

# Nasopharyngeal Colonization Elicits Antibody Responses to Staphylococcal and Pneumococcal Proteins That Are Not Associated with a Reduced Risk of Subsequent Carriage

Sabine M. P. J. Prevaes,<sup>a,c</sup> Willem J. B. van Wamel,<sup>b</sup> Corné P. de Vogel,<sup>b</sup> Reinier H. Veenhoven,<sup>c</sup> Elske J. M. van Gils,<sup>a,c</sup> Alex van Belkum,<sup>b,d</sup> Elisabeth A. M. Sanders,<sup>a</sup> and Debby Bogaert<sup>a</sup>

Department of Pediatric Immunology and Infectious Diseases, Wilhelmina Children Hospital, University Medical Center, Utrecht,<sup>a</sup> Department of Medical Microbiology and Infectious Diseases, Erasmus MC, Rotterdam,<sup>b</sup> and Research Center Linnaeus Institute, Hoofddorp,<sup>c</sup> The Netherlands, and BioMérieux, Route de Port Michaud, La Balme-Les-Grottes, France<sup>d</sup>

**Knowledge of the immunological correlates of *Staphylococcus aureus* and *Streptococcus pneumoniae* colonization is required for the search for future protein vaccines. We evaluated natural antibody levels against pneumococcal and staphylococcal proteins in relation to previous bacterial colonization with both pathogens. In a randomized controlled trial, nasopharyngeal samples were obtained from children at 1.5, 6, 12, 18, and 24 months and cultured for *S. aureus* and *S. pneumoniae*. Approximately 50% of the children were PCV7 vaccinated. Serum IgG against 18 pneumococcal and 40 staphylococcal proteins was semiquantified by Luminex technology from 111 12 month olds and 158 24 month olds. Previous culture-proven *S. aureus* colonization was associated with higher IgG levels against 6/40 staphylococcal proteins (ClfB, ClfA, Efb, CHIPS, LukD, and LukF [ $P \leq 0.001$ ]) compared to noncarriers. Previous pneumococcal colonization was associated with increased IgG levels against 12/18 pneumococcal proteins compared to noncarriers ( $P \leq 0.003$ ). Increasing age was associated with higher levels of antibodies to most pneumococcal proteins and lower levels of antibodies to over half the staphylococcal proteins, reflecting natural colonization dynamics. Anti-*S. pneumoniae* and anti-*S. aureus* protein antibodies at the age of 12 months were not negatively correlated with subsequent colonization with the homologous species in the following year and did not differ between PCV7-vaccinated and nonvaccinated children. Colonization with *S. aureus* and *S. pneumoniae* induces serum IgG against many proteins, predominantly proteins with immune-modulating functions, irrespective of PCV7 vaccination. None of them appeared to be protective against new acquisition with both pathogens, possibly due to the polymorphic nature of those proteins in the circulating bacterial population.**

*Streptococcus pneumoniae* and *Staphylococcus aureus* are both important causes of bacterial infections in children in the first years of life (14, 21). Nasopharyngeal colonization is a prerequisite for the development of diseases. Preventive strategies may aim for protection against acquisition and colonization or subsequent infection. Current pneumococcal conjugate vaccines are based on the capsule as antigen and therefore restricted to the present epidemiologically predominant capsular serotypes in children (11). Although the 7-valent pneumococcal conjugate vaccine (PCV7) has been shown to effectively eradicate the vaccine serotype pneumococci from the nasopharynx, the vacant niche is immediately filled by nonvaccine pneumococci, which may also cause disease (7, 35). For this reason, there has been growing interest in vaccines against common and conserved pneumococcal proteins, since they may target all pneumococcal strains irrespective of capsule (32). In mice, immunization with pneumococcal proteins, such as the pneumococcal histidine triad (Pht) proteins, as well as PspA, PdbD, PmpA, CbpA (PspC), and PsaA, has been proven effective against pneumococcal colonization or invasive disease (3, 11, 25, 26, 32).

Comparable strategies are needed to prevent *S. aureus* colonization and infection, where multidrug (methicillin) resistance has become a serious problem (19). Several *S. aureus* proteins, e.g., clumping factor A (ClfA), clumping factor B (ClfB), and IsdB, have been shown to protect against colonization and invasive disease in mice (13, 24, 39).

To predict which proteins might be of special interest to prevent disease in humans, we investigated the dynamics, immunogenicity, and (cross)protectiveness of virulence proteins of both species in relation to nasopharyngeal colonization. In a randomized controlled trial (RCT) setting, we determined IgG levels against 18 pneumococcal and 40 *S. aureus* virulence factors, which included 21 newly tested *S. aureus* proteins.

