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Natural Antibodies against Several Pneumococcal Virulence Proteins in Children during the Pre-Pneumococcal-Vaccine Era: the Generation R Study▽

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The currently available pneumococcal vaccines do not protect against all serotypes of *Streptococcus pneumoniae*. A shift toward nonvaccine serotypes causing colonization and invasive disease has occurred, and studies on protein-based vaccines have been undertaken. We assessed the association between specific antibodies against pneumococcal virulence proteins and colonization and respiratory tract infections (RTIs). Additionally, we assessed the extent to which colonization induces a humoral immune response. Nasopharyngeal swabs collected from children at 1.5, 6, 14, and 24 months of age were cultured for pneumococcus. Serum samples were obtained at birth and at 6, 14, and 24 months ($n = 57$ children providing 177 serum samples). Data were collected prior to the pneumococcal vaccine era. IgG, IgA, and IgM levels against 17 pneumococcal protein vaccine candidates were measured using a bead-based flow cytometry technique (xMAP; Luminex Corporation). Information regarding RTIs was questionnaire derived. Levels of IgG against all proteins were high in cord blood, decreased in the first 6 months and increased again thereafter, in contrast to the course of IgA and IgM levels. Specific antibodies were induced upon colonization. Increased levels of IgG against BVH-3, NanA, and SP1003 at 6 months, NanA, PpmA, PsaA, SlrA, SP0189, and SP1003 at 14 months, and SlrA at 24 months were associated with a decreased number of RTIs in the third year of life but not with colonization. Maternal antipneumococcal antibodies did not protect against pneumococcal colonization and infection. Certain antibodies against pneumococcal virulence proteins, some of which are induced by colonization, are associated with a decreased number of RTIs in children. This should be taken into account in future pneumococcal vaccine studies.

Streptococcus pneumoniae (pneumococcus) is a commensal organism but also a pathogen that plays an important role in the pathogenesis of respiratory tract infections (RTIs) such as pneumonia and otitis media in infants and young children. In addition, the pneumococcus may cause invasive diseases such as meningitis and sepsis (24). Morbidity and mortality in infants and young children worldwide are frequently caused by this pathogen. The World Health Organization generated estimates on child mortality due to invasive pneumococcal infection ranging from 700,000 to more than a million children per annum (24). Healthy children may be colonized with the pneumococcus in the nasopharynx; the frequency of this colonization increases in the first year of life from approximately 8% to 45% (20). This pathogen often presents as a commensal, causing no harm due to the adequate innate and adaptive immune reactions of the host. However, asymptomatic nasopharyngeal carriage is the primary source of pneumococcal infection (2).

More than 90 different pneumococcal serotypes have been identified on the basis of variability in the capsular polysaccharides. The current vaccines are based on antibodies against these polysaccharides; hence, only some these serotypes are covered. Presently, significant research is focused on improving pneumococcal vaccines in order to generate broader protection against pneumococcal disease. The current 7-valent pneumococcal conjugate vaccine (PCV-7), which has now been introduced in national immunization programs in most developed countries, is up to 90% effective in reducing vaccine serotype-specific invasive pneumococcal disease. However, the net vaccine benefit has been negatively affected by a 71% increased rate of invasive pneumococcal disease caused by nonvaccine serotypes (30). Recently, a 13-valent pneumococ-

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TABLE 1. Functions of the 17 pneumococcal proteins

Pneumococcal virulence protein	Main role
BVH-3 (PhtE)	Pneumococcal histidine triad; possibly a role in complement inhibition
PspC (CbpA)	Binds to human secretory component on a polymeric Ig receptor during the first stage of translocation across the epithelium
PdbD	Double mutant of PLY
Enolase (Eno)	Binds to plasminogen, which is subsequently activated to the serine protease plasmin by tissue or urokinase plasminogen activator
Hyaluronidase (Hyl)	Breaks down hyaluronan-containing extracellular matrix components
IgA-1protease	Cleaves human IgA1
NanA	Removes sialic acids and cleaves terminal sugars from various glycoconjugates, which might reveal receptors for adherence
PLY	Pneumolysin; cytolytic toxin that also activates complement; an important determinant of virulence in *in vivo* models of disease; wide range of effects on host immune components at sublytic concentrations
PpmA	Induces opsonophagocytosis *in vitro*
PsaA	Component of the ABC transport system, which is involved in resistance to oxidative stress and transport of $Mn2^{+}$
PspA	Prevents binding of C3 onto pneumococcal surface; also binds lactoferrin
SlrA	Cyclophilin-type PP_iase can catalyze the *cis-trans* isomerization of proline-containing tetrapeptides; modulates the biological function of important virulence proteins
SP0189	Hypothetical protein
SP0376	Response regulator (intracellular location)
SP1003 (BVH-11-2/PhtD)	PhtD (histidine triad protein)
SP1633	Response regulator (intracellular location)
SP1651	Thiol peroxidase (intracellular location)

