference was only observed approximately 3 months post-booster,[12] and disappeared at 24 months of age.[14] A study investigating the effects of the licensed PHiD-CV, using a naïve, age-matched non-randomized control group, showed no consistent effect on NTHi colonization.[15] Importantly, carriage rates were low in both studies.[14, 15] In the present study, we found high colonization rates and applied molecular methods to quantify the presence of *H. influenzae*. We observed no efficacy of PHiD-CV immunization against NTHi colonization or acquisition, at 3 months post-booster or at any other time point. It is unknown if anti-PD antibodies correlate with protection against NTHi-AOM or colonization. Anti-PD antibody concentrations in the present study[19] were lower as those measured in POET,[12] but comparable with other PHiD-CV trials. Our use of 7vCRM as non-active control contrasts

with previous studies in which controls did not receive a pneumococcal conjugate vaccine.[12, 15] It has been shown that multiple strains of *S. pneumoniae* and *H. influenzae* can coexist in the upper respiratory tract.[24] Therefore, immunization with a pneumococcal conjugate vaccine could exert a bystander effect on the presence of *H. influenzae*. However, overall and serotype-specific pneumococcal colonization patterns were similar in both vaccine groups. Additionally, no effect on *H. influenzae* colonization was observed in a recent study evaluating reduced-dose schedules with 7vCRM.[21] Therefore, any possible indirect effects of PHiD-CV on NTHi through effects on *S. pneumoniae* are unlikely to have influenced our results.

Our study raises the question of how a PD-conjugate vaccine induces protection against NTHi-caused AOM, while not affecting presence of NTHi in the nasopharynx—generally regarded the point from which respiratory tract infections originate. We propose several possible explanations.

First, although PD may play a role in pathogenesis of NTHi-caused respiratory infections in animal models,[25,26] intervening with its function may not fully prevent a complex biological process such as colonization.[26] Virulence factors other than PD may contribute to colonization of this unencapsulated pathogen. For instance, *H. influenzae* is highly adaptive and selection of certain phase variants occurs during colonization.[27] Also, specific properties could differ between colonizing *H. influenzae* strains and those found in disease, such as previously shown for the expression of IgA protease.[28]

Second, in a chinchilla model for AOM, Johnson and colleagues found that abrogation of protein D's activity reduced NTHi adherence in the middle ear, but not in the nasopharynx—suggesting a compartment-specific effect.[26] Third, the efficacy of the 11Pn-PD vaccine against NTHi-AOM could merely reflect prevention of the first pneumococcal AOM. In general, the first otitis episode is more frequently caused by pneumococcus, while recurrent episodes are more often associated with NTHi etiology.[4,5] Therefore, preventing the first pneumococcal AOM episode can be hypothesized to prevent sequelae involving NTHi. This hypothesis seems to contradict results of the Finnish Otitis Media trial (FinOM), in which a statistically nonsignificant increase in *H. influenzae* AOM was observed after a 3+1-dose schedule of 7vCRM.[29] However, although differences in case definitions did not explain the different study results,[30] comparing POET with FinOM is fraught with difficulties, due to dissimilarities in study design and case ascertainment—further complicated by differences in the distribution of AOM-causing pathogens. Nonetheless, surveillance is warranted to monitor bacterial colonization patterns and pathogens involved in AOM following nationwide initiation of pneumococcal conjugate vaccination. Clinical trials assessing efficacy of PHiD-CV against AOM (NCT00839254 and NCT00466947) are currently ongoing.

It is important to emphasize that this study was undertaken at the time of herd immunity with respect to vaccine pneumococcal serotypes.[16] As expected, no major differences were observed between groups with respect to pneumococcal acquisition, colonization and density. Consistent with previous reports,[31,32] serotypes 1, 5, and 7F, known for their high invasiveness or case-to-carrier ratio, were found to be rarely carried. Also, colonization with potentially cross-reactive serotypes like 6A and 19A and other, non-PHiD-CV pneumococcal serotypes was similar in both groups. Regardless of the administered vaccine and detection method, serotype 19A was the predominant colonizer throughout follow-up. Finally, no major differences were observed between groups in the prevalence of *M. catarrhalis* and *S. aureus* in nasopharyngeal samples.

Some limitations need to be addressed. First, we have focused in this study on NTHi colonization in healthy children. Therefore, we cannot speculate on effects of PHiD-CV during NTHi disease or, for example, viral infections, when overgrowth of NTHi could be somewhat contained in children immunized with PHiD-CV. In addition, this study was not powered to detect pneumococcal serotype-specific differences.
Strengths of our study include the longitudinal randomized controlled study design with an adequate sample size to assess the efficacy of PHiD-CV on NTHi colonization, virtual absence of loss to follow-up and a low rate of protocol deviations. In addition, we had relatively high nasopharyngeal bacterial colonization rates compared to other countries. Finally, we applied molecular methods to measure the densities of *H. influenzae* and *S. pneumoniae* in the nasopharynx.

In conclusion, PHiD-CV immunization had no differential effect on nasopharyngeal NTHi colonization, acquisition or density compared to 7vCRM in healthy children in the Netherlands up to two years of age. This implies that herd effects on NTHi are not to be expected following introduction of PHiD-CV. Similar nasopharyngeal pneumococcal, *M. catarrhalis* and *S. aureus* colonization rates were also observed in children vaccinated with either PHiD-CV or 7vCRM.

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Supplementary material

	PHiD-CV group			7vCRM group			Vaccine efficacy
	n	N	% (95% CI)	n	N	% (95% CI)	% (95% CI)
Any *H. influenzae*[a]							
Age 11–13 mo	172	512	33.6 (29.5 to 37.9)	83	256	32.4 (26.7 to 38.5)	-3.6 (-36.3 to 20.7)
Age 14–16 mo	95	514	18.5 (15.2 to 22.1)	58	257	22.6 (17.6 to 28.2)	18.1 (-15.6 to 41.5)
Age 18–20 mo	126	510	24.7 (21.0 to 28.7)	64	256	25.0 (19.8 to 30.8)	1.2 (-35.7 to 27.4)
Age 23–25 mo	148	509	29.1 (25.2 to 33.2)	71	254	28.0 (22.5 to 33.9)	-4.0 (-40.1 to 22.1)
Across all visits	391	515	75.9 (72.0 to 79.6)	199	258	77.1 (71.5 to 82.1)	1.6 (-17.3 to 17.2)
Nontypeable *H. influenzae*[a]							
Age 11–13 mo	161	512	31.4 (27.4 to 35.7)	78	255	30.6 (25.0 to 36.6)	-2.8 (-36.5 to 22.0)
Age 14–16 mo	80	513	15.6 (12.6 to 19.0)	45	257	17.5 (13.1 to 22.7)	10.9 (-31.3 to 38.9)
Age 18–20 mo	88	509	17.3 (14.1 to 20.9)	45	254	17.7 (13.2 to 23.0)	2.4 (-43.0 to 32.6)
Age 23–25 mo	104	505	20.6 (17.1 to 24.4)	61	254	24.0 (18.9 to 29.8)	14.2 (-19.6 to 38.1)
Across all visits	367	515	71.3 (67.1 to 75.1)	190	258	73.6 (67.8 to 78.9)	3.2 (-15.9 to 19.0)

***Supplementary Table 1*: Acquisition of culturable *H. influenzae* in children (Total Vaccinated Cohort).** For each visit, N indicates the number of subjects with swabs cultured and n (%) indicates the number (percentage) of subjects with new acquisition of the specified bacterium. For across all visits, N indicates the number of subjects with swabs cultured after at least one visit; n (%) indicates the number (percentage) of subjects with new acquisition of the specified bacterium after at least one visit. Vaccine efficacy of PHiD-CV compared to 7vCRM was estimated as ([1 – relative risk] x 100). [a] Data include only results from samples confirmed by PCR as *H. influenzae* after discrimination from *H. haemolyticus*. Samples with invalid test results were excluded from the analysis. Abbreviations: mo, months; CI, confidence interval. For each visit, N indicates the number of subjects with swabs cultured and n (%) the number (percentage) of swabs positive for the specified bacterium. For across all visits, N indicates the number of subjects with swabs cultured after at least one visit and n (%) the number (percentage) of swabs positive for the pecified bacterium after at least one visit. Vaccine efficacy of PHiD-CV compared to 7vCRM was estimated as ([1 – relative risk] x 100). [a] Data include only results from samples confirmed by PCR as positive for *H. influenzae* after discrimination from *H. haemolyticus*. Samples with invalid test results were excluded from the analysis. c Two-sided conditional exact test. Abbreviations: mo, months; CI, confidence interval.

	PHiD-CV + DTPa-HBV-IPV/Hib group		PHiD-CV + DTPa-IPV-Hib group		7vCRM + DTPa-IPV-Hib group	
	N=1042		N=1058		N=1039	
***H. influenzae*[a,b]**	**Culture**		**Culture**		**Culture**	
qPCR	**Positive**	**Negative**	**Positive**	**Negative**	**Positive**	**Negative**
Positive	629 (60%)	118 (11%)	632 (60%)	120 (11%)	608 (59%)	114 (11%)
Negative	24 (2%)	271 (26%)	23 (2%)	283 (27%)	25 (2%)	292 (28%)
	N=1106		N=1113		N=1112	
***S. pneumoniae*[a,b]**	**Culture**		**Culture**		**Culture**	
qPCR	**Positive**	**Negative**	**Positive**	**Negative**	**Positive**	**Negative**
Positive	532 (48%)	265 (24%)	547 (49%)	241 (22%)	517 (47%)	240 (22%)
Negative	4 (0%)	305 (28%)	9 (1%)	316 (28%)	4 (0%)	351 (32%)
	N=797		N=788		N=756	
Serotype 19A	**Culture**		**Culture**		**Culture**	
PCR	**Positive**	**Negative**	**Positive**	**Negative**	**Positive**	**Negative**
Positive	78 (10%)	86 (11%)	79 (10%)	79 (10%)	80 (11%)	62 (8%)
Negative	1 (0%)	632 (80%)	2 (0%)	628 (80%)	2 (0%)	612 (81%)

***Supplementary Table 2*: Link between conventional culture[a] and PCR assay[b] results across visits (Total Vaccinated Cohort).** Data are n (%), indicating the number (percentage) of swab samples with the identified category. N indicates the number of swab samples with results available for both culture and PCR. [a] Culture indicates results from the primary culture of the original nasopharyngeal swab (and for *H. influenzae* confirmed by PCR as true *H. influenzae*). [b] qPCR indicates results from real-time qPCR targeting the lgtC gene with values of ≥ 200 genomic equivalents per millilitre (GE/mL) defined as positive for *H. influenzae*; values of ≥ 900 GE/mL of lytA defined as positive for *S. pneumoniae*. [c] PCR indicates results from serotype-specific PCR for serotype 19A; only samples that were tested positive for lytA by qPCR and samples with available conventional culture results (including serotyping) were included.

	PHiD-CV group			7vCRM group		
	n	N	% (95% CI)	n	N	% (95% CI)
Any *S. pneumoniae*[a]						
Age 11–13 mo	217	514	42.2 (37.9 to 46.6)	107	258	41.5 (35.4 to 47.7)
Age 14–16 mo	181	514	35.2 (31.1 to 39.5)	90	257	35.0 (29.2 to 41.2)
Age 18–20 mo	233	514	45.3 (41.0 to 49.7)	125	258	48.4 (42.2 to 54.7)
Age 23–25 mo	245	512	47.9 (43.5 to 52.3)	110	257	42.8 (36.7 to 49.1)
Across all visits	457	515	88.7 (85.7 to 91.3)	224	258	86.8 (82.1 to 90.7)
Serotypes common to both vaccines[b]						
Age 11–13 mo	25	514	4.9 (3.2 to 7.1)	12	257	4.7 (2.4 to 8.0)
Age 14–16 mo	15	514	2.9 (1.6 to 4.8)	7	257	2.7 (1.1 to 5.5)
Age 18–20 mo	19	514	3.7 (2.2 to 5.7)	6	258	2.3 (0.9 to 5.0)
Age 23–25 mo	9	512	1.8 (0.8 to 3.3)	6	257	2.3 (0.9 to 5.0)
Across all visits	59	515	11.5 (8.8 to 14.5)	27	258	10.5 (7.0 to 14.9)
Serotype 19A						
Age 11–13 mo	29	514	5.6 (3.8 to 8.0)	16	257	6.2 (3.6 to 9.9)
Age 14–16 mo	28	514	5.4 (3.6 to 7.8)	16	257	6.2 (3.6 to 9.9)
Age 18–20 mo	45	514	8.8 (6.5 to 11.5)	22	258	8.5 (5.4 to 12.6)
Age 23–25 mo	22	512	4.3 (2.7 to 6.4)	14	257	5.4 (3.0 to 9.0)
Across all visits	114	515	22.1 (18.6 to 26.0)	65	258	25.2 (20.0 to 31.0)

Supplementary Table 3: Acquisition of culturable *S. pneumoniae* in children (Total Vaccinated Cohort). For each visit, N indicates the number of subjects with swabs cultured and n (%) indicates the number (percentage) of subjects with new acquisition of the specified bacterium. For across all visits, N indicates the number of subjects with swabs cultured after at least one visit and n (%) indicates the number (percentage) of subjects with new acquisition of the specified bacterium after at least one visit. [a] Data include any *S. pneumoniae* as identified by conventional culture methods, excluding nontypeable strains. [b] Pneumococcal serotypes common to both vaccines are serotypes 4, 6B, 9V, 14, 18C, 19F, and 23F. Abbreviations: mo, months; CI, confidence interval.

	PHiD-CV group N=520		7vCRM group N=260	
	n	% (95% CI)	n	% (95% CI)
Serotypes common to both vaccines				
4	1	0.2 (0.0 to 1.1)	0	0.0 (0.0 to 1.4)
6B	28	5.4 (3.6 to 7.7)	8	3.1 (1.3 to 6.0)
9V	3	0.6 (0.1 to 1.7)	4	1.5 (0.4 to 3.9)
14	3	0.6 (0.1 to 1.7)	1	0.4 (0.0 to 2.1)
18C	11	2.1 (1.1 to 3.8)	4	1.5 (0.4 to 3.9)
19F	30	5.8 (3.9 to 8.1)	15	5.8 (3.3 to 9.3)
23F	25	4.8 (3.1 to 7.0)	13	5.0 (2.7 to 8.4)
Additional serotypes in PHiD-CV				
1	2	0.4 (0.0 to 1.4)	2	0.8 (0.1 to 2.8)
5	2	0.4(0.0 to 1.4)	1	0.4 (0.0 to 2.1)
7F	2	0.4 (0.0 to 1.4)	6	2.3 (0.9 to 5.0)
Non-PHiD-CV serotypes[a]				
3	24	4.6 (3.0 to 6.8)	17	6.5 (3.9 to 10.3)
6A	44	8.5 (6.2 to 11.2)	16	6.2 (3.6 to 9.8)
6C	49	9.4 (7.1 to 12.3)	17	6.5 (3.9 to 10.3)
10A	49	9.4 (7.1 to 12.3)	21	8.1 (5.1 to 12.1)
11A	83	16.0 (12.9 to 19.4)	40	15.4 (11.2 to 20.4)
15B	43	8.3 (6.0 to 11.0)	22	8.5 (5.4 to 12.5)
15C	36	6.9 (4.9 to 9.5)	24	9.2 (6.0 to 13.4)
16F	66	12.7 (10.0 to 15.9)	25	9.6 (6.3 to 13.9)
19A	146	28.1 (24.3 to 32.2)	80	30.8 (25.2 to 36.8)
21	38	7.3 (5.2 to 9.9)	21	8.1 (5.1 to 12.1)
22F	37	7.1 (5.1 to 9.7)	16	6.2 (3.6 to 9.8)
23A	50	9.6 (7.2 to 12.5)	26	10.0 (6.6 to 14.3)
23B	49	9.4 (7.1 to 12.3)	17	6.5 (3.9 to 10.3)
33F	35	6.7 (4.7 to 9.2)	24	9.2 (6.0 to 13.4)
35B	27	5.2 (3.4 to 7.5)	18	6.9 (4.2 to 10.7)
35F	35	6.7 (4.7 to 9.2)	16	6.2 (3.6 to 9.8)

Supplementary Table 4: **Serotype-specific nasopharyngeal colonization of culturable *S. pneumoniae* in children across all visits (Total Vaccinated Cohort).** N indicates the number of subjects with swab cultured after at least one visit and n (%) indicates the number (percentage) of subjects with swabs positive for the specified serotype after at least one visit. Abbreviation: CI, confidence interval. [a] Data included are all serotypes not included in PHiD-CV with a prevalence of at least 5% across visits in either group.

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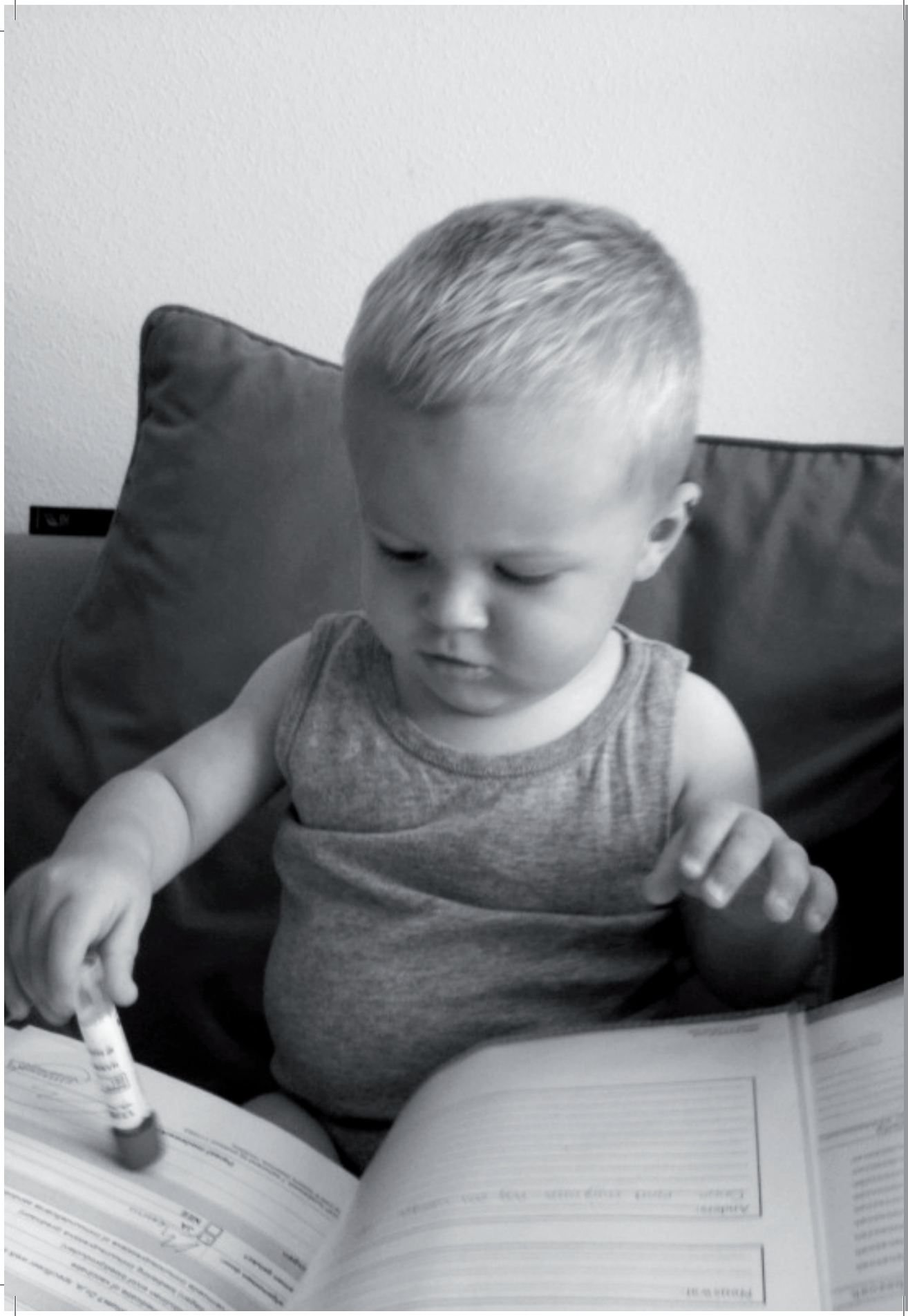

CHAPTER 5

Associations between pathogens in the upper respiratory tract of young children: interplay between viruses and bacteria

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Abstract

Background High rates of potentially pathogenic bacteria and respiratory viruses can be detected in the upper respiratory tract of healthy children. Investigating presence of and associations between these pathogens in healthy individuals is still a rather unexplored field of research, but may have implications for interpreting findings during disease.

Material and methods We selected 986 nasopharyngeal samples from 433 6- to 24-month-old healthy children that had participated in a randomized controlled trial. We determined the presence of 20 common respiratory viruses using real-time PCR. *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Moraxella catarrhalis* and *Staphylococcus aureus* were identified by conventional culture methods. Information on risk factors was obtained by questionnaires. We performed multivariate logistic regression analyses followed by partial correlation analysis to identify the overall pattern of associations.

Results *S. pneumoniae* colonization was positively associated with the presence of *H. influenzae* (adjusted odds ratio 1.60, 95% confidence interval 1.18-2.16), *M. catarrhalis* (1.78, 1.29-2.47), human rhinoviruses (1.63, 1.19-2.22) and enteroviruses (1.97, 1.26-3.10), and negatively associated with S. aureus presence (0.59, 0.35-0.98). *H. influenzae* was positively associated with human rhinoviruses (1.63, 1.22-2.18) and respiratory syncytial viruses (2.78, 1.06-7.28). *M. catarrhalis* colonization was positively associated with coronaviruses (1.99, 1.01-3.93) and adenoviruses (3.69, 1.29-10.56), and negatively with *S. aureus* carriage (0.42, 0.25-0.69). We observed a strong positive association between *S. aureus* and influenza viruses (4.87, 1.59-14.89). In addition, human rhinoviruses and enteroviruses were positively correlated (2.40, 1.66-3.47), as were enteroviruses and human bocavirus, WU polyomavirus, parainfluenza viruses, and human parechovirus. A negative association was observed between human rhinoviruses and coronaviruses.