These antibody levels were all examined in relation to nasopharyngeal colonization and PCV7 vaccination by Luminex multiplex technology.

## MATERIALS AND METHODS

**Design, sample collection, and processing.** Between July 2005 and February 2006, before nationwide implementation of PCV7, 1,003 infants were enrolled in a randomized controlled trial, investigating the effects of reduced-dose PCV7 schedules on pneumococcal colonization during the

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Address correspondence to Debby Bogaert, d.bogaert@umcutrecht.nl.

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**TABLE 1** Baseline characteristics of participants receiving 2 + 1 doses (PCV7<sup>+</sup>) or no PCV7 vaccinations (controls)

Parameter	No. (%)		
	All children	PCV7 <sup>+</sup>	Controls
Total	207	116 (56)	91 (44)
Male gender	107 (52)	60 (56)	47 (44)
Serum samples ( <i>n</i> = 269)			
12 mo olds	111	82 (74)	29 (26)
24 mo olds	158	78 (49)	80 (51)
Paired	62	44 (71)	18 (30)
Nasopharynx cultures ( <i>n</i> = 1,030) <sup>a</sup>			
Children culture positive for <i>S. aureus</i>			
6 wk	96 (46)	53 (46)	43 (47)
6 mo	25 (12)	15 (13)	10 (11)
12 mo <sup>b</sup>	22 (11)	15 (13)	7 (8)
18 mo	16 (8)	9 (8)	7 (8)
24 mo <sup>b</sup>	17 (8)	11 (10)	6 (7)
Children culture positive for <i>S. pneumoniae</i>			
6 wk	30 (15)	20 (17)	10 (11)
6 mo	99 (48)	56 (48)	43 (47)
12 mo <sup>b</sup>	125 (66)	70 (60)	55 (60)
18 mo	132 (64)	66 (57)	66 (73)
24 mo <sup>b</sup>	123 (59)	66 (57)	57 (63)

<sup>a</sup> Missing 1 and 2 cultures at 12 and 18 months, respectively.

<sup>b</sup> Culture times at which blood samples were obtained.

first 2 years of life (NCT00189020) (35). In brief, after obtaining written informed consent from both parents or a guardian, healthy participants were randomly assigned to receive PCV7 (i) PCV7 at 2 and 4 months of age (2-dose group); (ii) PCV7 at 2, 4, and 11 months (2- + 1-dose group); or (3) no PCV7 (control group). Part of this cohort was asked to participate in the immunogenicity arm of the study on a voluntary basis (for details, see reference 31). The present study was performed in the 2- + 1-dose (*n* = 116) and control (*n* = 91) groups (baseline characteristics are shown in Table 1).

During house visits, 269 serum samples, 111 samples from 12 month olds, and 158 samples from 24 month olds, including 62 consecutive serum samples, were collected. Sera were separated within 24 h and stored at -20°C until they were assayed. For each child, swabs were obtained by approaching the nasopharynx transnasally at 6 weeks and 6, 12, 18, and 24 months of age with a flexible, dry cotton-wool swab. Transport, isolation, and identification of pneumococci and *S. aureus* in these 1,013 samples were done using standard methods (35, 36). A national ethics committee from The Netherlands approved the trial. The study was performed in accordance with the European Statements for Good Clinical Practice.

**Detection of anti-protein antibodies.** Antibodies to pneumococcal and *S. aureus* proteins were measured in serum by a multiplex fluorescent-bead-based immunoassay (xMap; Luminex). This is a validated technique for both protein panels (34, 37), where validation was repeated upon expansion of the panels, as well. We confirmed for IgG directed against TSST-1 that there is a correlation between median fluorescence intensity (MFI) values and the *in vitro* neutralizing capacity of antibodies (37). The antigens used in this study were selected based on (i) immunological importance, as indicated by the current scientific literature; (ii) their potential role in vaccine development; and (iii) availability (2, 6, 9, 10, 23, 29, 33, 34, 37). Isolation, purification, coupling of the antigens and determination of IgG antibodies by Luminex were performed as described previously (34, 37). In each experiment, control beads (not protein coupled) were included to determine nonspecific antibody binding. In case of nonspecific binding, these MFI values were subtracted from the antigen-specific values. We used human pooled sera (HPS) from 36 healthy donors as a standard tool to test for interassay variation. Tests were accomplished as

independent duplicates. The MFI values represent antibody levels in a semiquantitative way. Since MFIs are not normally distributed, we averaged and log transformed them.