cal conjugate vaccine (PCV-13) was developed to further improve protection (9, 19) by covering the serotypes that most frequently cause infection and colonization. However, the increase in carriage of nonvaccine serotypes, and the associated increase in invasive disease, could ultimately outweigh the benefits of the current PCV (21). Since PCV is also quite expensive and therefore not extensively used in developing countries where it is needed the most, there is an urgent need to develop alternative pneumococcal vaccines to cover these gaps. Novel vaccines with expanded coverage and immunogenicity are urgently needed for optimal prevention of pneumococcal infections. Most promise is offered by the addition of protein-based vaccines to the current PCV, which may provide protection regardless of serotype (3). Several protein antigens, such as Ply, CbpA, PspA, PsaA, PiaA, PhtB, PhtE (BVH-3), and NanA, have been identified as vaccine candidates (1, 6, 8, 12, 14, 18, 22, 23, 26, 27, 29, 35). There is evidence that immunization with certain combinations of virulence proteins provides additive or even synergistic protection (5, 25). The combination of Ply, PspA, and CbpA provided protection in mouse models especially successfully (7, 11, 26). These murine studies demonstrated a protective effect of immunoglobulins directed against these primarily but not exclusively surface-located pneumococcal proteins. However, prospective studies on the protectiveness of antibodies against pneumococcal proteins in humans are lacking. Since infection with *S. pneumoniae* is supposed to start with colonization, it seems rational to aim for prevention of colonization and thus search for antipneumococcal antibodies providing protection against colonization (31).

Our primary objective was to assess, in children from the pre-pneumococcal-vaccine era, the level of protection provided by antibodies against 17 pneumococcal proteins for infant pneumococcal colonization and RTIs. Additionally, we assessed the extent to which colonization induces a humoral immune response.

MATERIALS AND METHODS

Study design and population. This study was part of the Generation R Study. The Generation R Study is a population-based prospective cohort study following pregnant women and their children. Further details on this cohort study have been described previously (15, 16). The present study was performed in a subgroup of 1,079 Dutch women and their single-born children. Detailed assessments were conducted of this subgroup. All of these children were born between February 2003 and August 2005. This period was before the introduction of the pneumococcal vaccination in the Netherlands in 2006. The Medical Ethics Committee of the Erasmus Medical Center, Rotterdam, Netherlands, approved the study, and written informed consent was obtained.

Antipneumococcal antibodies. A cord blood sample was obtained after delivery, and infant blood samples were obtained during visits at the research center at the ages of 6, 14, and 24 months. Of the 1,079 infants in the postnatal cohort of analyses, 57 were selected for this particular study. They were selected on the basis of the availability of biological samples. Seventeen pneumococcal proteins were selected based on importancek, as indicated by current scientific literature, their potential role in vaccine development, and availability. Antigens included putative protease maturation protein A (PpmA), pneumococcal surface adhesin A (PsaA), pneumococcal surface protein A (PspA), and choline binding protein A (PspC/CbpA), as well as neuraminidase (NanA), pneumolysin (PLY), a double mutant of pneumolysin (PdbD), the pneumococcal histidine triad (Pht) family (BVH-3), streptococcal lipoprotein rotamase A (SlrA), alpha-enolase (Eno), IgA1 protease, hyaluronidase (Hyl), and the *Streptococcus pneumoniae* proteins SP0189 (hypothetical protein), SP0376 (response regulator, intracellular location), SP1003 (PhtD/BVH11-2, histidine triad protein), SP1633 (response regulator, intracellular location), and SP1651 (thiol peroxidase, intracellular location). Isolation and purification methods were as described previously (33). Table 1 provides the functions of the studied proteins (17). The levels of IgG, IgA, and IgM against these proteins were measured using a recently described 17-plex assay based on pneumococcal proteins (28) with the (bead-based) flow cytometry technique (xMAP; Luminex Corporation, Austin, TX). Here we used this novel multiplex assay. The median fluorescence intensity (MFI) values, reflecting semiquantitative antibody levels, were averaged. Tests were performed in independent duplicates, and control beads (where no protein was coupled) were included to determine nonspecific binding. In cases of nonspecific binding, the nonspecific MFI values were subtracted from the antigen-specific results.