Conclusions Our data revealed high viral and bacterial prevalence rates and distinct bacterial-bacterial, viral-bacterial and viral-viral associations in healthy children, hinting towards the complexity and potential dynamics of microbial communities in the upper respiratory tract. This warrants careful consideration when associating microbial presence with specific respiratory diseases.

Introduction

Koch's original postulates, designed to link one causative microbe to one specific disease, have been subject to reconsideration since they were formulated in 1884.[1–3] In fact, Koch himself abandoned his first postulate when he discovered that the causative agent of cholera could also be carried asymptomatically.[1] Since then, it is increasingly acknowledged that human diseases, including respiratory tract infections like otitis media and pneumonia, are polymicrobial—resulting from synergistic and antagonistic interactions between pathogens.[4,5]
The human nasopharynx is considered the niche from which respiratory tract infections originate.[6] Several residents of the nasopharyngeal microbiome, including *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Moraxella catarrhalis* and *Staphylococcus aureus*, are major contributors to disease in childhood. However, they are also common, transient colonizers of the nasopharynx of healthy children, especially in the youngest, whose immune systems are still maturing. Colonization of this niche in the upper respiratory tract appears to be a dynamic process of acquisition and elimination of various microbes, during which they interact with the host, its maturing immune system and each other.[6] In a balanced state, this bacterial ecosystem is assumed to be beneficial for health, for example by stimulating the immune system and functioning as a protective barrier against invading pathogens.[7]
Viruses can also be frequently detected in nasopharyngeal samples of healthy children.[8–10] Episodes of new bacterial or viral acquisition potentially disturb the equilibrium of this ecosystem, upon which pathogens could have an opportunity to invade, disseminate and cause diseases like acute otitis media, pneumonia or even meningitis.[4] Importantly, dynamics in exposure to viruses and bacteria are influenced by environmental factors, like crowding at day care or siblings living in the same household.[6] Knowledge about the prevalence of bacteria and viruses in the nasopharynx of healthy children as well as specific associations between these pathogens is important to interpret findings during disease and to ultimately better understand pathogenesis of respiratory infections. However, this is still a rather unexplored field of research.
Here, we describe the results of a post-hoc analysis in 986 nasopharyngeal swab samples from healthy 6- to 24-month-old children who had participated in a pneumococcal vaccination trial. We aimed to evaluate the prevalence of a wide range of common respiratory viruses as well as co-occurrence patterns with four of the most commonly detected bacterial pathogens in clinical practice (*S. pneumoniae*, *H. influenzae*, *M. catarrhalis* and *S. aureus*), while taking well described epidemiologic and environmental determinants into account.

Material and methods

Ethics statement

An acknowledged Independent Ethics Committee from the Netherlands (Stichting Therapeutische Evaluatie Geneesmiddelen) approved the study protocol. The trial was undertaken in accordance with the European Statements for Good Clinical Practice, which include the provisions of the Declaration of Helsinki. Written informed consent was obtained from each subject's parent(s) or legal guardian(s) before enrolment.

Study population and design

We selected 986 nasopharyngeal samples obtained from children who had participated in a randomized controlled trial (ClinicalTrials.gov Identifier NCT00189020).[11] This trial, de-

signed to assess the effects of reduced-dose schedules of 7-valent pneumococcal conjugate vaccine (PCV-7) on nasopharyngeal pneumococcal colonization, was conducted in the Netherlands between July 2005 and February 2008. Details of the trial and bacterial carriage rates have been reported previously.[11-13] In short, a total of 1003 healthy infants were enrolled and randomly assigned to receive either (1) PCV-7 at 2 and 4 months of age, (2) PCV-7 at 2, 4 and 11 months of age, or (3) no dosage (control group). Children were visited at home to obtain nasopharyngeal samples at the age of 6 weeks, 6, 12, 18 and 24 months. Children were generally healthy at the time they were visited—i.e., visits were postponed when parents deemed their child unfit for the study procedures, for example in case of fever or acute symptoms of an infection. At each visit, a questionnaire collecting information on day care attendance, the presence of siblings, and administration of antibiotics was obtained from the parents.
For the present study, nasopharyngeal samples were selected from children in the unvaccinated control group and the 2+1-dose schedule group based on availability of sufficient quantity of remaining materials. Ultimately, 497 samples from the 2+1-dose schedule group and 489 samples from the control group were analyzed. These 986 samples were taken from a total of 433 children: 212 were paired samples from the same children at 6 and 18 months of age, 121 were paired samples from the same children at 12 and 24 months of age, samples from 74 children were collected at four consecutive time points, while from another 26 children only one sample was used.

Nasopharyngeal samples and laboratory procedures

Nasopharyngeal samples were taken transnasally with a flexible, sterile swab (Transnasal Pernasal Plain, Medical Wire and Equipment Co, Corsham Wiltshire, England) by trained study staff according to World Health Organization standard procedures.[14] Culture and bacterial identification occurred according to standard procedures, as previously described in detail.[11–13] After plating, the swabs were rinsed in 1 mL of saline and stored at -80°C until further analysis. Nucleic acids were extracted from one aliquot of 200 µL swab 'rinse' solution using the MagNa pure LC total nucleic isolation (Roche Diagnostics, Basel, Switzerland), as previously described.[15] Samples were tested using real-time PCR specific for human bocavirus, polyomaviruses (WU and KI), respiratory syncytial virus (A and B), human influenza virus A and B, parainfluenza virus 1–4, human rhinoviruses, adenoviruses, human coronavirus OC43, NL63, HKU and 229E, human metapneumovirus, human parechovirus, and enteroviruses. Primers, probes and PCR assay conditions used for this study have been previously reported in detail.[15–17] The presence of human parechovirus and enteroviruses was determined in a subgroup of 831 samples due to limited amounts of nucleic acids that had remained available to run these final tests.

Statistical analysis

The bacterial colonization rate was defined as the proportion of nasopharyngeal samples positive for a particular bacterium by conventional culture. Likewise, the viral detection rate was defined as the proportion of samples positive for a particular virus by real-time PCR. For convenience of statistical analyses, cycle threshold (Ct) values were dichotomized and the different subtypes of viruses belonging to a specific group (e.g., parainfluenza viruses) were pooled. Ct values < 45 were defined as positive, i.e., if the sample did not become positive after 45 cycles, viruses assayed for were defined to not be present.
First, we explored univariate associations amongst the four bacteria and between each bacterium and risk factors for bacterial colonization—i.e., age, presence of siblings, day care attendance, recent antibiotic use (i.e., within two months before sampling), and vaccination

with PCV-7. We calculated the risk and 95% confidence interval (CI) for each bacterium to co-occur with another bacterium (or risk factor) relative to the presence of that bacterium in absence of another bacterium (or risk factor). Similarly, we assessed the co-occurrence of each of the bacteria with a particular virus (or pooled group of subtypes). Again, we calculated the risk and 95% CI for each of the bacteria to be present when a particular virus (or pooled group of subtypes) co-occurred relative to the presence of each bacterium in absence of that virus.
Next, we used logistic regression models to examine independent associations between the isolation of bacteria, detection of viruses, and risk factors. All associations with a P value of < 0.1 in univariate regression analysis were subsequently entered in multivariate regression models. We verified the age-related distribution of each of the covariates included in the various models, which turned out to be linear. Age was therefore entered into the model as continuous variable. Adjusted odds ratios with their 95% CIs were computed. In order to retain sufficient statistical power in models that included enterovirus and human parechovirus, missing values were imputed by the single imputation procedure, which suffices in case the number of missing values is limited as in our study.[18] We verified our primary analyses using a repeated measurements model taking more than one measurement per child into account using generalized linear models with an autoregressive correlation structure.[19] Results were virtually the same, indicating that potential within-person dependency was not substantially affecting the precision of our estimates. Results from the primary analyses are presented here. All these analyses were performed with SPSS Statistics version 17.0.
Finally, all variables for which statistically significant associations were found in multivariate analysis were subsequently entered into a single partial correlation matrix. This analysis identifies all independent correlations between any two given parameters in the matrix after correcting for the remaining variables with P-values of 0.01 and 0.05 used as cut-off. This analysis was performed in software package R 2.7 (function cor2por [package for R; available at: http://cran.r-project.org/web/packages/corpcor/index.html]) and visualized using the complex network analysis in Cytoscape (version 2.7).[20] Rather than correcting for multiple comparisons, correlations at different levels of significance are visualized. Still, significant results should be interpreted with caution.

Results

Characteristics of the children, nasopharyngeal bacterial colonization and viral detection rates are shown in Table 1. Bacterial colonization rates in the present study were similar to results of the main trial.[11–13] Respiratory viruses were detected in almost 70% of samples, with 29% of samples showing evidence of multiple viruses. Human rhinoviruses were detected most frequently, ranging from 31% to 50% of samples (Table 1).
Results of univariate analyses are shown in detail in the Supplementary material (Figures S1 and S2). To summarize, *S. pneumoniae*, *H. influenzae* and *M. catarrhalis* were positively associated with each other (Figure S1A-C), whereas each of them was negatively associated with *S. aureus* (Figure S1D). Day care attendance and presence of siblings in the household were associated with an increased risk of colonization with all bacteria except *S. aureus* (Figure S1). Recent use of antibiotics was associated with a significant decreased risk of pneumococcal colonization as was PCV-7 vaccination, which corresponds to previously described results.[11] In general, *S. pneumoniae*, *H. influenzae*, and *M. catarrhalis* were more likely to be present in the nasopharynx in combination with (multiple) respiratory viruses (Figure S2A-C).
In multivariate analyses, we observed persistent positive associations between *S. pneumoniae* colonization and the presence of *H. influenzae* and *M. catarrhalis*, the presence of siblings,

day care attendance, human rhinoviruses, and enteroviruses (Table 2). *S. aureus* carriage, recent antibiotic use, and PCV-7 vaccination remained inversely related with pneumococcal colonization in multivariate analysis. We found no major differences between the risk of co-occurrence of the most prevalent respiratory viruses with pneumococcal vaccine or non-vaccine serotypes. Likewise, we found no differences upon stratification of the analyses for vaccination status. *H. influenzae* colonization was positively associated with the presence of *S. pneumoniae*, human rhinoviruses and respiratory syncytial viruses, the presence of siblings, and day care attendance. *M. catarrhalis* colonization remained positively correlated with the presence of *S. pneumoniae*, coronaviruses, adenoviruses and the presence of siblings. A negative association was found between *M. catarrhalis* and *S. aureus*, with an adjusted odds ratio being even more profound than that between *S. aureus* and *S. pneumoniae* colonization (0.42 vs 0.56, respectively). The positive association between the presence of *S. aureus* and the pooled group of influenza viruses persisted in the multivariate model (Table 2).

Characteristic	6-mo-old children	12-mo-old children	18-mo-old children	24-mo-old children	Total
	No. (%)	No. (%)	No. (%)	No. (%)	No. (%)
Number of samples	288	198	298	202	986
Age, months (SD)	6.1 (0.39)	12.1 (0.41)	18.1 (0.35)	24.3 (0.69)	NA
Period of sampling					NA
From	November 2005	October 2006	December 2006	September 2007	
To	June 2006	January 2007	July 2007	January 2008	
Male sex	162 (56)	105 (53)	162 (54)	107 (52)	536 (54)
PCV-7 vaccination	146 (51)	104 (53)	149 (50)	98 (49)	497 (50)
Day care attendance[a]	159 (55)	127 (64)	186 (62)	149 (74)	621 (63)
Presence of siblings	142 (49)	106 (54)	157 (53)	130 (64)	535 (54)
Recent antibiotic use[b]	22 (8)	23 (12)	35 (12)	11 (5)	91 (9)
Symptoms of URTI[c]	71 (25)	79 (40)	102 (34)	76 (38)	328 (33)
Bacterial colonization					
Any bacterium	250 (87)	185 (93)	278 (93)	187 (93)	900 (91)
Multiple bacteria	173 (60)	150 (76)	204 (69)	142 (70)	639 (65)
S. pneumoniae	143 (50)	139 (70)	191 (64)	134 (66)	607 (62)
H. influenzae	99 (34)	103 (52)	179 (60)	115 (57)	496 (50)
M. catarrhalis	190 (66)	158 (80)	215 (72)	148 (73)	711(72)
S. aureus	47 (16)	13 (7)	14 (5)	11 (5)	85 (9)

Table 1: **Characteristics of the children, nasopharyngeal bacterial colonization and viral detection rates.** Abbreviations: SD, standard deviation; NA, not applicable; PCV-7, 7-valent pneumococcal conjugate vaccine; URTI, upper respiratory tract infection. [a] Defined as more than 4 hours per week with at least one child from another family (yes/no). [b] Defined as use of an antibiotic, orally or intravenously administered with start date within 2 months before sampling date (yes/no). Of those, the prescribed antibiotic was amoxicillin (n=69), penicillin (n=1), amoxicillin/clavulanic acid (n=3), a macrolide (n=14; claritromycin (n=8), azitromycin (n=5), erythromycin (n=1), a cephalosporin (n=1, unknown type), and 3 unknowns. [c] Parent-reported presence of mild symptoms of an upper respiratory tract infection (eg, a runny nose) at the time of sampling (yes/no). [d] Presence of enteroviruses and human parechovirus was determined in a subgroup of samples (N=831) due to insufficient amounts of remaining nasopharyngeal swab material or nucleic acids to run these tests. Missing values were imputed by the single imputation procedure in multivariate analysis models in which these viruses were included to retain statistical power. —***Table continues on next page***

Characteristic	6-mo-old children	12-mo-oldchildren	18-mo-old children	24-mo-old children	Total
	No. (%)	No. (%)	No. (%)	No. (%)	No. (%)
Viral detection rates					
Any virus	168 (58)	146 (74)	209 (70)	140 (69)	663 (67)
Multiple viruses	53 (18)	76 (38)	91 (31)	65 (32)	285 (29)
Human rhinovirus	88 (31)	98 (50)	104 (35)	80 (40)	370 (38)
Enterovirus[d]	6 (2)	42 (21)	37 (26)	43 (21)	128 (15)
Human bocavirus	26 (9)	17 (9)	53 (18)	27 (13)	123 (12)
Polyomaviruses	30 (10)	33 (17)	43 (14)	41 (20)	147 (15)
WU	16 (6)	28 (14)	34 (11)	33 (16)	111 (11)
KI	14 (5)	5 (3)	9 (3)	8 (4)	36 (4)
Human coronaviruses	23 (8)	6 (3)	31 (10)	20 (10)	80 (8)
OC43	6 (2)	5 (3)	22 (7)	0 (0)	33 (3)
NL63	6 (2)	1 (1)	6 (2)	3 (1)	16 (2)
HKU	5 (2)	0 (0)	3 (1)	7 (3)	15 (2)
229E	2 (1)	0 (0)	0 (0)	5 (2)	7 (1)
Unknown	4 (1)	0 (0)	0 (0)	5 (2)	9 (1)
Parainfluenza viruses	14 (5)	10 (5)	31 (10)	7 (4)	62 (6)
Type 1	9 (3)	0 (0)	20 (7)	1 (0)	30 (3)
Type 2	1 (0)	0 (0)	0 (0)	2 (1)	3 (0)
Type 3	3 (1)	1 (1)	6 (2)	0 (0)	10 (1)
Type 4	0 (0)	9 (5)	5 (2)	3 (1)	17 (2)
Unknown	1 (0)	0 (0)	0 (0)	1 (0)	2 (0)
Human adenovirus	15 (5)	11 (6)	23 (8)	9 (5)	58 (6)
Human parechovirus[d]	14 (5)	29 (15)	9 (6)	19 (9)	71 (9)
Respiratory syncytial virus	6 (2)	5 (3)	9 (3)	5 (3)	25 (3)
Influenza virus	11 (4)	2 (1)	3 (1)	1 (1)	17 (2)
Human metapneumovirus	2 (1)	1 (1)	1 (0)	0 (0)	4 (0)

Table 1: **Continued.**

Multivariate models for independent associations with the most frequently detected viruses are shown in Table 3. Human rhinoviruses were positively associated with the presence of siblings as well as with enteroviruses, and negatively associated with coronaviruses. In addition, enteroviruses were positively associated with the presence of human bocavirus, WU polyomavirus, parainfluenza viruses and human parechovirus. Human bocavirus was also associated with day care attendance and the presence of WU polyomavirus (Table 2).
Figure 1 graphically summarizes the results of partial correlation network analysis. All significant associations shown by multivariate analysis persisted in partial correlation network analysis (Figure 1A). When simultaneously adjusting for driving risk factors (Figure 1B) all correlations remain, except for those between *H. influenzae* and *M. catarrhalis* (P=0.17), and between *M. catarrhalis* and coronaviruses (P=0.064).

Discussion

To our knowledge, this is the first study in which co-occurrence patterns of four potentially pathogenic bacteria and 20 common respiratory viruses in nasopharyngeal samples from otherwise healthy young children are investigated by multivariate and partial correlation network analysis. The latter enables a helicopter-view of possible interrelations between microbes in a complex community, simultaneously correcting for the influence of important epidemiologic and environmental determinants.[21] Even in a healthy state, we found numerous specific associations between viral and bacterial pathogens which could have an important role in local ecosystem dynamics. These findings may guide future studies in pursuing possible underlying mechanisms and their role in pathogenesis of respiratory disease.

Covariate	*Streptococcus pneumoniae*			*Haemophilus influenzae*		
	Without covariate	With covariate	aOR (95% CI)	Without covariate	With covariate	aOR (95% CI)
	No. (%)	No. (%)		No. (%)	No. (%)	
S. pneumoniae		NA		139 (37)	357 (59)	1.54 (1.15–2.06)
H. influenzae	250 (51)	357 (72)	1.60 (1.18–2.16)		NA	
M. catarrhalis	122 (44)	485 (68)	1.78 (1.29–2.47)	103 (37)	393 (55)	1.27 (0.91–1.76)
S. aureus	573 (64)	34 (40)	0.59 (0.35–0.98)	468 (52)	28 (33)	0.72 (0.43–1.23)
Antibiotic use	574 (64)	33 (36)	0.24 (0.15–0.40)	450 (50)	46 (51)	NA
Presence of siblings	236 (52)	371 (69)	2.31 (1.69–3.15)	181 (40)	315 (59)	2.42 (1.80–3.26)
Day care attendance	183 (50)	424 (68)	1.70 (1.23–2.36)	130 (36)	366 (59)	2.52 (1.83–3.46)
PCV-7 vaccination	326 (65)	281 (58)	0.68 (0.51–0.91)	248 (50)	248 (51)	NA
Human rhinovirus	335 (54)	272 (74)	1.63 (1.19–2.22)	267 (43)	229 (62)	1.63 (1.22–2.18)
Enterovirus	471 (58)	136 (79)	1.97 (1.26–3.10)	382 (47)	114 (66)	1.25 (0.84–1.85)
Human bocavirus	527 (61)	80 (65)	NA	424 (49)	72 (59)	1.11 (0.73–1.70)
WU polyomavirus	529 (60)	78 (70)	1.32 (0.82–2.14)	430 (49)	66 (59)	1.17 (0.75–1.83)
Human coronavirus	550 (61)	57 (71)	1.38 (0.80–2.38)	456 (50)	40 (50)	NA
Parainfluenza virus	568 (60)	39 (63)	NA	470 (51)	26 (42)	NA
Adenovirus	561 (60)	46 (79)	1.91 (0.95–3.84)	462 (50)	34 (59)	NA
Human parechovirus	530 (60)	77 (76)	NA	434 (49)	62 (61)	1.19 (0.75–1.90)
KI polyomavirus	586 (62)	21 (58)	NA	482 (50)	14 (39)	NA
RSV	590 (61)	17 (68)	NA	478 (50)	18 (72)	2.78 (1.06–7.28)
Influenza virus	596 (62)	11 (65)	NA	487 (50)	9 (53)	NA

Table 2:* Distribution and adjusted odds ratios[a] for nasopharyngeal bacterial colonization, co-occurrence with each of the other bacteria, respiratory viruses and risk factors.** Abbreviations: aOR, adjusted odds ratio; CI, confidence interval; NA, not applicable (i.e., not included in the model for that particular bacterial pathogen), RSV, respiratory syncytial virus. [a] Adjusted for age and all variables with a P value of < 0.1 in univariate analysis. —Table continues on next page***

Covariate	*Moraxella catarrhalis*			*Staphylococcus aureus*		
	Without covariate	With covariate	aOR (95% CI)	Without covariate	With covariate	aOR (95% CI)
	No. (%)	No. (%)		No. (%)	No. (%)	
S. pneumoniae	226 (60)	485 (80)	1.73 (1.26–2.38)	51 (13)	34 (6)	0.56 (0.34–0.91)
H. influenzae	318 (65)	393 (79)	1.24 (0.89–1.72)	57 (12)	28 (6)	0.72 (0.44–1.21)
M. catarrhalis		NA		45 (16)	40 (6)	0.42 (0.25–0.68)
S. aureus	671 (74)	40 (47)	0.42 (0.25–0.69)		NA	
Antibiotic use	643 (72)	68 (75)	NA	75 (8)	10 (11)	NA
Presence of siblings	309 (69)	402 (75)	1.59 (1.14–2.22)	46 (10)	39 (7)	NA
Day care attendance	202 (55)	509 (82)	3.22 (2.30–4.51)	47 (13)	38 (6)	0.74 (0.45–1.21)
PCV-7 vaccination	372 (75)	339 (70)	NA	40 (8)	45 (9)	NA
Human rhinovirus	420 (68)	291 (79)	1.21 (0.86–1.69)	54 (9)	31 (8)	NA
Enterovirus	568 (70)	143 (83)	1.27 (0.79–2.05)	74 (9)	11 (6)	NA
Human bocavirus	615 (71)	96 (78)	NA	78 (9)	7 (6)	NA
WU polyomavirus	629 (72)	82 (74)	NA	78 (9)	7 (6)	NA
Human coronavirus	642 (71)	69 (86)	1.99 (1.01–3.93)	82 (9)	3 (4)	NA
Parainfluenza virus	662 (72)	49 (79)	NA	83 (9)	2 (3)	NA
Adenovirus	657 (76)	54 (93)	3.69 (1.29–10.56)	83 (9)	2 (3)	NA
Human parechovirus	630 (71)	81 (80)	1.17 (0.67–2.05)	76 (9)	9 (9)	NA
KI polyomavirus	683 (72)	28 (78)	NA	84 (9)	1 (3)	NA
RSV	694 (72)	17 (68)	NA	83 (9)	2 (8)	NA
Influenza virus	701 (72)	10 (59)	NA	79 (8)	6 (35)	4.87 (1.59–14.89)

Table 2: **Continued.**

The bacterial detection rates in our study are comparable to previous studies in young children.[11–13] Also, viral detection rates in our study were comparable to previous reports.[9,10] The abundant presence of viruses in samples from asymptomatic children raises the question whether a positive result could be regarded as causal in case of respiratory disease symptoms. It seems clear that certain viruses, such as respiratory syncytial viruses and influenza viruses, are capable of causing disease on their own.[9] For the more commonly "carried" viruses (eg, rhinovirus, human bocavirus, WU polyomavirus), it has been suggested that not merely presence but rather a certain viral load is needed above which respiratory symptoms may occur.[22] However, considering both their high detection rates and their associations with bacterial colonization, we feel it becomes even more complex to identify an individual microbe or a certain viral load as single cause of a respiratory tract infection in young children.