All MFI values above zero were defined as measurable antibody titers. Abbreviations of all tested *S. aureus* and *S. pneumoniae* virulence factors are listed in Table S1 in the supplemental material. IgG levels against 18 pneumococcal antigens were determined: toxins PLY and PdbD, which is a double mutant of PLY; pneumococcal cell surface proteins CbpA (PspC), PspA, PpmA, SlrA, PsaA, Hyl, NanA, BvH-3, Pht-D (Sp1003; BVH-11-2; histidine triad protein), and Pilus A; enzymes ENO and IgA-1; and proteins Sp0189 (hypothetical protein), Sp0376 (response regulator; intracellular location), Sp1633 (response regulator; intracellular location), and Sp1651 (thiol peroxidase; intracellular location) (15, 18, 23). The data on the double mutant, PdbD are not shown, since children are not exposed to this protein in nature. In addition, IgG levels against 40 *S. aureus* antigens were determined: the microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) ClfA, ClfB, SasG, IsdA, IsdH, FnbpA, FnbpB, SdrD, and SdrE; staphylococcal enterotoxins (SE) SEA to SEE, SEG to SEJ, SEM to SEO, SEQ, and SER; and staphylococcal superantigen-like 1 (SSL-1), SSL-3, -5, -9, and -11 and TSST-1; exfoliative toxins ETA and ETB; leukocidins LukD, LukE, LukF, and LukS; alpha-toxin; HlgB; and immunomodulatory proteins SCIN, chemotaxis inhibitory protein (CHIPS), and extracellular fibrinogen binding protein (Efb) (39).

**Outcome measures.** The immunogenicity of proteins was analyzed by studying the correlation between antibody levels at 12 months of age and colonization in the previous year (measured as culture positive at the age of 6 weeks and/or 6 months) and the correlation between antibody levels at the age of 24 months and colonization in the previous year (measured as culture positive at 12 and/or 18 months of age) and in the whole previous period of 2 years.

To reflect general protein antibody responses to each organism, we performed a combined measurement. First, for each age group, titers were ranked for each protein (from low to high MFI), followed by a combined ranking for all pneumococcal or staphylococcal protein responses per child. These values were correlated with previous colonization status.

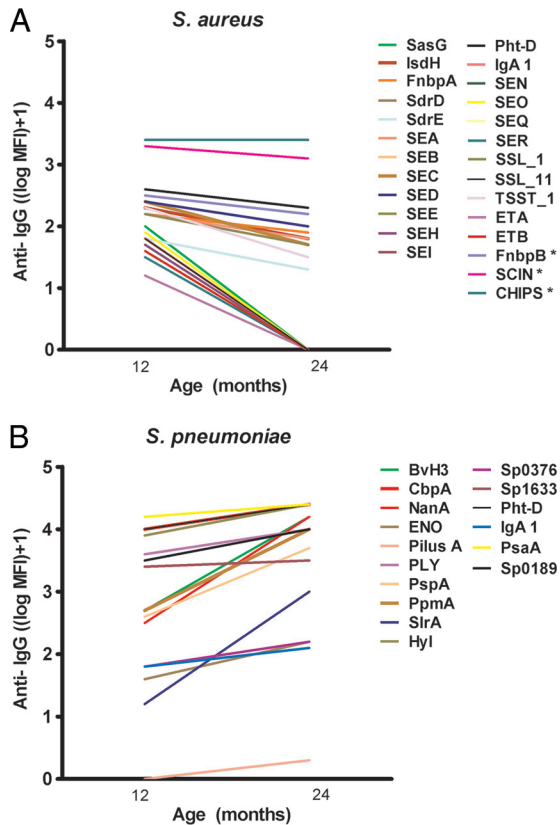
The potential protectiveness of anti-protein antibodies was studied by correlating antibody levels at 12 months of age with colonization with the homologous species in the following year (as measured at the age of 18 and/or 24 months).

**Statistical analysis.** Mann-Whitney U tests were used to study immunogenicity, overall "species" antibody responses, potential protectiveness, and PCV7 vaccine effects. Wilcoxon signed rank tests were used to study the dynamics of antibodies in time for children for whom we had paired samples available. Antibody levels were determined as continuous variables. Kruskal-Wallis tests were used to test the impact of colonization frequency.

For all analysis, a Bonferroni correction was applied to adjust for multiple comparisons. In this study, we tested 40 *S. aureus* and 18 pneumococcal proteins; therefore, the null hypothesis was rejected for comparisons regarding anti-staphylococcal-protein and anti-pneumococcal-protein antibodies with *P* values of ≤0.001 and ≤0.003, respectively. The statistical analyses were performed using the Statistical Package of Social Sciences version 17.0 for Windows (SPSS Inc., Chicago, IL).

## RESULTS

IgG levels against 18 pneumococcal and 40 staphylococcal virulence proteins were determined in 269 serum samples collected from 111 12-month-old and 158 24-month-old children (Table 1). In the serum samples of all 12-month-old children, the IgG levels (MFI units) against the *S. aureus* proteins SSL-3, SSL-9, and HlgB remained nonconcordant when tested in duplicate and were consequently excluded from analyses. We excluded data on 2, 6, and 11 samples of all *S. aureus* proteins (12 months), pneumococcal proteins (12 months), and pneumococcal proteins (24



**FIG 1** Dynamics of humoral responses against both pathogens in time. Proteins in which significant changes were observed are depicted. (A) We observed a decrease in anti-IgG levels against half the *S. aureus* proteins between the ages of 12 and 24 months ( $P \leq 0.001$ ; \*,  $P \leq 0.004$ ). (B) We observed an increase in anti-IgG levels against almost all *S. pneumoniae* virulence factors between the ages of 12 and 24 months ( $P \leq 0.003$ ).

months), respectively, because of nonconcordant results. Therefore, statistical analyses are based on 105/111 (12 months) and 147/158 (24 months) samples of pneumococcal proteins and 109/111 (12 months) and 158/158 (24 months) samples of *S. aureus* proteins. Consequently, of the 62 paired samples, 56 were available for anti-pneumococcal protein and 60 for anti-*S. aureus* protein analyses.

**Natural humoral responses against *S. pneumoniae* and *S. aureus* proteins in infancy.** To study the natural immunogenicity of *S. pneumoniae* and *S. aureus* proteins, we determined in the paired serum samples the number of children with detectable natural antibodies against these proteins at the ages of 12 and 24 months irrespective of colonization status. In general, all anti-pneumococcal-protein antibody titers increased between the ages of 12 and 24 months ( $P \leq 0.003$ ), except for Sp1651. In contrast, for over half of the *S. aureus* proteins (23/40), we observed a decrease in antibody titers between 12 and 24 months of age ( $P < 0.001$ ). The other 17 anti-staphylococcal-protein antibodies remained at a more or less stable level (Fig. 1). Nevertheless, at 12 months of age, we already observed in more than three-quarters of infants measurable antibodies against most pneumococcal proteins, whereas at 24 months of age, almost all children had detectable antibodies against those proteins. The only exception was

observed for IgG against Pilus A antigen, which was found in only 10% of 12-month-old children, although this increased rapidly to 87% in 24 month olds. With respect to the antibody response against *S. aureus* proteins, we observed antibodies directed against all but one staphylococcal protein in almost all 12-month-old children. However, at 24 months of age, detectable anti-protein IgG dropped, depending on the protein measured. For more detailed information regarding the dynamics of the natural humoral responses in infancy, see Tables S2 and S3 in the supplemental material.

**Immunogenicity of *S. pneumoniae* and *S. aureus* virulence proteins in relation to proven previous colonization in infancy.** We investigated the immunogenicities of all *S. pneumoniae* and *S. aureus* proteins in relation to recent challenge with the homologous species, defined as culture-proven carriage at the preceding two sample times.

In children with proven challenge at the age of 6 weeks and/or 6 months or at 12 and/or 18 months of age, the levels of IgG against the proteins PLY, PdbD, CbpA (PspC), PspA, PpmA, PsaA, NanA, Pht-E (BVH-3), ENO, Pht-D (Sp1003), and Sp0189 were significantly higher than those in children without preceding positive cultures at the ages of 12 and 24 months, respectively (Table 2). In addition, SlrA IgG antibodies were higher in 12 month olds with recent pneumococcal challenge than in culture-negative children. Calculations of immunogenicity at 24 months in relation to proven previous colonization in the preceding 2 years showed similar data (Table 2).

We performed identical calculations for immunogenicity of *S. aureus* proteins. At 12 months of age, we found significantly higher antibody titers against *S. aureus* proteins ClfB and Efb in children colonized at 6 weeks and/or 6 months of age compared to noncolonized children. At 24 months of age, we observed higher antibody titers against ClfA and Efb (38) and also higher antibody titers against CHIPS, LukF, and LukD in children with culture-proven colonization at 12 and/or 18 months of age ( $P \leq 0.001$ ) (Table 2).

Repeating the calculations, including “current” colonization (immunogenicity at 12 months based on culture results at 6 weeks, 6 months, and 12 months of age and immunogenicity at 24 months based on culture results at 12, 18, and 24 months), did not change the results (Table 2).

Repeating the 24-month calculations, taking into account culture results from all sample times in the previous 2 years, showed results similar to those taking into account culture results from the preceding year (Table 2).