In general, the risk of *S. pneumoniae*, *H. influenzae* and *M. catarrhalis* colonization seemed to increase in the presence of particular respiratory viruses. The magnitude of this relative risk differed per virus and per bacterium. The presence of influenza viruses in general was associated with an increased risk for colonization by *S. aureus* as well as *S. pneumoniae*, both of which are also supported by observations during flu pandemics. While H1N1 influenza A was associated with *S. aureus* pneumonia in the 2009 pandemic, an association with pneumococcus prevailed during others, depending on the subtype of virus.[5,23] In the present study, only a positive association between influenza viruses and *S. aureus* persisted in multivariate analysis, which could be a reflection of differential effects of various influenza virus subtypes on these bacterial pathogens.[24]

Covariate	Human rhinovirus			Enterovirus		
	Without covariate	With covariate	aOR (95% CI)[a]	Without covariate	With covariate	aOR (95% CI)[a]
	No. (%)	No. (%)		No. (%)	No. (%)	
S. pneumoniae	98 (26)	272 (45)	1.70 (1.25–2.31)	36 (9)	136 (22)	1.64 (1.05–2.54)
H. influenzae	141 (29)	229 (46)	1.52 (1.14–2.03)	58 (12)	114 (23)	1.26 (0.85–1.87)
M. catarrhalis	79 (29)	291 (41)	1.22 (088–1.70)	29 (11)	143 (20)	1.30 (0.80–2.11)
S. aureus	339 (38)	31 (36)	NA	161 (18)	11 (13)	NA
Antibiotic use	343 (38)	27 (30)	NA	153 (17)	19 (21)	NA
Presence of siblings	146 (32)	224 (42)	1.41 (1.05–1.90)	75 (17)	97 (18)	NA
Day care attendance	108 (30)	262 (42)	1.37 (0.99–1.89)	34 (9)	138 (22)	1.56 (0.98–2.49)
PCV-7 vaccination	191 (38)	179 (37)	NA	79 (16)	93 (19)	NA
Human rhinovirus		NA		69 (11)	103 (28)	2.50 (1.72–3.63)
Enterovirus	267 (33)	103 (60)	2.40 (1.66–3.47)		NA	
Human bocavirus	318 (37)	52 (42)	NA	127 (15)	45 (37)	2.78 (1.75¬–4.42)
WU polyomavirus	320 (34)	50 (45)	1.11 (0.72–1.72)	136 (16)	36 (32)	1.70 (1.03–2.80)
Human coronavirus	355 (39)	15 (19)	0.27 (0.15–0.49)	153 (17)	19 (24)	NA
Parainfluenza virus	350 (38)	20 (32)	NA	155 (17)	17 (27)	2.06 (1.06–3.99)
Adenovirus	339 (37)	31 (53)	1.53 (0.86–2.74)	154 (17)	18 (31)	1.76 (0.93–3.33)
Human parechovirus	317 (36)	53 (52)	1.40 (0.89–2.20)	134 (15)	38 (38)	2.77 (1.69–4.55)
KI polyomavirus	360 (38)	10 (28)	NA	166 (17)	6 (17)	NA
RSV	364 (38)	6 (24)	NA	168 (17)	4 (25)	NA
Influenza virus	367 (38)	3 (18)	NA	170 (18)	2 (12)	NA

Table 3*: Distribution and adjusted odds ratios[a] for nasopharyngeal presence of the most common viruses, co-occurrence with each of the other respiratory viruses, bacteria and risk factors.** Abbreviations: aOR, adjusted odds ratio; CI, confidence interval; NA, not applicable (i.e., not included in the model for that particular virus or pooled group of viruses), RSV, respiratory syncytial virus. [a] Adjusted for age and all variables with a P value of < 0.1 in univariate analysis. ***—Table continues on next page

Covariate	Human bocavirus			WU polyomavirus		
	Without covariate	With covariate	aOR (95% CI)[a]	Without covariate	With covariate	aOR (95% CI)[a]
	No. (%)	No. (%)		No. (%)	No. (%)	
S. pneumoniae	43 (11)	80 (13)	NA	33 (9)	78 (13)	1.30 (0.82–2.06)
H. influenzae	51 (10)	72 (15)	1.07 (0.71–1.63)	45 (9)	66 (13)	1.13 (0.73–1.75)
M. catarrhalis	27 (10)	96 (14)	NA	29 (11)	82 (12)	NA
S. aureus	116 (13)	7 (8)	NA	104 (12)	7 (8)	NA
Antibiotic use	107 (12)	16 (3)	NA	101 (12)	10 (11)	NA
Presence of siblings	62 (14)	61 (11)	NA	51 (11)	60 (11)	NA
Day care attendance	25 (7)	98 (16)	2.04 (1.26–3.31)	32 (9)	79 (13)	1.07 (0.67–1.71)
PCV-7 vaccination	59 (12)	64 (13)	NA	47 (9)	64 (13)	1.46 (0.97–2.20)
Human rhinovirus	71 (12)	52 (14)	NA	61 (10)	50 (14)	1.16 (0.76–1.78)
Enterovirus	78 (10)	45 (26)	2.48 (1.59–3.88)	75 (9)	36 (21)	1.71 (1.05–2.78)
Human bocavirus		NA		82 (10)	29 (24)	2.38 (1.44–3.91)
WU polyomavirus	94 (11)	29 (26)	2.34 (1.42–3.87)		NA	
Human coronavirus	115 (13)	8 (10)	NA	101 (11)	10 (13)	NA
Parainfluenza virus	111 (12)	12 (19)	1.55 (0.77–3.10)	103 (11)	8 (13)	NA
Adenovirus	115 (12)	8 (14)	NA	106 (11)	5 (9)	NA
Human parechovirus	110 (12)	13 (13)	NA	95 (11)	16 (16)	NA
KI polyomavirus	115 (12)	8 (22)	2.28 (0.96–5.40)	108 (11)	3 (8)	NA
RSV	120 (12)	3 (12)	NA	108 (11)	3 (12)	NA
Influenza virus	122 (13)	1 (6)	NA	110 (11)	1 (6)	NA

***Table 3:* Continued.**

The previously reported negative association between *S. pneumoniae* and *S. aureus* during nasopharyngeal carriage was confirmed in the present study.[12,25,26] However, we found an even stronger negative association between *M. catarrhalis* and *S. aureus*. Whether this reflects true microbial interference or, for instance, results from an indirect effect through other (commensal) bacteria or immune modulation warrants further investigation.

Partial correlation analysis provides a two-dimensional view compared to the unidimensional view of classical multivariate analysis.[27] As such, it does not put more value to a single variable over others, and allows for independent associations tested between all parameters simultaneously.

The observed associations are, however, mathematical and need further investigation to unravel their underlying biological mechanisms and to determine whether they are direct or dependent on other (unknown) determinants. Nevertheless, several of the observed statistical associations are supported by an overwhelming body of evidence indicating virus-mediated susceptibility for bacterial infection in the respiratory tract. For instance, research on animal models as well as in vitro studies provide biological clues to the positive association between

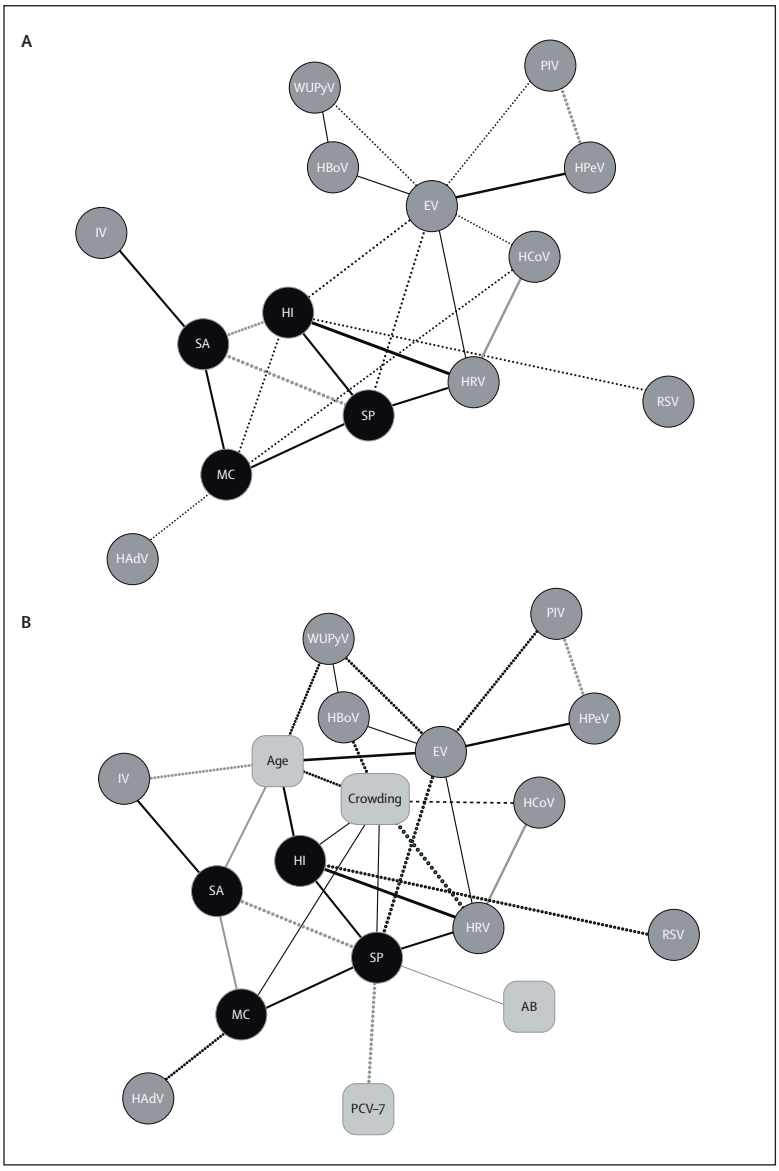

Figure 1: **Graphical representation of interaction patterns.** Visualization of the partial correlations between bacteria and viruses (A) and epidemiologic drivers (risk factors) of those interactions (B). The patterns depicted here result from partial correlation network analysis and are visualized by Cytoscape. Bacteria are shown in blue, respiratory viruses in orange and risk factors in grey boxes. The solid lines represent associations with a p-value less than 0.01, the dashed lines represent associations with a p-value between 0.01 and 0.05. Green lines indicate positively correlated variables; red lines indicate negative correlations. The thickness of the line indicates the magnitude of the correlation.Abbreviations: SP, *S. pneumoniae*; HI, *H. influenzae*; MC, *M. catarrhalis*; SA, *S. aureus*; HRV, human rhinovirus, EV, enterovirus; HBoV, human bocavirus; WUPyV, WU polyomavirus; HCoV, human coronavirus; PIV, parainfluenza virus; HAdV, human adenovirus; IV, influenza virus; HPeV, human parechovirus; RSV, respiratory syncytial virus; AB, antibiotic use within 2 months before sampling; 'crowding' was entered into the model as a variable combining the presence of siblings (yes/no) and day care attendance (yes/no); 0 = no siblings and no day care attendance, 1 = siblings present, but not attending day care, or vice versa, and 2 = siblings present and attending day care.

rhinoviruses and *S. pneumoniae*.[28,29] Of interest was a recently described temporal association between these microbes in disease.[30] This adds to a previously demonstrated association between circulation of influenza and respiratory syncytial viruses, and invasive pneumococcal and meningococcal disease.[31]

It is of great interest to gain more insight into the synergism and competition among members of microbial communities of the upper respiratory tract to better understand progression toward disease. A balance in its polymicrobial composition and diversity is assumed to be important for maintenance of a healthy state.[4,6] For example, it has previously been shown that *S. pneumoniae* can kill *S. aureus* by remote-control bacteriophage induction, possibly accounting for the negative association between those species observed in carriage studies.[32] Research on microbiota of the intestinal tract has made clear that symbiotic bacteria are co-dependent because of shared metabolic pathways.[33] Possibly, a similar mechanism by which bacteria interfere with each other's presence could also be at play in the upper respiratory tract. We should also note the enormous heterogeneity of highly adaptive bacteria such as *S. pneumoniae* and *H. influenzae* in this context. Although the observed positive association in our study has been previously reported,[34] antagonistic effects have also been described to occur.[35]

With respect to respiratory disease, it has been shown that pneumococcal conjugate vaccination also reduced virus-associated pneumonia in general, and cases associated with seasonal viruses such as influenza and parainfluenza viruses in particular.[36] This suggests that presumed 'virus-associated pneumonia' is actually polymicrobial in nature. The same may be true for acute otitis media, a disease that can be caused by individual pathogens but could also be an end stage of true polymicrobial pathogenesis. In either case, it appears that the old paradigm on viruses predisposing to secondary bacterial disease is an oversimplification of the complexity and dynamics of potential interactions. For example, interdependence between viruses and bacteria occurring in the gut was recently described: some viruses cover themselves with molecules from bacterial residents to make a viral infection possible.[37,38] This also contradicts the predominant view that resident microbes merely protect against new viral infection.

Some limitations need to be mentioned. First, we modeled the results of the current standard for detecting bacteria (conventional culture) with that for detecting viruses (qPCR). Use of PCR instead of conventional culture may have increased the detection of (less abundant) bacteria. We restricted analysis to four culturable bacterial pathogens, since they are generally considered to be the major contributors to respiratory disease in childhood. Additionally, since bacteria need to be present in sufficient abundance to be detected by culture, these results may reveal the most clinically relevant associations. Also, the prevalence rates of cultured *S. pneumoniae*, *H. influenzae* and *M. catarrhalis* were already high in our study. We recognize that the microbial communities in the upper respiratory tract are inherently complex and include non-culturable, less abundant commensal bacterial species.[39,40] How these fit into the picture and relate to pathogenesis is an interesting topic of current and future research. Second, by assessing the prevalence of viruses and bacteria in a cross-sectional manner, we only describe associations and cannot speculate on causality. Future studies are needed to show the importance of these associations and possible underlying mechanisms. Third, we focused on the crude presence or absence of viruses and bacteria, ignoring viral and/or bacterial load. Finally, it should be noted that, besides possible interactions with members of the flora not assayed for in our study, the demonstrated associations between microbes could, at least partially, be influenced by host genetics and immune maturation status.

Strengths of our study include the large sample size of healthy children, a wide range of viruses included in the analyses and the availability of detailed data on risk factors. The relatively high bacterial colonization and viral detection rates allowed for solid statistical analyses, summarized as a "network model" of distinct associations between these microbes.
In conclusion, we have demonstrated that bacterial pathogens and respiratory viruses are abundantly present in the upper respiratory tract of otherwise healthy young children. The distinct associations between viruses and bacteria found in our study warrant careful consideration when associating microbial presence with specific respiratory diseases.

Acknowledgments

We thank the members of the research team, laboratory staff and all cooperating institutes for their dedication to the project. Most of all, we are indebted to all participating children and their families.

Supplementary material

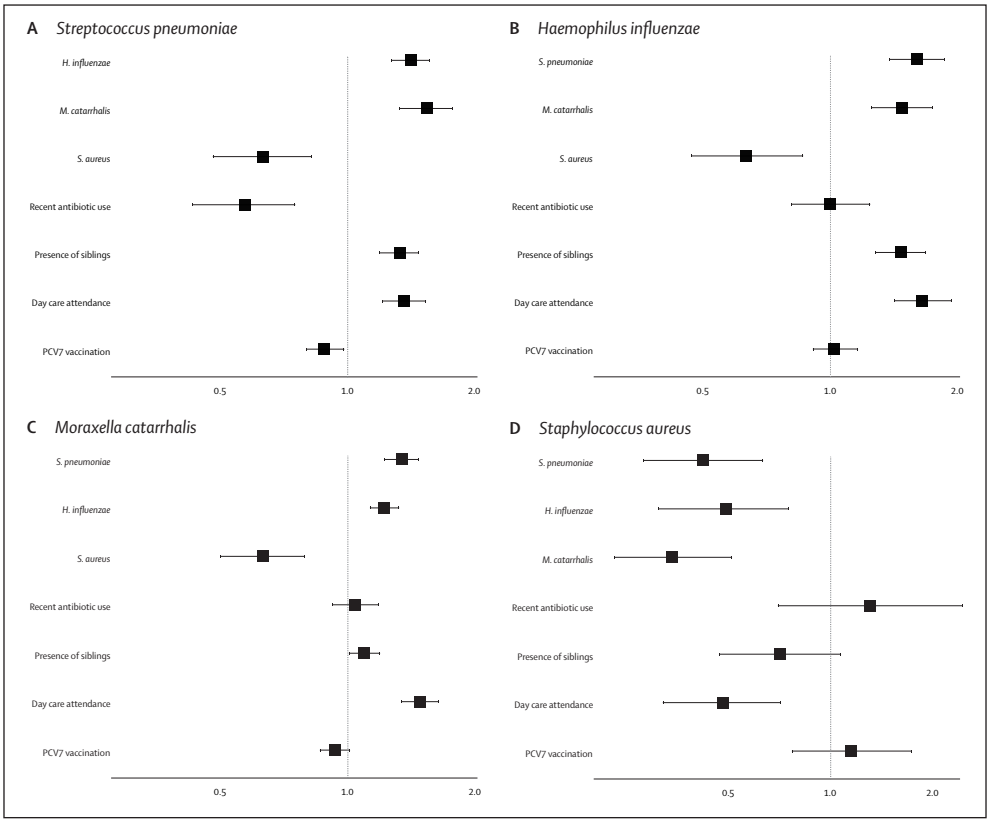

Figure S1: **Bacterial colonization in relation to co-occurrence with other pathogenic bacteria and risk factors.** Point estimates are shown for the risk of nasopharyngeal colonization of [A] *S. pneumoniae*, [B] *H. influenzae*, [C] *M. catarrhalis* and [D] *S. aureus* in the presence of another bacterium (or risk factor) relative to colonization in the absence of that particular bacterium (or risk factor). Relative risks are plotted on a logarithmic scale. The horizontal bars indicate 95% confidence intervals.

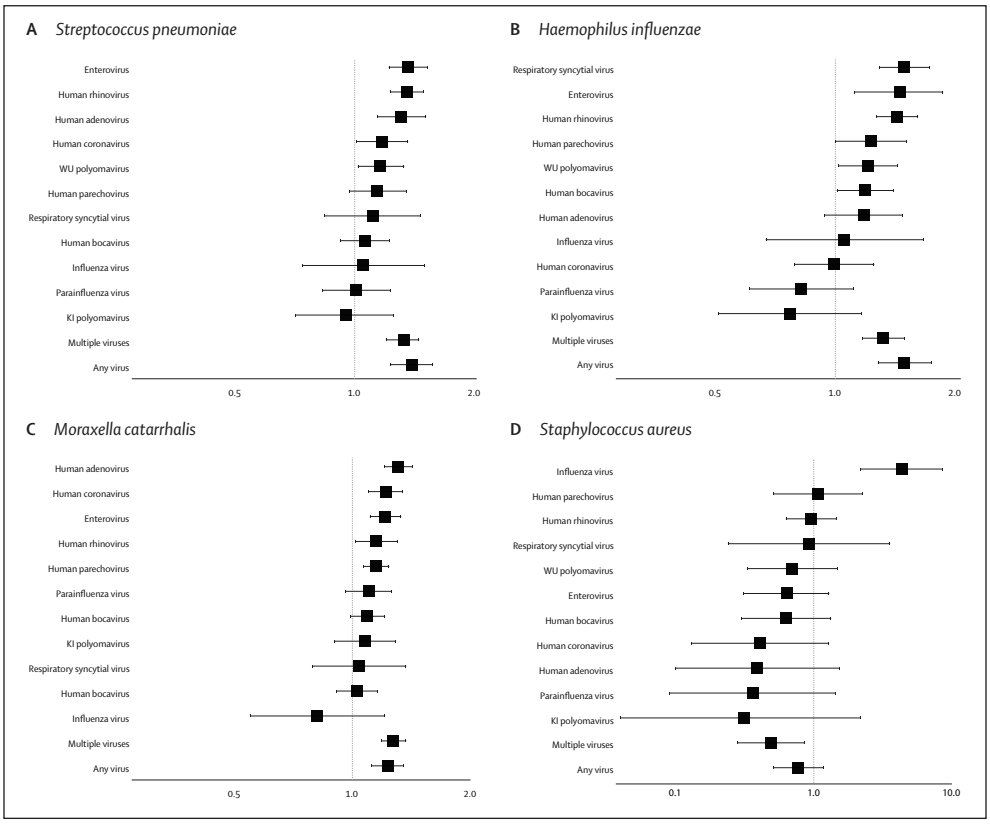

Figure S2: **Bacterial colonization in relation to the co-occurrence with respiratory viruses.** Point estimates are shown for the risk of nasopharyngeal colonization of [A] *S. pneumoniae*, [B] *H. influenzae*, [C] *M. catarrhalis* and [D] *S. aureus* in the presence of a respiratory virus-relative to colonization in the absence of that particular virus. Relative risks are plotted on a logarithmic scale. The horizontal bars indicate 95% confidence intervals. Note that the scale in the figure for *S. aureus* differs from the others.