Additionally, we assessed the impact of the number of positive cultures on antibody levels at 12 months and 24 months. Fifteen, 17, 35, 42, 29, and 7 children were positive for *S. pneumoniae* zero, one, two, three, four, and five times, respectively, in the preceding 2 years (5 sample times). *S. aureus* was cultured zero, one, two, three, four, and five times from 69, 57, 18, 9, 2, and 1 children 24 months of age, respectively (Table 3). We found a significant positive association between the number of cultures (0, 1, 2, and  $\geq 3$ ) positive for *S. pneumoniae* and *S. aureus* in the first 2 years of life, and antibody titers against pneumococcal proteins PspA, PpmA, SlrA, Sp0189, Pht-D (Sp1003), BvH3, ENO, Sp1633, NanA, PLY, PsaA, and CbpA ( $P \leq 0.003$ ) and *S. aureus* proteins ClfA, Efb, LukD, SCIN, CHIPS, HlgB, and FnbpB ( $P \leq 0.001$ ) (Table 3) measured at 24 months of age. Similar trends were observed at 12 months of age (Table 3).

TABLE 2 Immunogenic *S. pneumoniae* and *S. aureus* proteins determined by increase of antibody titers in previous culture-positive carriers compared to non-carriers

Protein	Ab (MFI) <sup>a</sup>											
	12 mo olds with previous carriage at:				24 mo olds with previous carriage at:				6 wks: 6, 12, 18 mo olds:			
	6 wks: 6 mo	6 wks: 6, 12 mo	12, 18 mo	12, 18, 24 mo	6 wks: 6, 12, 18 mo	6 wks: 6, 12, 18, 24 mo	6 wks: 6, 12, 18, 24 mo	6 wks: 6, 12, 18, 24 mo	6 wks: 6, 12, 18, 24 mo	6 wks: 6, 12, 18, 24 mo	6 wks: 6, 12, 18, 24 mo	6 wks: 6, 12, 18, 24 mo
<i>S. pneumoniae</i>												
Nana	1,127	191	947	94	2,914	968	2,907	407	2,906	885	2,907	279
Sp0189	5,817	1,572	4,526	824	10,482	5,839	10,258	2,749	9,932	3,952	10,062	2,430
Phc-D	20,307	2,357	17,598	1,179	24,449	20,031	24,398	17,392	24,419	18,685	24,399	11,560
PLY	6,586	2,458	5,532	933	12,393	7,463	12,413	4,132	11,988	5,729	12,216	3,512
Ppma	1,560	2	1,293	0	4,729	1,835	4,384	104	4,654	1,151	4,384	12
Psaa	20,446	10,924	19,536	6,997	22,678	20,000	22,488	7,733	22,510	15,422	22,460	5,771
BvH3	5,952	222	4,273	111	15,589	4,047	14,624	419	15,174	1,162	14,624	368
Cbpa	22,814	2,508	20,814	654	24,944	21,946	24,902	1,631	24,941	17,018	24,907	480
Pspa	891	214	676	107	3,186	758	3,364	214	3,100	615	3,228	113
ENO	127	26	119	15	337	150	334	120	337	143	334	119
Slra	159	0	88	0	537	204	542	176	489	180	532	143
Sp1633	2,369	1,863	2,307	1,759	3,635	2,832	3,632	2,504	3,635	2,729	3,632	2,288
Iga-1	118	63	114	48	738	174	745	81	726	158	731	81
Hyl	11,309	3,805	11,941	233	21,023	14,231	21,058	11,083	21,023	13,902	21,059	11,083
<i>S. aureus</i>												
ClaA	546	294	553	285	1,438	314	1,332	251	879	204	792	198
Efb	1,041	361	1,038	361	3,558	321	2,760	313	1,050	245	1,050	229
CiBb	1,051	203	1,029	200	2,122	257	1,387	243	568	243	568	155
CHiPS	4,921	269	5,112	247	9,897	1,672	9,262	1,290	5,314	324	5,681	275
LukD	2,186	614	2,094	610	6,707	1,224	3,660	1,130	2,653	775	2,653	757
LukF	396	275	399	275	532	257	479	239	309	239	319	208
LukS	4,119	1,060	4,154	1,059	5,689	1,930	5,163	1,863	3,245	1,658	3,423	1,513
SCIN	2,278	256	2,327	245	4,953	852	5,661	756	2,260	607	2,938	407
HlgB	NA	NA	NA	NA	6,224	1,873	6,110	1,820	3,260	1,072	3,394	1,018

<sup>a</sup> Median antibody (Ab) levels (MFI units). The shaded cells represent the immunogenic proteins (*S. aureus*,  $P \leq 0.001$ , and *S. pneumoniae*,  $P \leq 0.003$ ). NA, not applicable. For *S. pneumoniae*, 12 mo olds,  $n = 105$ ; 24 mo olds,  $n = 145$  (2 out of 147 children for whom serology data were available because of missing culture data [Table 1]). For *S. aureus*, 12 mo olds,  $n = 109$ ; 24 mo olds,  $n = 156$  (2 out of 158 children for whom serology data were available because of missing culture data [Table 1]).