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CHAPTER 6

Alternative sampling methods for detecting bacterial pathogens in children with an upper respiratory tract infection

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Abstract

Background Nasopharyngeal sampling is the current standard for detecting bacteria commonly involved in upper respiratory tract infections (URTI), but requires training and may not be well tolerated by the child, especially when conducted repeatedly. Therefore, we evaluated alternative sampling methods.

Material and methods Children (n=66) aged 0-4 years with rhinorrhea as symptom of an URTI were sampled by [1] a nasopharyngeal swab, [2] a nasal swab, and [3] blowing the nose into a paper tissue, that was [3a] sampled with a swab and [3b] transported as a whole in phosphate-buffered saline to the laboratory to detect *S. pneumoniae* (Spn) *H. influenzae* (Hi), *M. catarrhalis* (Mc) and *S. aureus* (Sa) using conventional culture methods. Bacterial density was measured semiquantitatively.

Results Spn was cultured in [1] 64%, [2] 64%, [3a] 65% and [3b] 74%, Hi in [1] 70%, [2] 67%, [3a] 64% and [3b] 65%, Mc in [1] 71%, [2] 74%, [3a] 68% and [3b] 68% and Sa in [1] 33%, [2] 27%, [3a] 23% and [3b] 38% of samples. Spn was detected more frequently from a paper tissue than from a nasopharyngeal swab (P=0.039). Concordance between methods ranged from 80% to 97%. Bacterial density for Hi, Mc and Sa was comparable across sampling methods. Spn showed more dense growth in cultures of a paper tissue than in each of the other sampling methods (P<0.001).

Conclusions In conclusion, culture of nasal secretion collected with a paper tissue in children with rhinorrhea allows reliable detection of common bacterial pathogens. This opens new opportunities for surveillance and transmission modeling studies.

Introduction

Streptococcus pneumoniae, *Haemophilus influenzae*, *Moraxella catarrhalis* and *Staphylococcus aureus* are the bacterial pathogens most commonly involved in upper respiratory tract infections in young children. The recommended sampling site for *S. pneumoniae* and *M. catarrhalis* is the nasopharynx,[11,10] while for *H. influenzae* and *S. aureus* this is the naso- and oropharynx[4] and the nasal vestibule,[17] respectively. However, sampling these sites requires training and may not be well tolerated by the child, especially when conducted repeatedly.
At the time of an upper respiratory tract infection, secretions from the nasopharynx, paranasal sinuses and nasal cavity accumulate and discharge spontaneously from the nose. Theoretically, bacteria residing at these various niches should be detectable in these nasal secretions. Leach and colleagues showed that pneumococci can indeed be detected in a swab of nasal secretions collected by blowing or wiping the nose into a paper tissue, but this was compared with a nasal rather than a nasopharyngeal swab.[9]
Therefore, we compared conventional culture results of nasal secretions collected by blowing or wiping the nose into a paper tissue to those of samples obtained by the current standards for *S. pneumoniae*, *H. influenzae*, *M. catarrhalis*, and *S. aureus* in a group of children with rhinorrhea as symptom of an upper respiratory tract infection.

Methods

Study design and subjects

Children (n=66) aged 0 to 4 years with nasal secretions as a symptom of an upper respiratory tract infection were eligible for the present cross-sectional study. Children were recruited from the ear-nose-throat (ENT) outpatient clinic of the Wilhelmina Children's Hospital, University Medical Center Utrecht, from two local daycare centers, and from a population of children who had previously participated in a randomized trial.[14] Parents whose child had an appointment at the ENT clinic were informed about the study in writing two weeks prior to the appointment. For children attending the daycare centers, parents were informed in writing and offered the possibility to participate in the study. At both locations, informed consent was obtained when a child presented with nasal secretions and parents had expressed an interest to participate. For the children who had previously participated in the randomized trial, parents were instructed to contact the study team when their child developed an upper respiratory tract infection with visible nasal secretions and a home visit was subsequently scheduled. Children with craniofacial abnormalities were excluded, as well as children who had received antimicrobial therapy in the previous two weeks.
The sequence of sampling was randomized, determined by a computer-generated list and linked to a subject number upon enrolment. The study was approved by the Institutional Review Board of the University Medical Center Utrecht (available at: http://www.umcutrecht.nl/metc) and undertaken in accordance with the European Guidelines for Good Clinical Practice, which incorporate the provisions of the Declaration of Helsinki. Written informed consent was obtained from both parents.

Sampling and laboratory methods

Pediatric swabs with a flocked nylon fiber tip were used (Eswab 482CE; Copan, Brescia, Italy). A sample from the nasal vestibule, hereafter referred to as a "nasal swab", was taken by inserting a swab one centimeter into the nostril and rotating it three times. A nasopha-

ryngeal sample was obtained by passing the swab parallel to the floor of the nasopharynx to the posterior wall, according to World Health Organization guidelines.[11] Nasal secretions were obtained with a paper tissue as described by Leach and colleagues[9] by blowing or wiping the nose, depending on the child's age and cooperation. This tissue was taken from a standard (non-sterile) cardboard box (Scott Tissue box; VWR International BV, Amsterdam, the Netherlands), which was kept in a sealed bag. To minimize contamination, the first few paper tissues were discarded and disposable (non-sterile) gloves were worn during and hands disinfected between procedures. After taking a swab sample, the paper tissue was placed as a whole in a container with 10 mL of phosphate-buffered saline (PBS). All swabs were immediately inoculated in 1 mL of modified liquid Amies transport medium, stored at room temperature and transferred to the laboratory. All samples were cultured within 24 hours on the following agar media plates: Trypticase Soy Agar supplemented with 5% defibrinated sheep blood (TSA-SB; Oxoid, Badhoevedorp, the Netherlands), Hektoën Enteric Agar 2 (HEA2; Oxoid, Badhoevedorp, the Netherlands), Mannitol Salt Agar (MSA; Oxoid, Badhoevedorp, the Netherlands), and Trypticase Soy Agar supplemented with 7% defribinated sheep blood and 5 mg/L gentamicine (SB7-GENT; Oxoid, Badhoevedorp, the Netherlands). Plates were incubated at 37°C overnight; the MSA plate culture aerobically and the TSA, HEA2 and SB7-GENT plate cultures at 5% CO2. Both TSA-SB and SB7-GENT plate cultures were screened for the presence of *S. pneumoniae* colonies, TSA-SB and MSA plate cultures for *S. aureus*, HEA2 plate culture for *H. influenzae*, and TSA-SB plate culture for *M. catarrhalis*. Identification of *S. pneumoniae*, *H. influenzae*, *M. catarrhalis* and *S. aureus* was based on colony morphology and conventional methods of determination.[16]

All plates were inoculated using the original swab left in the Amies transport medium after sample collection. For the paper tissue samples stored in PBS, a cotton swab was used to inoculate the plates. The inoculum was placed onto the agar surface in a single, approximately two cm long streak and spread with a 10 µl disposable loop in a zigzag pattern until a quarter of the plate was covered. The latter procedure was repeated four times, slightly overlapping each previous streak, allowing to count colonies up to the fourth quadrant. The swab was dipped into the transport medium for each new plate inoculation. Resulting colonies were graded according to the distance from the point of inoculation, ranging from – (no growth) to ++++ (growth in fourth quadrant). This was used as a semiquantitative measure for the bacterial density in the original sample.

Statistical analysis

To detect a concordance rate of each bacterium at least 80% with 90% power and a two-sided alpha of 5%, a sample size of 66 subjects was required for this study. Concordance rates between the alternative and standard sampling methods as well as the specificity and sensitivity of these alternative methods were calculated. A nasopharyngeal swab was considered the gold standard for detecting *S. pneumoniae*, *H. influenzae* and *M. catarrhalis* and a nasal swab for *S. aureus*. Cohen's kappa statistic was computed to take chance agreement between pairs into account. A value of 1 indicates full agreement, while a value of 0 indicates merely chance. To assess differences between paired samples, we used McNemar's test to compare culture results and Wilcoxon signed rank test to compare the semiquantitative measure for bacterial density. A p-value of <0.05 was considered statistically significant. Data were analyzed with the statistical software package SPSS version 18.0 and Episheet.[13]

	N (%)	P value[c]	Concordance[d] (95% CI)	Cohen's Kappa	Sensitivity (95% CI)	Specificity (95% CI)
***Streptococcus pneumoniae*[a]**						
Nasopharyngeal swab	42 (64)	reference	NA	NA	NA	NA
Nasal swab	42 (64)	1.000	88 (78-94)	0.74	91 (77-97)	83 (62-95)
Swab from a paper tissue*	42 (65)	1.000	80 (69-88)	0.57	85 (70-94)	71 (49-87)
Paper tissue directly	49 (74)	0.039	86 (76-93)	0.69	98 (86-100)	67 (45-84)
***Haemophilus influenzae*[a]**						
Nasopharyngeal swab	46 (70)	reference	NA	NA	NA	NA
Nasal swab	44 (67)	0.500	97 (90-99)	0.93	96 (84-99)	100 (80-100)
Swab from a paper tissue	42 (64)	0.125	94 (86-98)	0.86	91 (78-97)	100 (80-100)
Paper tissue directly	43 (65)	0.250	95 (88-99)	0.90	94 (81-98)	100 (80-100)
***Moraxella catarrhalis*[a]**						
Nasopharyngeal swab	47 (71)	reference	NA	NA	NA	NA
Nasal swab	49 (74)	0.687	91 (82-96)	0.77	96 (84-99)	79 (54-93)
Swab from a paper tissue	45 (68)	0.727	88 (78-94)	0.71	89 (76-96)	84 (60-96)
Paper tissue directly	45 (68)	0.727	88 (78-94)	0.71	89 (76-96)	84 (60-96)
***Staphylococcus aureus*[b]**						
Nasopharyngeal swab	22 (33)	0.344	85 (75-92)	0.64	83 (58-96)	85 (72-94)
Nasal swab	18 (27)	reference	NA	NA	NA	NA
Swab from a paper tissue	15 (23)	0.508	86 (76-93)	0.64	67 (41-86)	94 (82-98)
Paper tissue directly	25 (38)	0.065	83 (73-91)	0.63	89 (64-98)	81 (67-91)

***Table 1:* Detection of bacterial pathogens in children with visible nasal secretions: prevalence, concordance, sensitivity and specificity between sampling methods.** N indicates the number of positive cultures for the specified bacterium. [a] Nasopharyngeal swab was considered the gold standard for detecting *S. pneumoniae*, *H. influenzae*, and *M. catarrhalis*. [b] Nasal swab was considered the gold standard for detecting *S. aureus*. [c] McNemar's test for paired samples; compared with the respective gold standards for detection. [d] Defined as the proportion of 'true' positives and negatives compared with the respective gold standards for detection. * Culture result for pneumococcus of one swab from a paper tissue was missing. Abbreviations: CI, confidence interval; NA, not applicable

Results

The study was conducted between April 2010 and November 2011. Mean age of the 66 included participants was 2.4 years (range 0-4 years) and 40 (61%) were boys. All four samples were collected from each child. *S. pneumoniae*, *H. influenzae*, *M. catarrhalis* and *S. aureus* were frequently cultured (Table 1). Concordance between each alternative sampling method and the respective gold standard was high for each of the four bacterial pathogens, ranging from 80% to 97% (Table 1). The agreement between pairs ranged from good (kappa statistic, 0.57) to excellent (0.93). *S. pneumoniae* was recovered even more frequently from a paper tissue directly than from a nasopharyngeal swab (p=0.039). Likewise, the detection rate of *S. aureus* was highest for culture of a paper tissue, albeit not statistically significant compared to a nasal swab. Detection rates for *H. influenzae* and *M. catarrhalis* were comparable across the

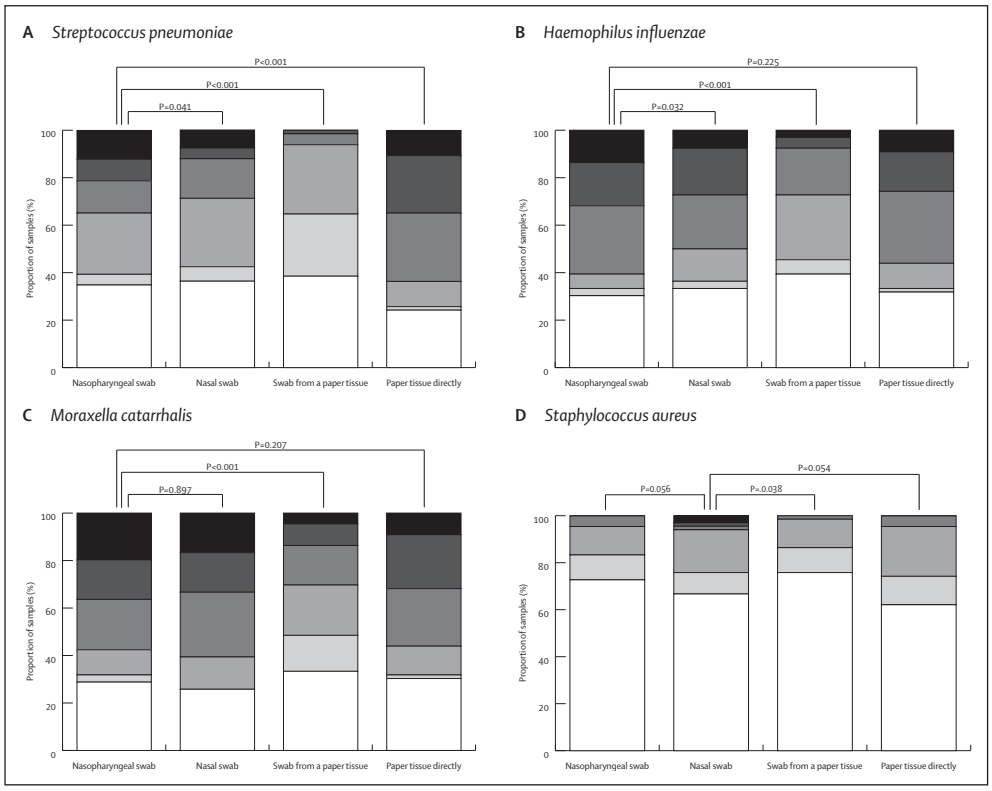

Figure 1: **Semiquantitative culture results of bacterial pathogens for different sampling methods.** Conventional culture results for *S. pneumoniae* (A), *H. influenzae* (B), *M. catarrhalis* (C) and *S. aureus* (D) in children with an upper respiratory tract infection, measured semiquantitatively. Colonies were graded according to the distance from the point of inoculation, ranging from – (no growth) to ++++ (growth at maximum distance). Sampling methods were compared to the "gold standard" by Wilcoxon Signed Rank test for paired samples. Nasopharyngeal sampling was considered the standard for *S. pneumoniae*, *H. influenzae* and *M. catarrhalis*,[4, 10, 11] and nasal sampling for *S. aureus*.[17]

sampling methods. The density of pneumococci (semiquantitative measure) was highest in cultures of a paper tissue directly compared to each of the other sampling methods (Figure 1). There was a trend for a higher density of *S. aureus* in cultures of nasal swabs compared to a paper tissue directly whereas the densities for *H. influenzae* and *M. catarrhalis* were comparable between culture of a nasopharyngeal swabs and a paper tissue (Figure 1).

Discussion

This is the first study comparing culture of nasal secretions, collected by wiping or blowing the nose into a paper tissue, with the current standards for detecting bacterial pathogens in children with an upper respiratory tract infection. We randomized the order in which samples were taken to minimize the risk of a potential bias introduced by a fixed sequence. Bacteria were detected at similar rates across sampling methods with an even higher detection rate

for *S. pneumoniae* in culture of a paper tissue. Most importantly, concordance between sampling methods was high, indicating that in children with an upper respiratory tract infection culture of a paper tissue collected by simply blowing or wiping the nose reliably detects four common bacterial pathogens. This may have major implications for research in this area as it is a familiar and less unpleasant procedure for the child than nasopharyngeal sampling and does not require training. This sampling method provides valuable information for studies on bacterial transmission or surveillance in children with upper respiratory tract infections.
Several authors have compared nasopharyngeal sampling with other sampling techniques, such as a nasal swab, oropharyngeal swab or nasopharyngeal wash,[1, 3, 4, 6, 12, 15] but none compared this with culture of a paper tissue directly. While Leach *et al.* found that detecting pneumococci in a swab from nasal secretions collected by blowing the nose into a paper tissue was highly sensitive in children with visible secretions, this was neither compared with a nasopharyngeal sample nor with culture of the paper tissue as a whole.[9] The latter is attractive since it does not require expensive swabs and is even simpler to perform. In our study, culture of a paper tissue directly had a higher yield than a swab from nasal secretions of a paper tissue, especially with regard to the bacterial densities. Also, pneumococci and, to a lesser extent, staphylococci were detected even more frequently in culture of a paper tissue directly than from a nasopharyngeal and nasal swab, respectively.

Whereas all swabs were inoculated in 1 mL of liquid Amies transport medium, the paper tissue was placed in 10 mL of PBS. PBS is a water-based salt solution commonly used in research, but contrary to modified liquid Amies, not specifically formulated to sustain the viability of microorganisms. We postulate that the higher detection rates of pneumococcus (and staphylococcus) are related to the (abundant) presence of mucus in samples collected by a paper tissue. The physiologic composition of mucus produced during an upper respiratory tract infection may provide a good substrate for bacteria to abide in.[2] Mucins have been shown to positively affect bacterial growth in vitro and in vivo.[8] The results of our study support the concept that in children with an upper respiratory tract infection, nasal secretions contain bacteria whose detection during asymptomatic episodes would be more constrained to a specific ecologic niche. Moreover, the nasopharynx, generally considered the primary niche for *S. pneumoniae*, is located at an anatomical site that normally prevents easy dispersion. Changes in the upper respiratory tract during infection may therefore be linked to enhancement of bacterial transmission among human hosts.
Some limitations of our study should be addressed. The presence of nasal secretions was a specific criterion for participation. Therefore, our results apply to a specific population of children with an upper respiratory tract infection and cannot be generalized to assessment of colonization in asymptomatic children.[11] Additionally, mild respiratory infections are mostly attributed to a viral infection.[5] In this context, it needs to be noted that the presence of bacterial pathogens under such circumstances may differ from a situation in absence of an infection.[7]
In conclusion, our data show that culturing nasal secretions from a paper tissue can reliably detect bacterial pathogens in children with symptoms of an upper respiratory tract infection. This offers great opportunities for research in children with upper respiratory tract infections.

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CHAPTER 7

S. pneumoniae colonization in asymptomatic elderly, the Netherlands

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Submitted

CHAPTER 7

Streptococcus pneumoniae colonization in asymptomatic elderly, the Netherlands

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Abstract

Nasopharyngeal colonization with *Streptococcus pneumoniae* is reported to be very low in the elderly, but most studies only use conventional culture. We sampled the nasopharynx in 330 asymptomatic elderly. Conventional culture alone detected *S. pneumoniae* in 25 subjects (8%). A quantitative PCR (qPCR) was positive in 71 subjects (22%), predominantly in transoral nasopharyngeal swabs. As compared to qPCR, conventional culture underestimates *S. pneumoniae* colonization.

Introduction

The incidence of invasive pneumococcal disease (IPD) and pneumonia typically follows a U-shaped curve, peaking at the extremes of life.[1] In the elderly, *Streptococcus pneumoniae* is the leading cause of community-acquired pneumonia (CAP) with up to 40% mortality in bacteremic CAP cases.[1] Children under-five have high pneumococcal colonization rates and are prone to pneumococcal infections, owing to their immature immune systems. In contrast, data on pneumococcal colonization in the elderly are limited and reported to be very low as measured by conventional culture methods, ranging from virtually non-existent to 5%.[2-4]. Molecular techniques, advocated to detect low-density *S. pneumoniae* colonization,[5] have rarely been applied in the elderly. Knowledge about the prevalence of *S. pneumoniae* in the upper respiratory tract in the elderly is important, not only to better understand the pathogenesis of pneumococcal infections like CAP, but also to assess possible vaccine and herd effects in this population.

Material and methods

A study evaluating nasopharyngeal *S. pneumoniae* colonization in 330 asymptomatic, community-dwelling adults ≥ 65 years was undertaken prior to the start of a randomized controlled trial assessing the efficacy of the 13-valent pneumococcal conjugate vaccine (Prevenar-13®, Pfizer, Inc.) against vaccine serotype CAP in elderly (ClinicalTrials.gov Identifier NCT00744263). Between December 10, 2007 and January 30, 2008 we sampled the nasopharynx using two transnasal and two transoral swabs. At the time, the 7-valent vaccine had been introduced for newborns (without a catch-up campaign) for 1.5 years (start June 2006). Until 2008 no herd effects on IPD in elderly were observed.[6] Antibiotic use in the preceding month was an exclusion criterion. Samples were collected by trained study staff, immediately stored in transport medium at room temperature and transferred to the Regional Laboratory (Haarlem, the Netherlands) within 8 hours. Here, the first of two transnasal and transoral swabs were used to detect *S. pneumoniae* by conventional culture methods.[7] The remaining transnasal and transoral swabs were stored in transport medium at -70°C until transferred to Pfizer's laboratories for further analyses. A real-time quantitative PCR (qPCR) was used to detect *S. pneumoniae*, targeting conserved regions of the autolysin (*lytA*) gene.[8] After thawing, DNA was extracted from 100 µl of each specimen using an ABI 6700 Nucleic Acid Workingstation (Applied Biosystems, CA) and eluted in a final volume of 100 µl. DNA fragments were amplified using the 7900 Fast Real-Time PCR System. Briefly, 10 µl of each purified DNA was added to 10 µL 2X Fast PCR mix (Applied Biosystems) containing primers and probes in a 96-well plate. PCR results were considered positive only if the *lytA* gene was amplified (cycle threshold [CT] <45).[9]

Results

The median age of the 330 participants was 72.7 years (interquartile range: 68.7-79.0), and 156 (47%) were male; only 4 subjects (1%) had previously received the 23-valent pneumococcal polysaccharide vaccine. Of all subjects, 25 (8%, 95% confidence interval (CI): 5-11%) carried *S. pneumoniae* as measured by conventional culture methods (Table). Based on qPCR alone, pneumococci were detected in 65 transnasal and/or transoral samples (22%, 95% CI: 18-27%). The mean Ct values were 36.05 (range: 24-40) and 36.31 (range: 25-44) for trans-

nasal and transoral samples, respectively. Combined, conventional culture and qPCR were positive for *S. pneumoniae* in either transnasal or transoral specimens in 75 subjects (23%, 95% CI: 19-28%). Whereas an equal number of individuals (15 of 25) would be identified as *S. pneumoniae* carriers by conventional culture if only transnasal or transoral samples would be collected, there were significantly more transoral (n=58; 18%) than transnasal specimens (n=32; 10%) positive for *S. pneumoniae* by qPCR (P<0.001).