<sup>b</sup> Col, culture positive ( $n$ , number of children culture positive at  $\geq 1$  of the time points for *S. pneumoniae*/*S. aureus*); Non-c, culture negative ( $n$ , number of children culture negative at all time points for *S. pneumoniae*/*S. aureus*).



TABLE 3 Anti-protein antibody levels of children in relation to *S. pneumoniae* and *S. aureus* colonization frequency

Age group <sup>a</sup>	n	No. of times culture positive <sup>b</sup>	Ab (MFI) <sup>c</sup>												
			<i>S. pneumoniae</i>												
			PspA	PpmA	SlrA	Sp0189	Pht-D	Pht-E	ENO	Sp1633	NanA	PLY	PsaA	CbpA	
12 mo	31	0	107	0	0	824	1,179	111	15	1,759	94	933	6,997	654	
	35	1	493	603	0	3,175	10,694	3,346	75	1,949	577	4,173	16,558	17,897	
	28	2	1,049	1,560	221	5,662	20,041	5,330	127	2,350	1,127	6,698	22,171	22,814	
	11	3	593	2,850	462	6,858	23,126	8,176	146	3,321	1,195	7,856	20,579	24,668	
24 mo	15	0	113	12	143	2,430	11,560	368	119	2,287	279	3,512	5,771	480	
	17	1	851	827	372	6,177	22,743	7,341	195	2,941	2,516	11,364	20,115	23,665	
	35	2	3,322	3,779	504	8,815	23,835	9,966	270	3,218	2,042	12,039	22,467	24,886	
	42	3	5,294	7,326	450	11,328	24,460	16,940	370	3,851	3,055	12,711	22,762	24,953	
	29	4	5,726	8,084	787	12,137	24,796	16,269	410	4,293	3,204	12,432	22,830	25,193	
	7	5	637	4,896	49	11,333	24,692	14,442	276	3,019	2,807	7,081	21,401	24,647	
			<i>S. aureus</i>												
			ClfA	ClfB	Efb	LukD	SCIN	CHIPS	HlgB	FnbpB					
12 mo	50	0	285	200	361					231					
	41	1	407	689	998					273					
	14	2	2,980	2,030	1,240					749					
	4	3	3,524	1,966	897					407					
24 mo	69	0	198		229	757	407	275	1,018	135					
	57	1	495		610	1,590	1,966	3,117	2,771	207					
	18	2	884		1,344	3,482	4,037	8,877	5,154	596					
	9	3	2,322		6,294	8,721	6,101	11,627	6,338	964					
	2	4	730		3,507	1,677	3,033	2,072	1,536	4,365					
	1	5	5,406		10,165	7,369	13,472	13,254	11,865	916					

<sup>a</sup> For *S. pneumoniae*, 12 mo olds,  $n = 105$ ; 24 mo olds,  $n = 145$  (2 out of 147 children for whom serology data were available were excluded because of missing culture data [Table 1]). For *S. aureus*, 12 mo olds,  $n = 109$ ; 24 mo olds,  $n = 156$  (2 out of 158 children for whom serology data were available were excluded because of missing culture data [Table 1]).

<sup>b</sup> Total number of times culture positive at 6 weeks and 6 and 12 months (12 mo) or at 6 weeks and 6, 12, 18, and 24 months (24 mo).

<sup>c</sup> Median antibody (Ab) levels (MFI units) against *S. pneumoniae* and *S. aureus* proteins. For the 12-month group, Kruskal-Wallis test in children 0 to  $\geq 2$  times culture positive,  $P < 0.003$  for *S. pneumoniae* and  $P \leq 0.001$  for *S. aureus*. For the 24-month group, Kruskal-Wallis test in children 0 to  $\geq 3$  times culture positive,  $P \leq 0.003$  for *S. pneumoniae* and  $P \leq 0.001$  for *S. aureus*.

Finally, we performed a combined measurement of responses to all proteins to reflect the overall antibody responses to the individual organisms. At both 12 months and 24 months of age, the combined antibody response against *S. pneumoniae* and *S. aureus* was significantly higher in children with previous culture-proven carriage than in noncolonized children (all  $P$  values  $\leq 0.002$ ).