	Conventional culture	qPCR[a]	Concordance[b]	P value[c]
Transnasal[d]	16 (5)	32 (10)	92%	P=0.001
Transoral	16 (5)	58 (18)	85%	P<0.001
Both transnasal and transoral	7 (2)	19 (6)	NA	NA
Either transnasal or transoral	25 (8)	71 (22)	NA	NA

Table 1: **Conventional culture and qPCR results for *S. pneumoniae* in nasopharyngeal samples of 330 adults ≥ 65 years of age in the Netherlands.** Data are n (%), unless otherwise stated. [a] Results for *lytA* qPCR; any signal for *lytA* (cycle threshold values < 45) was considered positive for the presence of *S. pneumoniae* (6, 8, 9). [b] Defined as the proportion of samples with corresponding results in both tests over the total number of samples tested. [c] McNemar test for paired samples. [d] Results from 6 transnasal specimens were unavailable; concordance was calculated for 324 samples. NA, not applicable

Discussion

The autolysin gene has been reported to be a highly sensitive and specific target to detect *S. pneumoniae*.[5, 8, 9] Our results confirm the superiority of molecular techniques over conventional culture in detecting the presence of *S. pneumoniae* in nasopharyngeal samples in general, and in transorally obtained nasopharyngeal specimens in particular (18% vs 5%, respectively; P<0.001). This lower sensitivity of conventional culture may reflect an inherent difficulty to process transoral samples for *S. pneumoniae* due to the presence of many competing species.[10] Of 32 culture-positive samples, seven were negative when tested by qPCR. There were also samples with low *lytA* Ct values (suggesting high bacterial density), with corresponding cultured samples negative for *S. pneumoniae*. This discordance can be partly explained by the fact that our culture and qPCR results were derived from separate swabs. Also, discordance would be particularly expected in case of low-density colonization. The relatively high Ct values observed by us seem to confirm overall low-density colonization, in line with reported low-density nasopharyngeal *S. pneumoniae* colonization in asymptomatic adults.[9]

In conclusion, our results indicate that, when compared to qPCR testing, conventional culture underestimates *S. pneumoniae* colonization rates in older adults. How colonization relates to disease in this population warrants further investigation.

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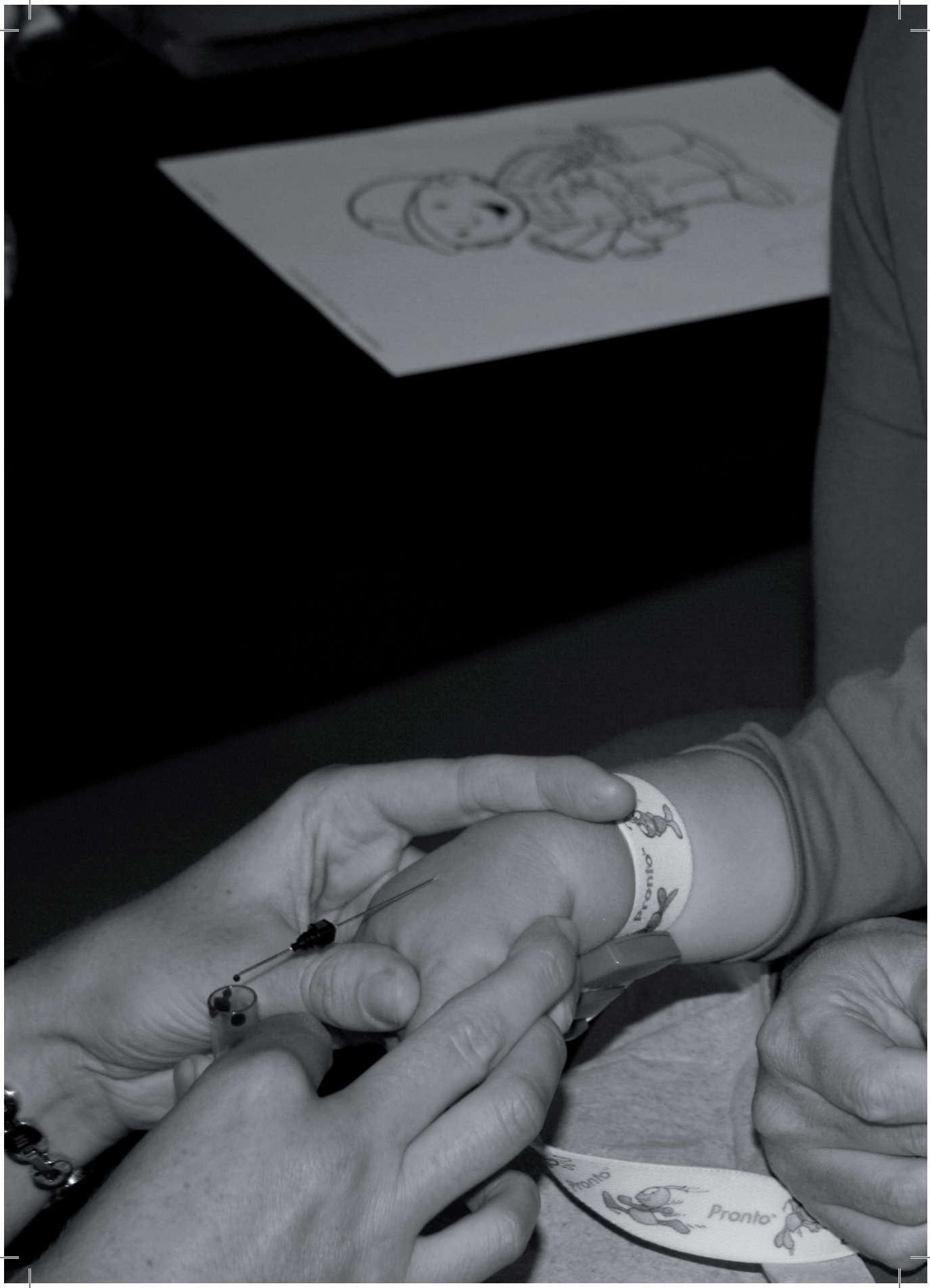
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CHAPTER 8

Summarizing discussion

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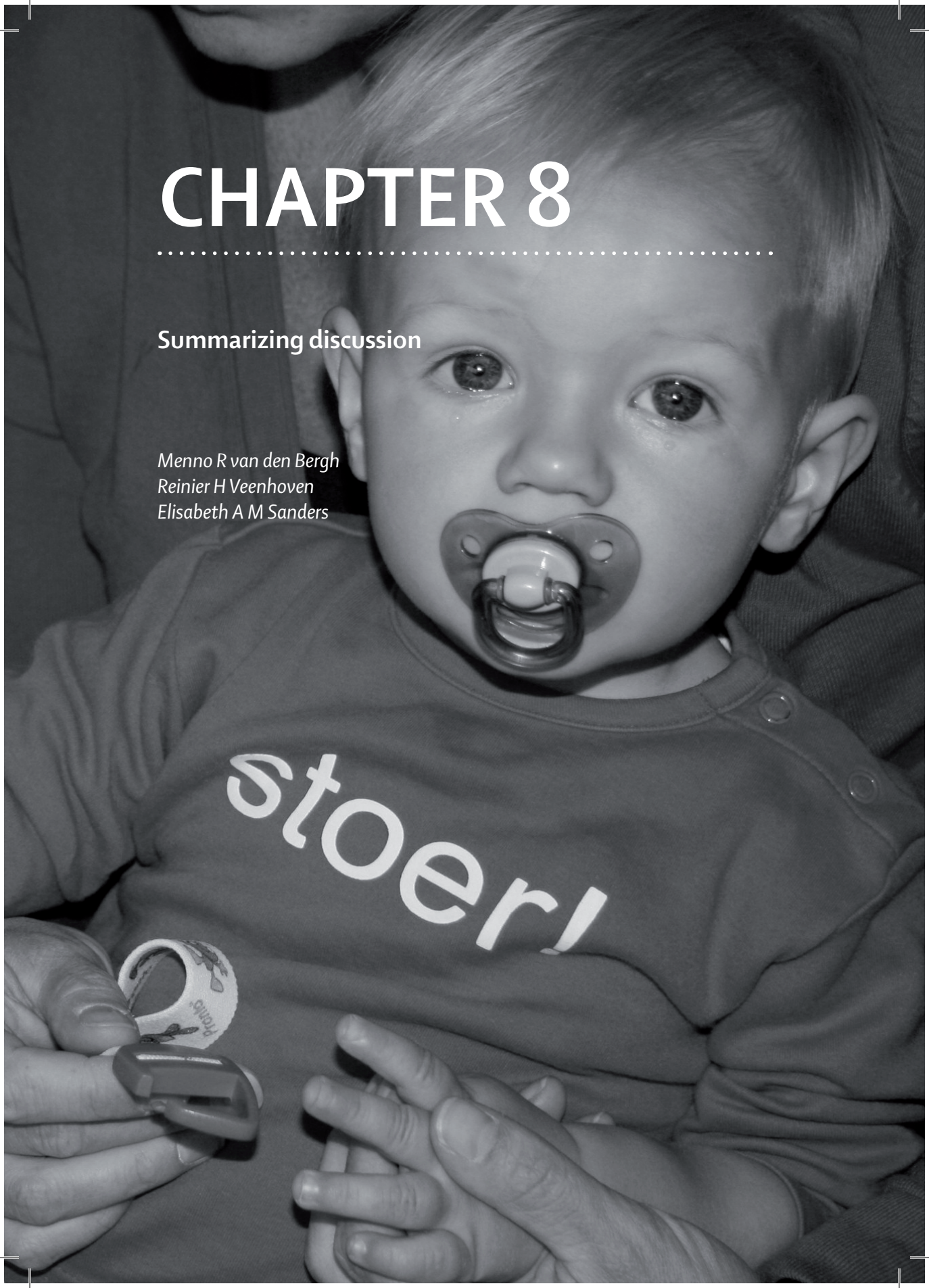

Pneumococcal conjugate vaccines (PCVs) have been introduced and shown to effectively prevent pneumococcal diseases and nasopharyngeal acquisition of vaccine pneumococcal serotypes. The great success of PCVs is, however, somewhat blunted by the emergence of nonvaccine serotypes, in particular 19A. This replacement has been far more outspoken for mucosal manifestations (colonization, AOM) than for invasive diseases. Effects of broader-coverage PVCs have been studied, including PCVs that use NTHi-derived protein D (PD) as a carrier protein. The experimental 11-valent PCV (with each serotype individually conjugated to PD) was shown to be efficacious against AOM caused by NTHi. Based on this 11-valent vaccine, a 10-valent formulation (with 8 of 10 serotypes conjugated to PD) was licensed in 2009. However, very limited and rather unrepresentative data were available with respect to effects of these PD-conjugate vaccines on nasopharyngeal bacterial colonization, and in particular NTHi. It has been established that investigating the effects of PCVs on nasopharyngeal colonization can be used as a measure for vaccine efficacy and a predictor for herd immunity. As a result, experts have propagated to adopt vaccine impact on nasopharyngeal colonization as an additional criterion for the licensure of new pneumococcal vaccines. Currently, licensure of PCVs relies fully relies on the measurement of functional anticapsular antibodies. In this regard, interference of immune responses could occur when PCVs are concomitantly administered with other routinely used vaccines in crowded immunization schedules. Consequently, regulatory authorities acknowledge that effects on immune responses should be thoroughly studied.
Some of the diseases aimed to be prevented by PCVs, i.e., respiratory tract infections such as pneumonia and AOM, are polymicrobial in nature. Clinically relevant interactions between respiratory viruses and bacteria are increasingly recognized to be important in the pathogenesis of these mucosal infections. This interplay could be reflected in the presence of viruses and bacteria in the nasopharynx—the point of origin for these infections.

Apart from children, *S. pneumoniae* causes a substantial burden of disease in the elderly population. In developed countries, community-acquired pneumonia (CAP) ranks among the top five causes of death in persons 65 years of age and older. PCVs could offer protection against CAP and presumably better than the 23-valent pneumococcal polysaccharide vaccine. However, little is known about the true prevalence of *S. pneumoniae* in the nasopharynx of older adults, although this is highly relevant not only with respect to pathogenesis of CAP but also in terms of possible vaccine impact.

In this thesis, we have focused on (1) results of a randomized controlled trial evaluating immunogenicity, safety and reactogenicity of the 10-valent pneumococcal nontypeable Haemophilus influenzae protein D-conjugate vaccine (PHiD-CV) and effects on nasopharyngeal bacterial colonization; (2) associations between respiratory pathogens present in the nasopharynx of healthy children; (3) alternative sampling methods for detecting bacterial pathogens in children with an upper respiratory tract infection; and (4) nasopharyngeal S. pneumoniae colonization in elderly. A review and discussion of the main findings shed new light into these research questions, and raise further issues for future research.

What is the immunogenicity of PHiD-CV and DTPa-IPV-Hib coadministration versus other combinations of pneumococcal conjugate and DTPa-combined vaccines? Is there evidence for immune interactions?

Coadministration of PCVs with other pediatric vaccines offers the advantage of preventing multiple infectious diseases in children at a time in which protection is most warranted.[1] However, immune interactions may occur when vaccines sharing antigens or carrier proteins are concomitantly administered,[2] resulting in either enhanced or impaired immune responses.[3-5] How coadministration of different vaccines affects immune responses is difficult to predict.[6] In their regulatory document, the European Medicines Agency states that the immunogenicity and safety of a new PCV does not need to be evaluated in coadministration with every other possible vaccine.[7] Rather, for the group with which coadministration is likely to occur, one representative vaccine suffices for the registration process.[7] For example, PHiD-CV was shown to be immunogenic and safe when coadministered with DTPa-HBV-IPV/Hib (Infanrix hexa™, GlaxoSmithKline Vaccines).[8] For licensure purposes, evaluation of coadministration with another vaccine from the same group (e.g., DTPa-IPV-Hib; Pediacel™, Sanofi Pasteur MSD, used in the Netherlands since 2006) was not strictly required. However, these DTPa-combination vaccines may not be interchangeable.[9]

Immunogenicity of PHiD-CV and DTPa-IPV-Hib compared to other combinations of vaccines
In **Chapter 2** and **Chapter 3**, we evaluated the immune responses to PHiD-CV and DTPa-IPV-Hib when coadministered in comparison to PHiD-CV combined with DTPa-HBV-IPV/Hib and compared to coadministration of 7vCRM and DTPa-IPV-Hib. Specifically, we assessed noninferiority of antibody responses to PHiD-CV (primary objective) and DTPa-IPV-Hib antigens (secondary objective). Noninferiority was defined to be reached if the upper limit of the 95% confidence interval of the ratio of antibody concentrations between groups was below 2. Our results showed that PHiD-CV and DTPa-IPV-Hib remain immunogenic and safe when coadministered as a 3+1-dose vaccination schedule, as compared to the other combinations of vaccines. From a strict statistical viewpoint, however, we could not conclude that post-primary antibody responses to PHiD-CV coadministered with DTPa-IPV-Hib were noninferior to those with DTPa-HBV-IPV/Hib coadministration. The reason for this was a higher antibody response to 18C (conjugated to tetanus toxoid) when PHiD-CV was coadministered with DTPa-HBV-IPV/Hib. This could be linked to the higher antitetanus response in the PHiD-CV + DTPa-HBV-IPV/Hib group. As a consequence, the predefined limit of noninferiority was marginally exceeded (**Chapter 2**). Pre- and post-booster, antibody responses to PHiD-CV remained within the noninferiority limit (**Chapter 3**). Nonetheless, the anti-18C responses in our study were in the lower ranges of those observed in other European PHiD-CV trials and comparable to those observed in a 2+1-dose PHiD-CV schedule.[10,11]

In another study in the Netherlands, 18C was also ranked among the serotypes with the lowest antibody concentrations.[12] Although the relatively low anti-18C antibody levels are as yet unexplained, the question is: how relevant is this? In our study, high and comparable proportions of PHiD-CV recipients reached the putative protective levels to all serotypes post-primary and post-booster, including 18C. Importantly, since colonization with vaccine pneumococcal serotypes had already declined dramatically due to the establishment of herd immunity,[13] we believe this difference in anti-18C response between groups not to be of clinical relevance.

With respect to antigens of the DTPa-combined vaccines, we found differences among groups in antibody titers to tetanus, Hib polysaccharide PRP, and diphtheria at one or more time points. Although most children after the primary series (**Chapter 2**) and following the booster dose (**Chapter 3**) reached antibody levels considered to be protective against disease (with no differences between groups), these differences in antibody GMCs suggest that immune interactions may be at play.

Long-term protection against *S. pneumoniae*

In the Dutch NIP, children receive their last dose of PCV around their first birthday. However, children are still at risk for IPD up to five years of age.[1] This raises the question whether PCVs still protect children in the time period after completion of the PCV schedule? In general, three mechanisms provide long-term protection against encapsulated bacteria: (1) persistence of functional antibodies, (2) maintenance of immunological memory, and (3) herd immunity.[14] In our study, we evaluated long-term immune responses to pneumococcal antigens (**Chapter 3**). We found that at 24 months of age, 12-13 months after the booster dose, serotype-specific anticapsular antibodies and opsonophagocytic titers had declined to near pre-booster levels. Long-term antipneumococcal antibody responses have been evaluated for experimental PCVs.[15,16] After priming and a booster dose of the 11-valent predecessor of PHiD-CV in the second year of life, a sharp decline in antibody levels was observed up until the age of 4.5 years for most pneumococcal serotypes.[16] However, for serotypes 6B, 14, 19F and 23F (previously the most common colonizers)[17] this drop was less outspoken. There was also a notable increase in antibody levels to the same serotypes in the unvaccinated control group.[16] These observations strongly suggest boosting of the immune system by natural exposure to circulating pneumococci.

Waning immunity is well described for protein-polysaccharide vaccines, including those targeting *Haemophilus influenzae* type b (Hib) and group C meningococci (MenC).[18] In fact, waning immunity was one of the driving factors for the surge of Hib and MenC vaccine failures in the United Kingdom after introduction of a 3-dose priming series without a booster dose.[14,19,20] With establishing herd effects and, thus, diminished circulation of vaccine-targeted serotypes,[13] seroepidemiology and kinetics of antibody responses may change, as previously noted following MenC introduction.[21] Waning of antibodies seems to proceed faster in the absence of natural exposure to vaccine serotype pneumococci, as observed in our study.

As mentioned, long-term protection also depends on maintenance of immunological memory and herd immunity. B-cell memory could provide long-term protection in individuals whose antibody levels have waned below the protective threshold. However, immunological memory does not guarantee protection.[22] This is particularly evident in case of a rapidly invading pathogen like Hib or MenC, since a B-cell response takes at least four days to be mounted.[23] However, protection against vaccine pneumococcal serotypes may now be achieved by herd immunity alone.

Of note, mechanisms other than serotype-specific antibodies are important for naturally acquired protection against pneumococcal colonization and disease.24 Interleukin-17A-secreting CD4+ T cells (TH17) mediate resistance to pneumococcal colonization in a serotype-independent manner that matures with age.[25]

On the basis of our results, we support the Dutch National Institute for Public Health and the Environment (RIVM) in recommending that serological surveillance should be conducted for every new vaccine introduced into the immunization program when coadministered with other vaccines.[26] In addition, surveillance of immune responses in the years following intro-

duction of a new PCV remains important, because pneumococcal seroepidemiology changes with establishing herd immunity.

Effects of PHiD-CV versus 7vCRM immunization on nasopharyngeal bacterial colonization, in particular nontypeable *H. influenzae* (NTHi)

The Pneumococcal Otitis Efficacy Trial (POET), evaluating the efficacy of the 11-valent predecessor (11Pn-PD) of PHiD-CV on acute otitis media (AOM), was the first study to demonstrate efficacy against AOM caused by NTHi (35% reduction),[27] thought to be due to utilization of NTHi-derived PD as the carrier protein in this vaccine.[28] Some reduction in NTHi colonization was evident following 11Pn-PD immunization was shown,[27] but only temporary after the booster dose.[29] For PHiD-CV, no consistent effect on NTHi colonization was found.[30] In contrast, the impact on colonization by protein-polysaccharide conjugate vaccines targeting encapsulated bacteria residing in the upper respiratory tract like Hib, MenC and *S. pneumoniae* has consistently been profound.[17,31-34] The colonization rates of NTHi and *S. pneumoniae* observed in PD-conjugate vaccine studies were also considerably lower than those observed in most other studies.[27,29,30] Consequently, effects of PD-conjugate vaccines on nasopharyngeal bacterial colonization has remained largely unknown, although highly relevant in terms of mechanisms of protection and possible herd effects.

Effects on *Haemophilus influenzae*

We found that compared to 7vCRM, immunization with PHiD-CV in infancy had no differential impact on either NTHi colonization or H. influenzae density (of which >90% was NTHi) in healthy Dutch children up to two years of age (**Chapter 4**). Therefore, no herd effects are to be expected for this bacterium after introduction of PHiD-CV in the Dutch NIP.

How do the results of our trial with PHiD-CV on nasopharyngeal NTHi colonization relate to the observed efficacy of 11Pn-PD against NTHi-AOM? Hypothetically, PD-conjugate vaccines could exert an effect on disease while not affecting colonization. Johnson, *et al* proposed a compartment-dependent effect (i.e., in middle ear but not in the nasopharynx) to explain similar findings to ours in a chinchilla model of AOM.[35] Yet, effective vaccines against mucosal infections usually also affect colonization.[31] In addition, besides POET, there is a lack of data to support direct protection of a PD-conjugate vaccine against human NTHi disease.The implications of assuming that PD-conjugate vaccines are not efficacious against NTHi are profound, particularly because the efficacy observed in POET has been extrapolated to invasive NTHi disease[36] and included in cost-effectiveness estimates.[37] If we would assume that a PD-conjugate vaccine does not confer protection against NTHi disease, how would the POET results be explained? As a concept, AOM evolution can be divided in primary and secondary events (Figure 1). The primary events of colonization with *S. pneumoniae* and/or *H. influenzae* (NTHi) early in life could lead to an early onset of the first AOM episode, which is in itself associated with an increased risk for recurrent disease.[38] *S. pneumoniae* is more pathogenic than NTHi and likely to be the otopathogen to cause the first AOM episode.[39] NTHi is particularly associated with recurrences,[40] and, thus, increasingly involved in secondary events (Figure 1). This has been suggested to result from biofilm formation.[41,42] PCVs effectively prevent both nasopharyngeal acquisition of vaccine serotypes (and, hence, early colonization) and vaccine serotype AOM. Designed to evaluate effects in randomized controlled trial settings, efficacy studies tend to be enriched by primary events (low burden disease). Effectiveness studies, by contrast, are designed to evaluate effects in routine clinical practice, and are more likely to be enriched by secondary events (high burden disease).

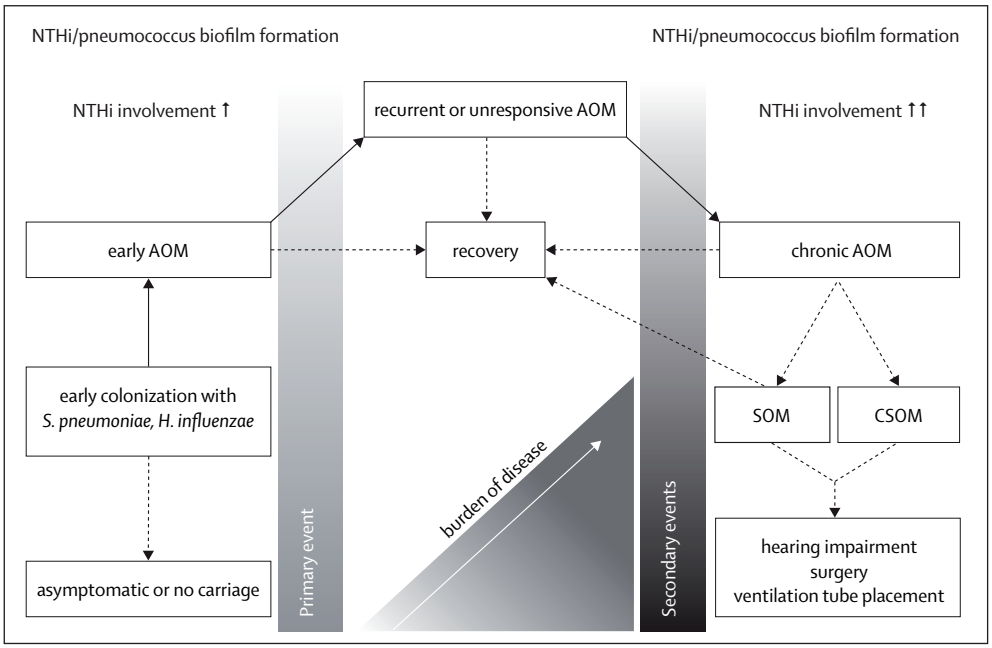

Figure 1: **Evolution of acute otitis media, a concept.** Courtesy of R. Dagan.
In this concept, acute otitis media evolution can be divided in primary and secondary events in which bacterial pathogens *S. pneumoniae* and nontypeable *Haemophilus influenzae* (NTHi) are mainly involved, respectively. Pneumococcal conjugate vaccines effectively prevent acquisition and thus (early) colonization and (early) vaccine serotype AOM. Therefore, prevention of the first pneumococcal AOM is hypothesized to prevent secondary events involving NTHi. Abbreviations: SOM, serous otitis media; CSOM, chronic suppurative otitis media.

We have learned from a randomized study in otitis-prone, 1- to 7-year-old children, that once colonization is established, pneumococcal conjugate vaccination shifts colonization from vaccine to nonvaccine serotypes, an effect that increases the likelihood of suffering from another AOM episode as compared to no vaccination.[43] Therefore, prevention of the first colonization episode is key. In this concept of AOM evolution, prevention or delay of the first pneumococcal AOM episode is hypothesized to prevent secondary cases involving NTHi. Supporting this hypothesis, unpublished data from Israel show a significant 22% decline in NTHi-AOM in children <24 months of age two years after 7vCRM introduction compared to a four year-period pre-7vCRM.[44] Results from POET may be explained by this concept: the observed effect on NTHi-AOM would be attributed to an effect on pneumococci rather than an effect mediated by PD. However, results from the FinOM trial,[45,46] appear to disprove the concept, as an 11% increase in AOM episodes caused by *H. influenzae* was noted in 7vCRM recipients compared to controls.[45]

Control groups constitute a notable difference between our trial and other PD-conjugate vaccine studies. In our study, the "control group" received 7vCRM, considered to be a non-active control with respect to effects on NTHi. In POET and other studies, children in control groups were PCV-naïve.[27,30] In vitro and animal studies have shown that these bacterial species interact with each other,[47] which may result in either interference or co-existence.[48] Effects of a pneumococcal conjugate vaccine on pneumococcal colonization patterns could indirectly affect NTHi colonization. In turn, this can be hypothesized to underlie the temporary decline

in *H. influenzae* colonization in POET. In contrast, AOM caused by *H. influenzae* tended to increase in FinOM, as previously mentioned.[45] Additionally, follow-up surveillance data from the Netherlands showed that *H. influenzae* colonization rates in healthy children were higher in 2010-2011 than before introduction of 7vCRM in 2006.[49]
In conclusion, our data indicate that targeting PD, a single protein antigen of unencapsulated NTHi strains, does not affect nasopharyngeal NTHi colonization or density. Further research is needed to learn if PD-conjugate vaccines prevent human NTHi disease. To this end, results of randomized clinical trials evaluating the efficacy of PHiD-CV against AOM should be awaited (ClinicalTrials.gov Identifier NCT00839254 and NCT00466947).

Effects on *Streptococcus pneumoniae*
Our trial (**Chapter 4**) was initiated approximately two years after introduction of 7vCRM in the Netherlands. A carriage surveillance study, conducted by our group a year later and in parallel to the trial, confirmed that herd effects were being established.[13] Consequently, we expected no major effects on *S. pneumoniae* colonization, especially since the additional serotypes 1, 5 and 7F, known for their invasiveness,[50] are rarely recovered from the nasopharynx.[51] In line, in our study, the additional serotypes were seldom detected and a further decline in vaccine serotypes common to both vaccines was observed during follow-up (from 8 to 3%) while the prevalence of nonvaccine serotypes was equally high in both vaccine groups.
Of particular interest was the effect of PHiD-CV on serotype 19A. Immunogenicity studies have shown cross-reactivity to serotypes related to those included in the vaccine, such as for 6A (due to 6B) and, to a lesser extent, 19A (due to 19F).[52-54] However, cross-*reactivity* is not synonymous with cross-*protectivity*. Vaccine efficacy and effectiveness point estimates against 19A IPD and AOM were consistently positive for modest protection, although not statistically significant in any study.[55] Nonetheless, serotype 19A emerged as the predominant replacing serotype both in colonization and disease.[49,56-58] Interestingly, acquisition of serotype 19A in the nasopharynx was found to be significantly higher in 7vCRM recipients compared to unvaccinated controls in a previous randomized study.[59]
In the optimized formulation of PHiD-CV, serotype 19F was conjugated to diphtheria toxoid. The conjugation method improved the immunogenicity to 19F,[60] which in turn enhanced the cross-reactive antibody response to 19A.[10] Functionally active anti-19A antibodies are also higher with PHiD-CV than 7vCRM (Chapter 2 and 3). In our study, serotype 19A was the most prevalent colonizer at every sampling moment during follow-up (6-10% and 20% as measured by conventional culture and PCR, respectively), with no differences between PHiD-CV and 7vCRM recipients (**Chapter 4**). It is unlikely that a modest effect of PHiD-CV on 19A colonization went undetected. Our results do not lend support to assume that 19F in PHiD-CV cross-protects against 19A colonization. Before introduction of PHiD-CV, a steadily increasing prevalence of 19A was found in the nasopharynx of Dutch children following implementation of 7vCRM.[13,49] Our group is currently conducting another follow-up surveillance study assessing nasopharyngeal colonization patterns in 11- and 24-month-old children, with the youngest having received 3-dose priming with PHiD-CV. Now, approximately seven years after the start of pneumococcal conjugate vaccination, colonization patterns may have reached a new equilibrium.
Finally, it should be noted that a (much) lower antibody concentration is needed to protect against invasive disease than to prevent nasopharyngeal acquisition.[61] It therefore remains to be seen whether PHiD-CV provides any cross-protection against 19A *disease*.

Prospects for new pneumococcal vaccines

Currently, licensure of new PCVs is solely based on a comparable immune response to an already registered product.[62] However, there are several limitations to this approach.[63] First, the serological licensing criteria are applicable to invasive disease only, not to any other endpoint, like mucosal infections and colonization. Second, the existing "correlate of protection" is a crude estimate and not serotype specific. Third, addressing only the direct effects (that is, immune responses in vaccine recipients) ignores the profound impact of herd effects. Especially for vaccines that are expensive and those that provide moderate protection, the ability to confer herd protection could be crucial to policy deliberations about vaccine introduction.[64] Finally, new vaccines containing antigens other than capsular polysaccharide are likely to confer protection in an antibody- and serotype-independent manner.[65] This has let to the strong case being made by the PneumoCarr consortium for vaccine efficacy against colonization to be included as an additional end point in the registration process of future pneumococcal vaccines.[66]

Current PCVs target a limited number of clinically important serotypes, while nonvaccine serotypes are increasing in both colonization and disease. Moreover, PCVs are costly, limiting their dispersion in low resource areas where the need for prevention is the greatest. Therefore, great interest exists to develop novel, more effective vaccines utilizing alternative or adjunctive means of protecting against pneumococcal colonization and disease. To overcome the threat of serotype replacement, identification of protein antigens common to all or most strains worldwide has been a major focus of research.[65] Vaccines utilizing multiple protein components may direct the immune system to attack the pneumococcus at various stages of its pathogenesis. If nasopharyngeal colonization is also affected, these vaccines could potentially result in a large herd effect. Protein antigens could also be used as alternative carriers in PCVs. A randomized study evaluating a new pneumococcal vaccine is currently being undertaken in The Gambia (ClinicalTrials.gov Identifier: NCT01262872). Since this study aims to evaluate nonvaccine pneumococcal serotype colonization in infants and toddlers, it is likely this experimental vaccine includes such protein antigens. Results are not expected before mid-2013.

Another candidate, pneumococcal whole-cell vaccine, induces immunity directed to a variety of conserved pneumococcal antigens and has the potential to be efficacious worldwide. When given intranasally to animals, whole-cell vaccines reduce nasopharyngeal *S. pneumoniae* colonization, otitis media and fatal pneumonia caused by various strains of encapsulated pneumococci. Protection against nasopharyngeal *S. pneumoniae* colonization is mediated by antibody- and T-cell immunity.[25,67] Whole-cell vaccines seem to be utilizing both immunological mechanisms, inducing cellular as well as humoral immunity.[68] However, *S. pneumoniae* is highly adaptable and capable of escaping antibiotic and vaccine pressure as a result of genomic plasticity.[69] Interestingly, using a mouse model of pneumococcal colonization, Li, *et al* have demonstrated that TH17 cell-based immunity almost equally reduces colonization by both an antigen-positive strain and a co-colonizing, antigen-negative strain.[70] DNA sequences of TH17 cell antigens demonstrated no detectable signs of selective pressure, unlike pneumococcal antigens known as strong antibody targets. These results suggest that evolution of escape from TH17 -based vaccines may be slower than from antibody-based vaccines.[70]

What will the future hold for *S. pneumoniae*?

What would the consequences be if future vaccines would effectively evict *S. pneumoniae* from its nasopharyngeal niche?[71,72] PCVs have already been shown to impact nasopharyngeal bacterial colonization, not only confined to a shift from vaccine to nonvaccine serotype strains. In a randomized trial setting in the Netherlands, *S. aureus* carriage was temporary doubled in children vaccinated with 7vCRM compared to unvaccinated controls.[73] The aforementioned Dutch carriage surveillance studies showed that, as compared to the same historical controls, *S. aureus* carriage remains substantially higher in 11-month-old, but not 24-month-old children in the years following introduction of 7vCRM.[49] Community-associated methicillin-resistant *S. aureus* (MRSA) has emerged in the USA and, although low, seems to be increasing in Europe as well.[74] Given these observations, a possible link between MRSA and pneumococcal conjugate vaccination warrants further investigation.[75]

In addition to *S. aureus*, nasopharyngeal *H. influenzae* colonization increased in the years following 7vCRM introduction in the Netherlands.[49] Although reduced-dose schedules of 7vCRM in a randomized controlled trial setting were not associated with an increase in *H. influenzae* colonization as measured by conventional culture methods,[76] a post-hoc study evaluating nasopharyngeal microbiota profiles of the same children showed trends toward increased presence and abundance of *Haemophilus* and *Staphylococcus* species in vaccinated children as compared to unvaccinated controls.[77] In fact, this study showed a temporary, though significant effect of 7vCRM on the stability, diversity and composition of the nasopharyngeal microbiota of young, healthy children.[77] If and how these effects relate to disease caused by nonpneumococcal species is currently unknown. *S. pneumoniae* is a well recognized human-restricted bacterial pathogen and a killer of children,[78,79] but also a highly prevalent commensal living harmoniously with its host most of the time.[72] With the (possible) effects on nonpneumococcal bacterial species mind, we caution that complete eradication of *S. pneumoniae* from the nasopharynx might result in unexpected adverse effects, although the outcome is rather unpredictable.

Specific associations among viral and bacterial pathogens in the nasopharynx of healthy children

Bacterial and viral pathogens are likely to interact in the human respiratory tract. Clinically important interactions between influenza virus and pneumococcus have been identified, as mentioned in Chapter 1. The potential benefit of PCVs during influenza pandemics was demonstrated in a clinical trial.[80,81] Children who received a 9-valent PCV and then developed laboratory-confirmed influenza were at 45% less risk of hospitalization due to the influenza-associated pneumonia than were children who had not received the PCV.[81]

Besides bacterial pathogens such as *S. pneumoniae* and *H. influenzae*, respiratory viruses are highly prevalent in the nasopharynx of asymptomatic children.[82,83] We found that 67% nasopharyngeal samples of healthy 6- to 24-month-old children contained detectable nucleic acids from at least one virus (**Chapter 5**). Furthermore, our findings indicate specific associations among respiratory pathogens. For some of these, a large and increasing body of evidence supports causality, with data derived from observational studies, animal and in vitro models (Table 1).[84]

Virus	Bacterium	Association	Human studies	Animal studies	*In vitro* studies
Human rhinovirus	*S. pneumoniae*	+	Healthy children	NA	Nasal Airway
	H. influenzae	+	Otitis-prone	NA	Nasal Primary airway Bronchial
RSV	*H. influenzae*	+	NA	Chinchillas	Nasopharyngeal Bronchial Small airway Alveolar basal
Influenza virus	*S. pneumoniae*	+	NA	Mice Tracheal explants	Bronchial Small airway Alveolar basal
	S. aureus	+	NA	Mice	NA
Adenovirus	*M. catarrhalis*	+	Healthy children	NA	NA

Table 1: **Selection of viruses and bacteria for which interactions have been described, including source of evidence: human studies (i.e., asymptomatic children), animal studies, or in vitro studies.** Adapted from Ref. 84. NA, not available from literature.

Singleton, *et al* divided respiratory viruses in two groups, based on disease potential. Group 1 consisted of viruses associated with disease and hospitalization, such as influenza and respiratory syncytial virus (RSV).[82] In contrast, group 2 consisted of rhinovirus, adenovirus and coronavirus; their role in respiratory infections is less clear. For instance, although assumed to cause the common cold,[85] rhinoviruses were found in up to 50% of asymptomatic children in our study. Nonetheless, correlations of human rhinoviruses, enteroviruses and others with bacterial pathogens may hint toward a possible facilitating role in bacterial acquisition and colonization. This is supported by Unger and colleagues' recent demonstration that human rhinovirus infection delays clearance of NTHi.[86] The effect was mediated by modulation of innate immune responses, which, in turn, may predispose to secondary bacterial infection.[86] Epidemiological studies also suggest that associations exist between circulation of respiratory syncytial viruses, influenza viruses,[103] but also human rhinoviruses and the incidence of invasive pneumococcal disease.[87,88]

The interaction between viral and bacterial pathogens may even be more complex than previously thought. This is exemplified by recent observations that enteric viruses cover themselves with molecules from commensal gut bacteria to make a viral infection possible.[89,90] These studies highlight the interdependence between viruses, bacteria and their host.[91]

Our study attempted to elucidate further the dynamics and complexity of the nasopharyngeal microbial communities. Additionally, our data can guide future studies in unraveling these potential interaction patterns, as has been done previously. For example, results of a randomized study showed an inverse relationship between *S. pneumoniae* and *S. aureus*.[43] This has been supported by epidemiological studies assessing colonization patterns in healthy subjects.[92,93] Since then, several mechanisms underlying this observation have been described.[94-96]

The human body harbors a riche ecological niche of an extraordinary amount and variety of microbes.[97] We are only just beginning to appreciate the extent to which bacteria and viruses present in and on our bodies outnumber the total number of human cells, what their functions are and how this relates to our health.[97,98] The Human Microbiome Project (http://commonfund.nih.gov/hmp/) and MetaHIT (http://www.metahit.eu/) invest significant effort to unravel the fundamentals of microbial communities at various niches in healthy individuals,[99] with the ultimate goal of unlocking new approaches to treat and prevent human diseases.

Using alternative sampling methods to detect bacterial pathogens in children with an upper respiratory tract infection

Nasopharyngeal sampling is the standard for detecting bacterial pathogens often involved in childhood respiratory tract infections, such as *S. pneumoniae* and *H. influenzae*. This method of sampling may not always be well tolerated. Studies aiming to investigate acquisition and transmission require a frequency of sampling to which few participants (or ethical committees) would agree. It is well known that crowding constitutes a risk factor for acquisition of respiratory pathogens. Parents of children attending day care experience this first hand, as their child develops one upper respiratory tract infection after the other. This was the point of origin of our research question: in children with a runny nose caused by an upper respiratory tract infection, could bacterial pathogens be reliably detected by simply wiping the child's nose? We found that culturing a paper tissue sample collected by wiping or blowing the nose of a child, *H. influenzae*, *M. catarrhalis*, and *S. aureus* were detected at comparable frequencies as the current standards of sampling (**Chapter 6**). Interestingly, *S. pneumoniae* was even detected more often in culture of a paper tissue than in culture of a nasopharyngeal swab. Our results indicate that in symptomatic children, these bacteria can be reliably detected in a simple way that does not require specific skills, swabs or expensive transport medium. Our findings offer great opportunities for transmission modeling studies.

Rhinorrhea was a specific inclusion criterion for this study. Consequently, the generalizability of our results and, thus, applicability of this method is limited to symptomatic individuals. In asymptomatic individuals, nasopharyngeal sampling is still the gold standard. Consequently, we do not propose to use this method in vaccine trials evaluating colonization patterns, since in such settings uniformity of sampling methods is important for sustaining comparability between studies.[100]

Prevalence of nasopharyngeal *S. pneumoniae* colonization in the elderly

The incidence of CAP is high among persons 65 years of age and older. *S. pneumoniae* is the most common cause of CAP in the elderly population, but nasopharyngeal *S. pneumoniae* colonization rates as measured by conventional culture methods are reported to be low. In Chapter 7 we explored nasopharyngeal *S. pneumoniae* colonization in the elderly by taking two transnasal and two transoral swabs in each subject. In addition to conventional culture, we applied a real-time quantitative PCR assay. Three main conclusions can be derived from our study (**Chapter 7**).

First, pneumococcal colonization is largely underestimated when using conventional culture methods exclusively. While in 25 of 330 subjects (8%) *S. pneumoniae* was detected by conventional culture alone (a figure already substantially higher when compared to other reports),

detection tripled to 75 of 330 (23%) when both conventional culture and qPCR were performed. This may still even be an underestimation, since our protocol at the time did not include an enrichment step.[101] Second, results from the qPCR targeting the pneumococcal autolysin gene suggest that the density of colonizing strains in the asymptomatic community-dwelling elderly is generally low. This is in line with a recent study in South African adults.[102] This may be the reason for the low detection rates observed when only conventional culture methods are used. Third, the transoral route is superior over the transnasal route of sampling the nasopharynx in this population.
It is important to test uniformly for the presence of *S. pneumoniae* to enable comparisons between studies. Assessment of nasopharyngeal pneumococcal colonization is subject to potential confounders that include low-density and multiple-strain colonization.[101] It has been established that autolysin is a highly sensitive and specific target in detecting pneumococci and used as the sole target in a recent study.[102] Including molecular techniques such as *lytA* qPCR in colonization studies is very useful to more accurately estimate the presence of *S. pneumoniae*, particularly in low-density settings like in (older) adults.

Both children and the elderly are at high risk for IPD, but the spectrum of diseases differs. Prior to the introduction of 7vCRM in the Netherlands, IPD in young children consisted of meningitis for 50%, while bacteremic pneumonia accounted for 80% of IPD cases in the elderly.[1] In the US, in the pre-PCV era, the proportion of so-called pediatric serotypes (serogroups 6, 19 and 23) causing IPD was observed to increase with age up to 50% in the very old.[103] Prolonged *S. pneumoniae* colonization was recently demonstrated in elderly mice compared to young-adults.[104] We hypothesized that colonization may also increase at older age. Although pneumococcal colonization rates in the elderly may indeed be higher than previously thought, they are not nearly as high as in children. Therefore, other factors such as immunoscenesence (i.e., decrease of immunological function with advanced age) and a more easily breached mucosal barrier may also play a role in the increased susceptibility of the elderly for pneumococcal diseases like CAP.