#### Protectiveness of *S. pneumoniae* and *S. aureus* antibodies.

We assessed the potential protectiveness of anti-protein antibodies by relating antibody levels at 12 months to subsequent colonization with the homologous species in the following year. Furthermore, we also explored whether these antibodies are protective against the heterologous species. We observed no negative correlation between antibodies to any of the *S. pneumoniae* and *S. aureus* proteins at the age of 12 months and pneumococcal and/or *S. aureus* colonization in the following year (data not shown).

#### Development of antibodies in relation to PCV7 vaccination.

To examine the effect of PCV7 on development of natural antibodies to pneumococcal and *S. aureus* antigens, we compared anti-IgG levels against pneumococcal and staphylococcal proteins between children previously immunized with PCV7 and nonimmunized children. In the 12-month-old group, serum antibodies

were measured for 76 vaccinees and 29 controls, and in the 24 month olds for 70 vaccinees and 77 controls. We observed no significant differences in anti-protein antibodies between vaccinees and controls for both pneumococcal and *S. aureus* antigens at both ages. Nevertheless, we did observe a small trend toward slightly lower pneumococcal antibody levels in the vaccinated group for NanA (MFI, 2,982 versus 2,177 [ $P = 0.025$ ]), Pilus A (MFI, 34 versus 19 [ $P = 0.034$ ]), PspA (MFI, 3,686 versus 1,060 [ $P = 0.015$ ]), and PsaA (MFI, 22,510 versus 20,281 [ $P = 0.054$ ]) at 24 months of age comparing the unvaccinated and the vaccinated group, respectively.

#### DISCUSSION

In a search for potential future protein vaccine candidates, we studied the immunogenicities of *S. aureus* and *S. pneumoniae* proteins and the potential protective levels of these antibodies against colonization in PCV-vaccinated and nonvaccinated infants. The study was performed in a randomized control setting before nationwide PCV7 introduction in The Netherlands. We examined 18 pneumococcal and 40 *S. aureus* proteins in total, including 21 *S. aureus* proteins that were tested for immunogenicity for the first time. Our experiments demonstrated the presence of natural an-

tibodies to all tested staphylococcal and pneumococcal proteins at both 12 and 24 months, with increasing anti-pneumococcal antibodies and declining anti-staphylococcal antibodies with age. These findings are in line with the literature (18, 38) and correspond to the natural dynamics of colonization, where nasopharyngeal colonization with pneumococcus increases and staphylococcal colonization decreases with age. Therefore, these data support the hypothesis that anti-protein antibodies are a result of natural exposure to the pathogens studied.

It is noteworthy that anti-Pilus A IgG was detectable in only 10% of the 12-month-old children but in over 80% of 24 month olds. This might be caused by lower immunogenicity of the protein, or it might be because the pilus is expressed in only a minority of strains. A previous study showed that the pilus frequency might be reduced by the conjugate pneumococcal vaccine (28), which was supported by our data showing a trend toward a lower antibody response against Pilus A in the PCV7-vaccinated group at 24 months of age.

Natural immunogenicity of many of the proteins was supported by the strong correlation between culture-proven colonization and consecutive development of antigen-specific antibodies to most pneumococcal virulence factors and several *S. aureus* proteins. We observed significantly higher IgG antibody levels against the *S. aureus* proteins Efb and CHIPS (34, 38) and against ClfA, ClfB, LukD, and LukF in children with culture-proven colonization in the preceding year(s), which underlines the immunogenicity of the organism and specifically of these proteins. Interestingly, ClfB appears to be a major determinant of persistent nasal *S. aureus* colonization in humans (37, 40). ClfA is known to reduce opsonophagocytosis of *S. aureus* by neutrophils (8). Secreted cytolytic toxins (LukD and LukF) interfere with neutrophil function by causing membrane damage (8). CHIPS blocks the neutrophil chemoattractant receptors FPR-1 and C5aR (8), while the Efb reduces neutrophil recruitment, as well as chemoattractant C5A (16). As a result, all of these proteins seem to have an immune-suppressive function, which potentially makes them ideal targets for vaccine development.