The efficacy of the 23-valent pneumococcal polysaccharide vaccine against CAP has been questioned and debated.[105] Consequently, the 23-valent pneumococcal polysaccharide vaccine is not routinely used in the Dutch elderly population. However, given the substantial proportion of pediatric serotypes in IPD of the elderly and presumed immunological superiority over the polysaccharide vaccine, pneumococcal conjugate vaccination may have its merit. In 2008, a large-scale randomized control trial was initiated in the Netherlands (CAPiTA; n=85,000 ClinicalTrials.gov identifier: NCT00744263) to evaluate the efficacy of the 13-valent pneumococcal CRM197-conju-

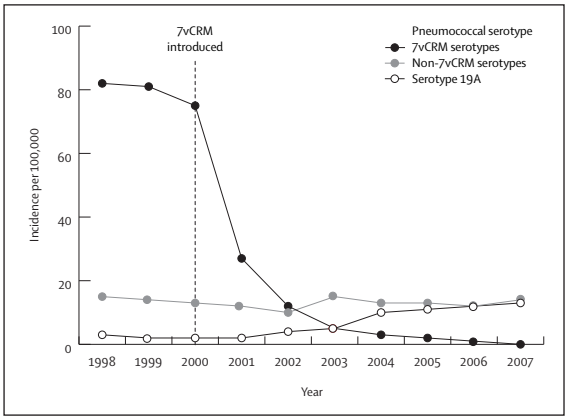

Figure 2: **Changes in invasive pneumococcal disease (IPD) incidence by serotype group among children aged <5 years.**
Adapted from Pilishvili T, et al. *J Infect Dis* 2010; 201: 32-41

gate vaccine (13vCRM; Prevnar-13®, Pfizer, Inc.) against CAP caused by vaccine pneumococcal serotypes. However, seven years after introduction of 7vCRM, herd immunity is in effect (Figure 2). In addition, now that PHiD-CV has been introduced, 13vCRM only protects against three additional serotypes (3, 6A and 19A). This raises the question of whether the elderly population needs to be vaccinated with 13vCRM when herd effects have been established.

To summarize, thorough evaluation of immune responses are important when new vaccines are introduced and coadministered with other vaccines in already crowded immunization schedules. In addition, the value of colonization studies with representative data has been established. Randomized controlled trials are pre-eminently suitable to study vaccine impact, mechanisms of action and predict herd effects. Furthermore, optimizing the methodology to assess colonization with respiratory pathogens at all ages, during illness and in health, will provide further insight into pathobiology and possibilities to prevent disease. Altogether, the studies described in this thesis have provided new insights into various aspects of nasopharyngeal bacterial colonization, but important questions remain. For example, can NTHi colonization and disease be targeted more effectively by including more than one distinct protein component of this bacterium? What are the underlying mechanisms of the associations among viruses and bacteria in the nasopharynx and how do these interactions relate to progression to disease? What will the effects on the nasopharyngeal microbiota be if future vaccines eradicate S. pnuemoniae? Some of these questions will not be answered in the near future. In the meanwhile, thorough monitoring of the effects of PCVs on immune responses, nasopharyngeal colonization (with S. pneumoniae, but also other pathogens and even commensals), and surveillance of disease remain of great importance.

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Nederlandse samenvatting

Luchtweginfecties komen vaak voor op de kinderleeftijd, variërend van een verkoudheid of een veel voorkomende middenoorontsteking (otitis media acuta (OMA)) tot een vervelende longontsteking, waarvoor zelfs een ziekenhuisopname nodig is. Longontstekingen zijn wereldwijd doodsoorzaak nummer één onder kinderen jonger dan vijf jaar oud. De Wereldgezondheidsorganisatie schat het aantal sterfgevallen op ongeveer één miljoen kinderen per jaar, voornamelijk in ontwikkelingslanden. In Westerse landen als Nederland zal een kind niet snel aan een longontsteking overlijden, dankzij goede zorg en het op tijd toedienen van antibiotica. Behalve zuigelingen en jonge kinderen met een nog lage afweer zijn ook ouderen boven de 65 jaar extra kwetsbaar voor een longontsteking. De bacteriën Streptococcus pneumoniae (pneumokok) en Haemophilus influenzae zijn de meest voorkomende verwekkers van luchtweginfecties. Soms lukt het deze bacteriën om door te dringen in de bloedbaan, dit noemt men invasieve infecties. Invasieve infecties kunnen uitmonden in ernstige ziektebeelden als bloedvergiftiging of hersenvliesontsteking, waaraan ook in Nederland kinderen kunnen overlijden of ernstige restverschijnselen houden na een infectie. Daarom is er in de laatste decennia veel onderzoek gedaan naar preventie van infecties door deze bacteriën en het ontwikkelen van vaccins. Er zijn echter nog vele onbeantwoorde vragen. Een aantal hiervan wordt in dit proefschrift beantwoord. Alvorens de bevindingen in de verschillende onderzoeken te bespreken volgt eerst een overzicht van de stand van zaken in dit onderzoeksgebied.

Van kolonisatie naar ziekte

Streptococcus pneumoniae

De mens is het natuurlijke reservoir voor de pneumokok, die hij regelmatig meedraagt in de neuskeelholte (nasopharynx). *S. pneumoniae* is een bacterie met een suikerkapsel (polysaccharide kapsel), waarvan inmiddels meer dan 94 verschillende varianten (serotypen) zijn herkend. Het kapsel beschermt de pneumokok tegen snelle opruiming door het immuunsysteem. Dankzij dit kapsel kan de pneumokok zich enige tijd in de nasopharynx nestelen. Dit komt veel voor en meestal zonder dat de gastheer er ziek van wordt. Men noemt dit ook wel asymptomatisch dragerschap of kolonisatie. Na enkele weken tot maanden wordt de bacterie opgeruimd, waarbij zowel antistoffen tegen het kapselpolysaccharide als specifieke cellen van het immuunsysteem (T cellen) een rol spelen. Kolonisatie met *S. pneumoniae* is sterk leeftijdsafhankelijk en de duur van kolonisatie neemt af met het ouder worden van een kind en het rijpen van het afweersysteem. Bij Nederlandse kinderen in hun tweede levensjaar kan met een diep afgenomen neuskeelwat bij zo'n 50-70% pneumokokkendragerschap worden aangetoond. Door de snellere klaring bij het ouder worden neemt deze kolonisatie af tot zo'n 10-20% op de volwassen leeftijd. Bij ouderen boven de 65 jaar zou pneumokokkendragerschap zeldzaam (<5%) zijn. Het is echter maar de vraag of dit werkelijk zo is, omdat een standaardkweek mogelijk niet de meest optimale manier is om bij deze populatie naar de aanwezigheid van *S. pneumoniae* te kijken.

Onder bepaalde omstandigheden kunnen pneumokokken van kolonisatie overgaan naar ziekte. Dit kan door lokale verspreiding naar het omliggende weefsel (zoals naar het middenoor via de buis van Eustachius) of naar de longen. Dit komt regelmatig voor als er bijvoorbeeld een virale infectie optreedt (verkoudheid) of iemand met weinig afweer een nieuw serotype tegenkomt. Bepaalde kapsels (serotypen) zijn sterk geassocieerd met het ontstaan van (invasieve) ziekte. Afweerstoffen tegen het kapsel beschermen tegen ziekte door het serotype met dat kapsel. Deze kapselpolysacchariden zijn daarom van oudsher het primaire doelwit geweest in de ontwikkeling van pneumokokkenvaccins.

Haemophilus influenzae

Haemophilus influenzae wordt ingedeeld in gekapselde en ongekapselde (niet-typeerbare) stammen (afgekort als NTHi). In het tijdperk vóór het beschikbaar komen van een effectief vaccin was de gekapselde *H. influenzae* type b (Hib) een gevreesde veroorzaker van hersenvliesontsteking, epiglottitis en longontsteking op de kinderleeftijd. NTHi veroorzaakt daarentegen slechts zelden invasieve ziekten bij gezonde kinderen en volwassenen, maar is wel een belangrijke oorzaak van luchtweginfecties als oorontsteking of bronchitis. Bij kinderen met recidiverende middenoorontstekingen wordt NTHi vaak als verwekker aangetroffen. Net als bij *S. pneumoniae* koloniseert NTHi frequent de nasopharynx, vooral bij jonge kinderen. Er zijn verschillende eiwitten van NTHi, die als kandidaten voor vaccinatie tegen ziekte door NTHi zijn overwogen. Eén daarvan is proteïne D (PD), een eiwit dat vrijwel alle *H. influenzae* stammen tot expressie brengen en waarvan in diermodellen is aangetoond dat anti-PD antistoffen beschermen tegen ziekte.

Microbiële interacties

Luchtweginfecties en otitis media acuta zijn meestal polymicrobiële ziekten. Dit betekent dat verscheidene micro-organismen, zowel virussen als bacteriën, in combinatie, kunnen leiden tot ziekte. Interacties tussen virussen en koloniserende bacteriën zijn van belang in het ontstaan van luchtweginfecties en invasieve ziekten. Uit observationele studies is al geruime tijd bekend dat een virale bovenste luchtweginfectie een voorbode kan zijn op een bacteriële OMA. De interactie die het best is bestudeerd is die tussen het griepvirus (influenza) en de pneumokok. Tijdens de grote Spaanse grieppandemie van 1918 bleek dat een aanzienlijk deel van de geschatte 20 tot 50 miljoen sterfgevallen het gevolg was van een longontsteking één tot twee weken na de start van de griep door het optreden van een secundaire bacteriële infectie, met name door pneumokokken. Hoe deze interacties tussen bepaalde virussen en bacteriën in de nasopharynx plaatsvindt is vaak nog niet geheel duidelijk. Kennis op dit gebied is van belang om uiteindelijk beter inzicht te krijgen in de gezonde situatie en hoe ziekten kunnen worden voorkomen.

Pneumokokkenconjugaatvaccins

Directe effecten op ziekte en kolonisatie

Kapselpolysacchariden wekken een slechte afweerreactie op bij kinderen onder de twee jaar en induceren geen immunologisch geheugen. Hierdoor zijn ze niet geschikt voor vaccinatie van de groep met het hoogste infectierisico. Als polysacchariden echter worden gekoppeld (geconjugeerd) aan een eiwit, vindt wel een goede afweerreactie tegen het eiwit én het polysaccharide plaats en wordt immunologisch geheugen opgebouwd, zelfs al op de zuigelingenleeftijd. Pneumokokkenconjugaatvaccins (PCVs) zijn zeer effectief in het induceren van beschermende antistoffen tegen het kapsel en het voorkómen van infecties die worden veroorzaakt door de serotypen die in het vaccin zitten (zogenaamde vaccintypen). PCVs voorkómen tussen de 77 en 100% van de invasieve ziekten veroorzaakt door vaccintypen, zo'n 20 tot 40% van de longontstekingen (gediagnosticeerd met behulp een röntgenfoto) en 50 tot 60% van de middenoorontstekingen door vaccintypen. Ook kolonisatie van de nasopharynx met vaccintypen neemt af met zo'n 40 tot 60% in gevaccineerde kinderen. Het eerste PCV dat in 2000 in de Verenigde Staten op de markt werd gebracht bevatte polysacchariden van zeven serotypen (4, 6B, 9V, 14, 18C, 19F en 23F), elk geconjugeerd aan cross-reactive material 197

(CRM197, een gemuteerd difterietoxine), 7vCRM (Prevenar™/Prevnar™, Pfizer, Inc.). Vervolgens werd een tweede conjugaatvaccin ontwikkeld waarbij het dragereiwit proteïne D was, zoals gezegd afkomstig van de bacterie *H. influenzae*.

Er werd een opvallend verschil gezien tussen de twee vaccins in onderzoeken naar het effect van vaccinatie op het voorkomen van middenoorontsteking. De Finnish Otitis Media trial (FinOM) vond dat 7vCRM 57% van de OMA gevallen die werden veroorzaakt door de 7 vaccintypen, maar over het geheel genomen namen middenoorontstekingen met slechts 6% af, doordat niet-vacccintypen vaker een oorontsteking veroorzaakten en ook oorontstekingen door H. influenzae leken toe te nemen. De Pneumococcal Otitis Efficacy Trial (POET) bestudeerde een 11-valent PCV (GlaxoSmithKline Vaccines), dat de additionele serotypen 1, 3, 5 en 7F naast de 7 typen van het andere vaccin bevatte en waarin elk polysaccharide was geconjugeerd aan PD. In POET werd eenzelfde afname gevonden van OMA veroorzaakt door vaccintypen (58%), maar men vond ook dat otitis door *H. influenzae* afnam en nieuwe niet-vaccin typen in deze studie niet opkwamen. Dit gaf een nettto reductie van 34% van alle oorontstekingen bij kinderen die waren gevaccineerd met dit 11-valente vaccin ten opzichte van de ongevaccineerde controle kinderen. De beide studies verschilden in selectie van patiënten en procedures. Het gemeten effect van vaccinatie op dragerschap van *H. influenzae* in de neus en keel was echter onduidelijk.

Indirecte effecten en typevervanging

Door de effecten van PCVs op nasopharyngeale kolonisatie wordt de transmissie van vaccintypen effectief onderbroken. Hierdoor worden ook niet-gevaccineerde personen van alle leeftijden in de samenleving beschermd, zogenaamde herd immunity of kudde-immuniteit. Belangrijk hierin was ook de afname van invasieve pneumokokkenziekte bij ouderen boven de 65 jaar. Dit indirecte effect van 7vCRM bleek zo omvangrijk, dat in de Verenigde Staten in 2005 twee derde van de afname van invasieve pneumokokkenziekte na invoering van het vaccin hieraan werd toegeschreven. Dit heeft een grote invloed gehad op de kosteneffectiviteit schattingen en het invoeren van 7vCRM in Nederlandse Rijksvaccinatieprogramma in 2006.

Een nadeel van PCVs die bescherming bieden tegen een beperkt aantal van alle serotypen is typevervanging, waarbij niet-vaccintypen de plaats innemen van de vaccintypen. Deze typevervanging is het meest uitgesproken op neus-keel niveau, maar geeft ook een afname van de effectiviteit van het vaccin op oorontstekingen. Bij invasieve ziekten treedt typevervanging ook op, maar vooralsnog zijn de vervangende typen minder vaak invasief en is er een netto daling van invasieve ziekte bij kinderen na vaccinatie met 7vCRM van 60%. Een paar vervangende serotypen vallen op. Het serotype dat het sterkst toenam was serotype 19A en dit gebeurde al snel na invoering van 7vCRM. In een gerandomiseerde studie in Nederland werd nasopharyngeale kolonisatie met 19A significant vaker gezien bij kinderen die gevaccineerd waren met 7vCRM dan bij de ongevaccineerde controlegroep, zelfs na vaccinatie met minder dan vier doses.

Studies naar de effecten van pneumokokkenvaccins op de nasopharyngeale kolonisatie zijn makkelijk uitvoerbaar. Dit soort onderzoek geeft zeer waardevolle informatie: (1) directe effecten op vaccin- en niet-vaccintypen (maar ook andere bacteriën in de nasopharynx), (2) voorspelling van kudde-immuniteit. Het PneumoCarr consortium heeft zich daarom ingezet om effecten op nasopharyngeale kolonisatie als additioneel criterium op te nemen bij de

registratie van nieuwe pneumokokkenvaccins, ook al omdat toekomstige vaccins mogelijk niet (uitsluitend) antistofafhankelijk en serotype-specifiek werken waardoor de huidige immunologische criteria (zie hieronder) niet meer van toepassing zijn.

Bemonsteringsmethoden

Een werkgroep van de Wereldgezondheidsorganisatie heeft een richtlijn opgesteld voor het bemonsteren van de nasopharynx met het doel pneumokokkenvaccinstudies met elkaar te kunnen vergelijken. Onderzoek zou zeker gemakkelijk uit te voeren zijn als snot een goede weerspiegeling zou zijn van de nasopharynx. Uit één van onze onderzoeken blijkt inderdaad dat bij kinderen met een snotneus pneumokokken ook uit slijm afgenomen met een zakdoek gekweekt konden worden. Deze methode is dus toepasbaar bij verkoudheden.

Licentie van nieuwe vaccins op basis van de immuunrespons

Het wordt steeds moeilijker om grootschalige effectiviteitsstudies op te zetten voor nieuwe PCVs. Behalve dat er een cohort van meerdere tienduizenden deelnemers nodig is (invasieve pneumokkenziekte is relatief zeldzaam), hebben steeds meer landen al een PCV ingevoerd. Voor het registreren van een nieuw PCV zijn daarom immunologische criteria opgesteld. Het kandidaatvaccin moet een vergelijkbare immuunrespons teweegbrengen als een geregistreerd product in de zin dat eenzelfde percentage kinderen een beschermende antistoftiter bereikt met het nieuwe vaccin als met het geregistreerde product. Een gemiddelde concentratie van 0.35 µg/ml gemeten met een standaard enzyme-linked immunosorbant assay (ELISA) of 0.20 µg/ml gemeten met een meer specifieke derde-generatie ELISA wordt internationaal als maatstaf genomen voor bescherming tegen invasieve ziekte.

Een belangrijk aspect is dat PCVs vrijwel altijd tegelijkertijd met andere kindervaccins worden toegediend. In Nederland werd 7vCRM in 2006 ingevoerd en op 2, 3, 4 en 11 maanden gelijktijdig toegediend met DKTP-Hib (Pediacel™, Sanofi Pasteur MSD). Uit onderzoek is duidelijk geworden dat als vaccins gelijktijdig worden toegediend, de afweerreactie tegen vaccincomponenten zowel in positieve als negatieve zin beïnvloed kan worden. De uiteindelijke effecten van gelijktijdige toediening van twee vaccins (versterkte of verminderde immuunrespons) blijkt echter complex en slecht te voorspellen. Het is dus van belang om de immuunrespons voorafgaand aan invoering van een nieuw PCV in een bestaand vaccinatieprogramma goed te onderzoeken. Hetzelfde geldt voor de veiligheid en bijwerkingen van de gelijktijdig toegediende vaccins.

Synflorix, een tweede-generatie pneumokokkenconjugaatvaccin

Het 10-valente pneumokokken niet-typeerbare *Haemophilus influenzae* proteïne D-conjugaat vaccin (PHiD-CV; Synflorix™, GlaxoSmithKline Vaccines) werd in 2009 geregistreerd en is gebaseerd op het niet-geregistreerde 11-valente vaccin dat werd onderzocht in POET. In PHiD-CV zijn 8 van de 10 serotypen geconjugeerd aan PD. Serotype 18C en 19F zijn gekoppeld aan respectievelijk tetanus-toxoïd en difterietoxoïd. Naast de effectiviteit tegen OMA veroorzaakt door NTHi suggereerde de POET-studie ook een tijdelijk, maar beperkt effect op nasopharyngeale NTHi kolonisatie.

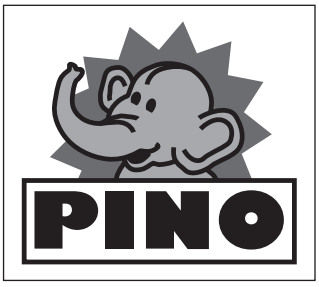
Voor het 10-valente PHiD-CV werden geen consistente effecten op NTHi kolonisatie gezien. Een duidelijk effect op NTHi kolonisatie zou niet alleen het resultaat van POET bevestigen en verklaren, het zou ook kudde-immuniteit op NTHi voorspellen.

Behalve een mogelijk effect op NTHi biedt PHiD-CV het potentiële voordeel van het voorkómen van immunologische interferentie, omdat PD niet in andere vaccins als dragereiwit wordt gebruikt. PHiD-CV werd al eerder werkzaam en veilig bevonden bij gelijktijdige toediening met verschillende andere vaccins, waaronder DKTP-Hib-HepB (Infanrix hexa™, GlaxoSmithKline Vaccines). De combinatie met DKTP-Hib zoals gebruikt in Nederland, werd niet eerder onderzocht.

Dit proefschrift beoogt verschillende vragen te beantwoorden met betrekking tot de hierboven genoemde onderwerpen:

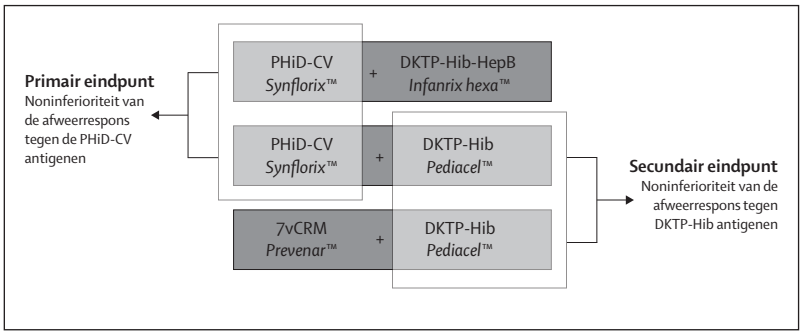
1. Wat is de immuunrespons tegen PHiD-CV en DKTP-Hib in vergelijking met andere combinaties van vaccins; is er sprake van immunologische interferentie?
2. Wat is het effect van PHiD-CV op nasopharyngeale bacteriële kolonisatie, in het bijzonder op NTHi?
3. Zijn er associaties tussen virale en bacteriële pathogenen in de neuskeelholte van gezonde kinderen?
4. Kunnen bacteriële pathogenen betrouwbaar gedetecteerd worden in slijm dat is eenvoudig is verzameld met een zakdoek bij kinderen met een bovenste luchtweginfectie?
5. Wat is de prevalentie van *S. pneumoniae* in de neuskeelholte bij 65-plussers, gemeten met conventionele kweek en moleculaire technieken?

In **Hoofdstuk 2** (primaire serie) en **Hoofdstuk 3** (boosterfase) worden de resultaten beschreven van het Pneumokokken Immunologie en NeusOnderzoek (PINO, figuur 1). Voor dit onderzoek werden 780 gezonde zuigelingen geïncludeerd en vanaf de leeftijd van twee maanden vervolgd tot de leeftijd van twee jaar. Figuur 2 geeft schematisch de drie studiearmen (grijze blokken) en de primaire en secundaire immunologische eindpunten (witte kaders) weer. Er was sprake van noninferioriteit als de bovengrens van het 95% betrouwbaarheidsinterval van de ratio van de antistoftiters tussen de groepen kleiner was dan twee. Het primaire eindpunt was de antistoftiter tegen de PHiD-CV antigenen wanneer PHiD-CV gelijktijdig met DKTP-Hib werd gegeven in vergelijking met gelijktijdige toediening met DKTP-Hib-HepB in serum één maand na de derde primaire dosis (Figuur 2).



Figuur 1: **Logo van het Pneumokokken Immunologie en NeusOnderzoek (PINO).**

Behalve anti-pneumokokken antistoffen werd ook de serotype-specifieke opsonophagocytische activiteit (OPA) van de antilichamen gemeten. In vitro OPA is een meer functionele uitleesmaat en opsonophagocytose wordt verondersteld het primaire afweermechanisme tegen pneumokokkeninfecties te zijn. Ook werd de afweerrespons tegen de DKTP-Hib antigenen geëvalueerd evenals de veiligheid en bijwerkingen van de verschillende combinaties van vaccins.



***Figuur 2*: Studiearmen en immunologische eindpunten.**

Eén maand na de primaire serie bleven de antistoftiters onder de limiet voor noninferioriteit voor alle PHiD-CV antigenen, met uitzondering van serotype 18C. Voor 18C (geconjugeerd aan tetanus-toxoïd) werd de vooraf gedefinieerde grens marginaal (2.03) overschreden. Dit kan in verband gebracht worden met de hogere anti-tetanus antistoftiter in de groep waarbij PHiD-CV samen met DKTP-Hib-HepB toegediend werd. Het percentage kinderen dat de drempel van 0.20 µg/ml haalde was vergelijkbaar tussen de PHiD-CV groepen, ook voor 18C. Hetzelfde geldt voor het percentage kinderen met functionele OPA titers. Daarom verwachtten we dat het verschil in antistoftiter tussen de groepen klinisch niet relevant zal zijn. Na de primaire serie en na de booster dosis bleef de afweerrespons tegen de DKTP-Hib antigenen binnen de vooraf gedefinieerde marge voor noninferioriteit. Toch werden tussen de groepen significante verschillen gemeten in de hoogten van de antilichamen tegen tetanus, Hib en difterie. Zo was bijvoorbeeld de anti-PRP (Hib) respons hoger in de groep waarbij DKTP-Hib samen met PHiD-CV werd gegeven in vergelijking met gelijktijdige toediening met 7vCRM. Ook dit kan gerelateerd worden aan de versterkte respons door tetanus en tetanus-toxoïd (dragereiwit voor zowel PRP-Hib en 18C). Desondanks waren in alle groepen de antistoftiters tegen deze antigenen ruim boven de drempel die is geassocieerd met bescherming tegen ziekte. Ook hierbij verwachtten we daarom dat de klinische relevantie van de verschillen in antistoftiters beperkt zal zijn.

Wat de respons tegen pneumokokken betreft, hebben we ook het persisteren van (functionele) antilichamen op de langere termijn bestudeert. Op tweejarige leeftijd (ongeveer een jaar na de booster dosis) waren de antistoffen weer gedaald tot ongeveer het niveau van net vóór de booster, hoewel er tussen serotypen en groepen wel verschillen werden gezien. Deze relatief snelle daling kan verklaard worden doordat vaccintypen al nauwelijks meer circuleerden (zie Hoofdstuk 4). Natuurlijke stimulering van het immuunsysteem door nasopharyngeale kolonisatie ontbreekt in de nieuwe situatie en dat beïnvloedt de sero-epidemiologie.

In **Hoofdstuk 4** beschrijven we de effecten van PHiD-CV op nasopharyngeale bacteriële kolonisatie in vergelijking met 7vCRM. Hierbij is door middel van conventionele kweek, typeringen en moleculaire technieken gedetailleerd gekeken naar de effecten op NTHi. Kolonisatie met NTHi (gemeten met conventionele kweek) nam toe met de leeftijd van rond de 33% op de leeftijd van 5 maanden tot 65% op tweejarige leeftijd. Deze getallen nemen toe op basis van moleculaire detectie: NTHi werd aangetroffen in de nasopharynx van 44% van de 5 maanden oude kinderen en in zo'n 80% van de 24 maanden oude kinderen. Vergeleken met de 7vCRM

groep was er op geen enkel moment een lagere NTHi kolonisatiegraad in de PHiD-CV groep. Ook de densiteit van NTHi in de nasopharynx was niet verschillend tussen de vaccingroepen. Op basis van ons onderzoek kan geconcludeerd worden dat kudde-immuniteit op NTHi na invoering van PHiD-CV in het Rijksvaccinatieprogramma niet mag worden verwacht. De vraag doet zich voor hoe een PD-conjugaatvaccin wel bescherming tegen OMA kan geven, zonder enig effect te hebben op nasopharyngeale kolonisatie. Hiervoor is nader onderzoek nodig en zal ook gewacht moeten worden op resultaten van gerandomiseerde studies naar de effecten van PHiD-CV op OMA.

Behalve NTHi werd tevens het dragerschap van pneumokokken bestudeerd, met een bijzondere interesse voor serotype 19A. Voor dit laatste doel werd dan ook een polymerase-kettingreactie (polymerase chain reaction (PCR)) ontwikkeld specifiek voor 19A. Pneumokokkendragerschap (gemeten met conventionele kweek) nam in onze studie toe met de leeftijd van 39% op de leeftijd van 5 maanden tot 57% in het tweede levensjaar. Op basis van moleculaire technieken was dat 50-60% en rond de 80%, respectievelijk. Er waren geen grote verschillen tussen de groepen wat betreft de verdeling vaccintypen en niet-vaccintypen. Dit hadden we ook niet verwacht, omdat ons onderzoek ongeveer twee jaar na invoering van 7vCRM in het Rijksvaccinatieprogramma werd gestart. Kudde-immuniteit was toen in ontwikkeling en de zeven vaccintypen van 7vCRM circuleerden al veel minder dan voorheen. Serotype 19A bleek het meest voorkomende serotype te zijn in de nasopharynx, zowel op basis van conventionele kweek (variërend van 6 tot 11% van de kinderen) als op basis van de serotype-specifieke PCR (20%), ongeacht welk vaccin de kinderen hadden gekregen. Dit percentage bleef stabiel tijdens follow-up. PHiD-CV lijkt nasopharyngeale kolonisatie met 19A niet terug te dringen. De vraag is of PHiD-CV wel invasieve ziekte door 19A voorkómt, aangezien hiervoor in het algemeen een (veel) lagere antistoftiter nodig is dan om kolonisatiereductie teweeg te brengen.

In **Hoofdstuk 5** wordt een studie beschreven van ongeveer 1000 neus-keelmonsters waarin de aanwezigheid van zo'n 20 respiratoire virussen (door middel van PCR) en de vier belangrijkste bacteriële pathogenen (*S. pneumoniae, H. influenzae, Morexella catarrhalis* en *Staphylococcus aureus*) werd bepaald. We hebben onderzocht of er een samenhang bestond tussen de onderlinge aanwezigheid van deze virussen en bacteriën. De monsters waren afkomstig van kinderen tussen de 6 en 24 maanden oud uit een eerder afgerond gerandomiseerd pneumokokkenvaccinonderzoek. Uit de onderzoeksresultaten kunnen een aantal conclusies worden getrokken. Ten eerste zijn virussen vaak terug te vinden in de bovenste luchtwegen van gezonde, asymptomatische kinderen. Bij gemiddeld twee op de drie de kinderen was ten minste één virus aanwezig en bij ongeveer één op de drie was meer dan één virus aanwezig. Het meest voorkomende virus was rhinovirus (een verkoudheidsvirus), gemiddeld aanwezig bij 38%. Ten tweede kwam in het algemeen kolonisatie met bacteriën vaker voor in aanwezigheid van respiratoire virussen. Er werden duidelijke associaties gevonden tussen bacteriën onderling, tussen de aanwezigheid van virussen en bacteriën en tussen virussen onderling. Hoewel deze studie geen oorzakelijk verband of richting van de associatie aangeeft, is er inmiddels substantieel ondersteunend bewijs voor enkele van de gevonden associaties. Het is uit eerder onderzoek bijvoorbeeld bekend dat rhinovirus de aanhechting van pneumokokken stimuleert door veranderingen van het slijmvlies in de luchtwegen. Uit recent onderzoek bleek ook dat na een infectie met rhinovirus klaring van *H. influenzae* vertraagd wordt. Deze bevindingen suggereren dat veelvoorkomende virussen een subtiele, maar belangrijke rol kunnen spelen in het ontstaan van luchtweginfecties. Er is aanvullend onderzoek nodig om de mogelijke onderliggende mechanismen voor de associaties te ontrafelen.

Een onderzoek naar alternatieve bemonsteringsmethoden voor de detectie van veelvoorkomende bacteriën wordt beschreven in **Hoofdstuk 6**. De standaardmethode om bacteriën op te sporen is een diepe neuskeelwat voor *S. pneumoniae*, *H. influenzae* en *M. catarrhalis*, en een ondiepe neuswat voor *S. aureus*. Als kinderen verkouden zijn verzamelt slijm zich in de bovenste luchtwegen voordat het wordt uitgestoten. Hierdoor is het theoretisch mogelijk dat in dit slijm bacteriën terug te vinden zijn die zich normaliter op verschillende plekken in de bovenste luchtwegen bevinden. In deze studie werd gekeken naar de mate van overeenkomst tussen kweekresultaten van een diepe neuskeelwat, een ondiepe neuswat en een directe kweek van een zakdoek bij 66 verkouden kinderen tussen de 0 en 4 jaar. Voor dit laatste werd slijm opgevangen door het verkouden kind simpelweg zijn of haar neus te laten snuiten of een snottebel af te vegen. *H. influenzae*, *M. catarrhalis* en *S.aureus* werden vrijwel net zo vaak in een zakdoek teruggevonden als via de standaardmethode. *S. pneumoniae* werd zelfs vaker gekweekt direct uit een zakdoek dan uit een neuskeelwat. De onderzoeksresultaten zijn veelbelovend voor studies naar bijvoorbeeld transmissie van bacteriën, waarbij veelvuldig monsters verzameld moeten worden. Deze eenvoudige methode is toepasbaar bij verkouden kinderen.

In **Hoofdstuk 7** bespreken we een onderzoek naar de prevalentie van *S. pneumoniae* in de nasopharynx bij 330 asymptomatische, zelfstandig wonende personen van 65 jaar en ouder. Bij ouderen wordt de ziektelast met name veroorzaakt door longontstekingen. In tegenstelling tot kinderen lijkt kolonisatie met *S. pneumoniae* maar weinig voor te komen bij ouderen (<5%). De meeste onderzoeken hebben echter alleen conventionele kweekmethoden gebruikt om pneumokokkendragerschap vast te stellen. In ons onderzoek namen we twee transnasale en twee transorale nasopharyngeale monsters af. De eerste van ieder werd op kweek gezet, terwijl de tweede met PCR werd onderzocht. Op basis van kweekresultaten werden 25 (8%) ouderen drager van pneumokokken. Kwantitatieve PCR detecteerde *S. pneumoniae* bij 71 (22%) van de ouderen. Bovendien bleek dat in vergelijking met kweek pneumokokken met behulp van PCR vooral werden gedetecteerd in transoraal verkregen nasopharyngeale monsters. Kortom, conventionele kweek onderschat bij ouderen het pneumokokkendragerschap in vergelijking met moleculaire detectietechnieken. Een openstaande vraag is hoe bij ouderen kolonisatie met *S. pneumoniae* zich verhoudt tot het uiteindelijk ontstaan van ziekte. In **Hoofdstuk 8** worden tenslotte de bevindingen bediscussieerd en in een breder kader geplaatst.

Samengevat is grondige evaluatie van de immuunrespons van vaccins die gelijktijdig worden toegediend belangrijk, zowel voorafgaand aan invoering van een nieuw PCV in een bestaand programma als op de lange-termijn door veranderde sero-epidemiologie ten tijde van kudde-immuniteit. Daarnaast wordt door ons onderzoek de waarde van representatieve dragerschapsstudies nogmaals onderstreept; gerandomiseerde, gecontroleerde studies zijn bij uitstek geschikt om de effecten en mechanismen van vaccins te bestuderen en om kudde-immuniteit te voorspellen. Bovendien zal het optimaliseren van de methodologie om kolonisatie met respiratoire pathogenen op verschillende leeftijden vast te stellen meer inzicht geven in de normale situatie, hoe progressie tot ziekte plaatsvindt en op welke manier nog betere ziektepreventie bereikt kan worden. Dit laatste zal nog veel onderzoek en vergen. In de tussentijd is het uitermate belangrijk om de effecten van PCVs op afweerresponsen, nasopharyngeale kolonisatie (met pneumokokken, maar ook andere pathogenen) en (invasieve) pneumokokkenziekte nauwgezet te blijven bestuderen.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Dankwoord

Na vijf jaar onderzoek is mijn proefschrift een feit! En dus is het de hoogste tijd voor het meest gelezen stuk tekst: het dankwoord. Het schrijven van wetenschappelijke artikelen is de afgelopen jaren al een grote uitdaging gebleken. Nu volgt mogelijk het moeilijkste deel van het proefschrift, want hoe zet je je dankbaarheid op papier? Alvorens een aantal mensen persoonlijk te bedanken, wil ik vooral de kinderen, hun ouders en de senioren bedanken die aan de verschillende onderzoeken hebben meegewerkt. Zonder jullie geen klinisch onderzoek! De betrokkenheid van de deelnemers was ongekend en hartverwarmend. Dit blijkt wel uit de teksten die ouders op de anonieme enquêtes plaatsten. De huisbezoeken waren vrijwel zonder uitzondering een feest. Voor sommige ouders van deelnemende kinderen voelde het zelfs alsof we op visite kwamen. En oh ja, daar hoorde dat neuswatje en die bloedafname ook nog bij. Eerlijk is eerlijk, zo voelde dat voor mij ook weleens. Enorm bedankt voor jullie bijdrage! Een speciaal dankwoord voor de families Hoekstra (Gwen), Hanique (Stef), van Harlingen (Daimy) en Hultzer (Olaf). Dank voor jullie deelname, gastvrijheid en de bereidheid om foto's van jullie kinderen in dit proefschrift te mogen opnemen.

Aan de onderzoeken en de totstandkoming van dit proefschrift hebben velen bijgedragen en zonder hen zou dit proefschrift niet zijn geworden tot wat het nu is. Een aantal mensen wil ik graag op deze plek in het bijzonder bedanken.

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Het wetenschapsbureau van het Linnaeusinstituut, Spaarne Ziekenhuis Hoofddorp. Het onderzoeksteam is door de jaren heen een prachtig voorbeeld geweest (en gebleven) hoe klantvriendelijkheid zijn uitwerking niet mist. Het gevoel van saamhorigheid, dat we als één team de klussen gingen klaren, was fantastisch! Het wetenschapsbureau is steeds verder geprofessionaliseerd en ik ben er trots op hier deel van te hebben uitgemaakt. Het onderzoeksteam voelde als een tweede familie en het "PINO kantoor" als een tweede huiskamer.

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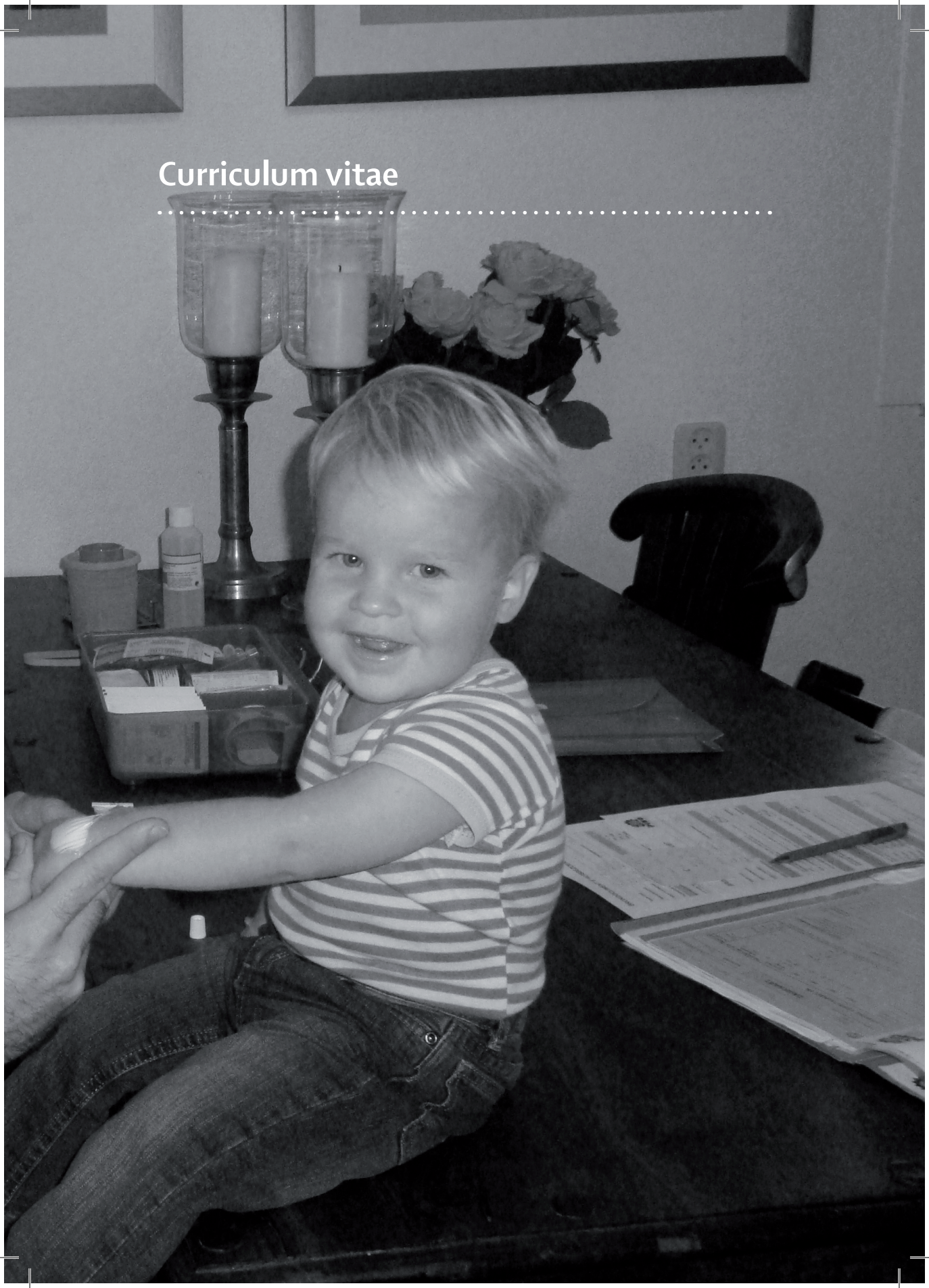
Curriculum vitae



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Menno Ruben van den Bergh was born on November 16, 1979 in Hoorn, the Netherlands. He graduated from secondary school (Atheneum) at the Atlas College, locatie Copernicus in Hoorn in 1997. From 1997 to 2000, he studied Medical Biology at the VU University in Amsterdam. In 2000, he switched to Medicine, the study of his first choice. During his medical training, he worked as a teaching assistant for several anatomy courses and supervised clinical problem-solving classes. After graduating his doctoral exam and obtaining his medical degree with honor (*cum laude*) in 2007, he worked as a research physician at the Spaarne Hospital Hoofddorp. In 2008, he started his PhD program and initiated with colleagues the PINO study, a collaboration project between GlaxoSmithKline (study sponsor), the University Medical Center Utrecht (Wilhelmina Children's Hospital) and the Spaarne Hospital Hoofddorp (Linnaeus Institute), under supervision of prof.dr. E.A.M. Sanders and dr. R.H. Veenhoven. The results of this study are described in this thesis. In 2012, he worked as a coordinator of clinical studies conducted at the Linnaeus Institute of the Spaarne Hospital Hoofddorp. In the same year, he was asked to train pediatric residents in Jakarta, Indonesia in how to obtain nasopharyngeal samples. Currently, he works as a pediatric resident (ANIOS) at the Wilhelmina Children's Hospital in Utrecht. He lives together with Barbara Hooghiemstra in Assendelft and they have two sons, Daniël and Robin.

Publications in this thesis

1. van den Bergh MR, et al. Immunogenicity, safety and reactogenicity of the 10-valent pneumococcal nontypeable Haemophilus influenzae protein D-conjugate vaccine and DTPa-IPV-Hib when coadministered as a 3-dose primary vaccination schedule in the Netherlands: a randomized controlled trial. *Pediatr Infect Dis J* 2011; 30: e170-179
2. van den Bergh MR, et al. Immunogenicity, safety, and reactogenicity of a booster dose of the 10-valent pneumococcal nontypeable *Haemophilus influenzae* protein D-conjugate vaccine (PHiD-CV) coadministered with DTPa-IPV-Hib in Dutch children. *Manuscript in preparation*
3. van den Bergh MR, et al. Effects of the 10-valent pneumococcal nontypeable Haemophilus influenzae protein D-conjugate vaccine (PHiD-CV) on nasopharyngeal bacterial colonization in young children: a randomized controlled trial. *Clin Infect Dis* 2013; 56: e30-39
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5. van den Bergh MR, et al. Associations between pathogens in the upper respiratory tract of young children: interplay between viruses and bacteria. *PLoS ONE* 2012; 7: e47711
6. van den Bergh MR, et al. *S. pneumoniae* colonization in asymptomatic elderly, the Netherlands. *Manuscript submitted*

Other publications

7. van den Bergh MR, et al. Pyomyositis: a limping diagnosis. *Eur J Pediatr* 2006; 166: 259-261
8. van den Bergh MR, et al. Radiofrequente ablatie: mogelijkheden en valkuilen van een lokale behandelmethode voor levertumoren. *Ned Tijdschr Oncol* 2005: 29-33