Known bacterial factors that contribute to pneumococcal colonization are, among others, CbpA, PspA, BvH-3, Hyl, and ENO (15, 23). CbpA binds to human secretory component on a polymeric Ig receptor during the first stage of translocation across the epithelium. PspA is known to prevent binding of C3 onto the pneumococcal surface, and BvH-3 is an inhibitor of the complement system as well. In our study, these proteins appeared immunogenic as well, underlining their need to be expressed at the time of colonization. The natural immunogenicity of all the proteins mentioned is further supported by the significant positive association between the frequency of culture-proven colonization and consecutive antibody titers. For SlrA and PpmA, we even observed unmeasurable antibody titers in 12-month-old children if their cultures were negative at all prior colonization time points. Children who were (repetitively) culture positive in the first year had higher titers against both proteins, which confirms previously published results (1).

Previously, we demonstrated in the same study population an increase in serotype-specific anti-capsular IgG antibodies in previous carriers compared to controls for the serotypes 19F, 23F, and 6B, as well, underlining the immunogenicity of most capsule polysaccharides (30). Since for each carrier event the host is exposed to a single or a few capsule polysaccharides but

to many surface-associated proteins at the same time, we also calculated a combined overall anti-protein antibody titer for the two species separately, which confirmed strong carriage-related general anti-protein immunity.

Our experiments show no protective effect of antibodies against any of the proteins on colonization in the following year. Possibly, this is caused by downregulation of protein expression by the bacterium under immune pressure or by the limited accessibility of opsonizing antibodies to proteins shielded by the capsule. Moreover, systemic antibodies might not reach the bacteria in the nasopharyngeal niche properly. The most plausible cause might be the polymorphic nature of *S. pneumoniae* and, to a lesser extent, *S. aureus* proteins, resulting in low cross-protectiveness of protein-specific antibodies among strains, which might be a serious problem for their use for future vaccine development (12, 22). Therefore, it might not be surprising that results from murine models strongly suggest CD4<sup>+</sup> Th17-mediated immunity rather than antibody-mediated immunity to be the primary (natural) mechanism of protection against pneumococcal colonization, although this still needs to be confirmed in humans (5, 20). Our immunogenicity data do not suggest cross-protection of *S. aureus* and *S. pneumoniae* anti-protein IgG against colonization with the heterologous bacterium, either (17). Consequently, the previously observed negative association between *S. aureus* and *S. pneumoniae* colonization cannot be explained by anti-protein IgG levels (4, 27).

To the best of our knowledge, the effect of PCV7 on early natural humoral responses to *S. pneumoniae* and *S. aureus* proteins is not yet known. No differences in responses to *S. pneumoniae* proteins between controls and vaccinees were observed, although we did notice a small trend toward lower pneumococcal antibody levels in the vaccinated group against NanA, Pilus A, PspA, and PsaA. This might be partly the consequence of the subtle temporary decrease in overall pneumococcal colonization that we observed at 18 months of age in the vaccinated group compared to the controls (in this subset of children, 57% versus 73%) or of the observed serotype replacement, both described in more detail previously (35, 36). The latter should imply that nonvaccine serotypes might express fewer (homologous) epitopes on their surfaces. The study was performed well before herd effects, after PCV7 introduction in the Dutch National Immunization Program. As a result, in the coming years, the impact of decreased vaccine serotype colonization and serotype replacement on natural anti-pneumococcal protein IgGs may need to be reevaluated (35). In addition, we observed no significant differences in IgG antibody responses against *S. aureus* between controls and vaccinees, which might be explained by similar colonization rates observed in the two groups.

Some limitations of our study need to be recognized. First, the results are derived from a *post hoc* analysis. It would have been ideal if we had sampled the children more frequently. Due to the 6-month sampling intervals in our study, we missed several colonization episodes in most individuals during their first 2 years of life, which skewed our results toward smaller effects. This is underlined by the presence of anti-protein antibodies to most proteins in children who remained culture negative for pneumococci at all sample times.

The strengths of our study include the randomized controlled and longitudinal design, which made it possible to estimate the effect of PCV7 vaccination on natural antibody responses against

virulence proteins in time in a controlled setting. In addition, this is the first study simultaneously analyzing IgG responses against *S. aureus* and *S. pneumoniae* in this large number of samples. Moreover, we corrected for multiple testing, probably resulting in lower numbers of false-positive associations. Finally, this multiplex fluorescent-bead-based immunoassay allowed us to analyze the humoral responses of 58 proteins simultaneously, which is highly advantageous when working with small volumes of pediatric serum samples.

In conclusion, natural anti-protein antibodies to pneumococci and *S. aureus* showed an inverse correlation with age, which is in line with colonization dynamics. Many pneumococcal and staphylococcal proteins appear to be immunogenic in children under 24 months of age. In contrast, none of the anti-protein antibodies appeared to be either protective or cross-protective against pneumococcal and *S. aureus* colonization in the second year of life. The consequences of these findings for protectiveness of the proteins when used as actual vaccine candidates should be further investigated.

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