***Streptococcus pneumoniae* colonization.** During the visits at ages 1.5, 6, 14, and 24 months, nasopharyngeal swabs for the isolation of *S. pneumoniae* were obtained. Methods of sampling were as described previously (20).

RTIs. Parentally retrieved questionnaires were obtained at 12, 24, 36, and 48 months. Questions regarding doctor visits (never, once or twice, three or four times, more than four times) because of fever and respiratory tract complaints were used to assess the burden of RTIs. We defined three subgroups: (i) child has not been to a doctor with fever and cough/runny or blocked nose/earache in the preceding year, (ii) child has been to a doctor with fever and cough/runny or blocked nose/earache once or twice in the preceding year, and (iii) child has been to a doctor with fever and cough/runny or blocked nose/earache three times or more in the preceding year. For the analyses, we compared children who frequently visited the doctor (at least three times) with the children who never visited the doctor for RTIs. Children scoring three or four times or four times or more on number of doctor visits were classified as visiting the doctor at least three times. Additionally, children scoring one or two times on doctor visits for at least two different symptoms (e.g., once or twice for fever with earache and once or twice for fever with a cough) were scored as visiting the doctor at least three times as well. The latter category may comprise children with only two doctor visits since we cannot distinguish between two and three or four doctor visits in this group.

Statistical analysis. Wilcoxon signed rank tests were used to compare antipneumococcal antibody levels in the groups of children at the four different ages. We compared IgG, IgM, and IgA levels between 0 and 6 months, 6 and 14 months, 14 and 24 months, and overall between 0 and 24 months.

Mann-Whitney *U* tests were used to compare differences in maternal antibody levels for colonized and noncolonized infants in the first year of life and to compare differences in maternal antibody levels for children with and without frequent RTIs in the first year of life.

Moreover, to assess whether levels of antibodies protect against later colonization, we used the Mann-Whitney *U* test to compare differences in antibody levels at the ages of 6, 14, and 24 months for children who were later colonized or were noncolonized. Additionally, we used this test to compare differences in antibody levels at the ages of 6, 14, and 24 months for children with and without frequent RTIs in the 3rd year of life. At age 14 months and older, the results will not be blurred by maternal antibodies.

Finally, Mann-Whitney *U* tests were used to compare differences in antibody levels between previously colonized and noncolonized children at the different measurement moments to assess whether these specific antibodies are induced upon colonization. The Wilcoxon signed rank tests and Mann-Whitney *U* tests were used for the same type of analyses described previously (34).

The results are presented as MFI values. *P* values of <0.05 were considered statistically significant. The statistical analyses were performed using the Statistical Package of Social Sciences version 17.0 for Windows (SPSS Inc., Chicago, IL).

RESULTS

Of the 57 children included for this study, 51 provided three serum samples and four serum samples were obtained from 6 children, for a total of 177 serum samples. Of these 177 samples, 54 (31%) were cord blood samples, 32 samples (18%) were obtained at 6 months, 46 (26%) at 14 months, and 45 (25%) at 24 months. Nasopharyngeal swabs were available for 40 children (70%) at 1.5 months, for 49 (86%) at 6 months, for 50 (88%) at 14 months, and for 48 (89%) at 24 months. At 1.5 months, 17.5% of the children ($n = 7$) were colonized with *S. pneumoniae*, which increased at 6, 14, and 24 months to 28.6% ($n = 14$), 36.0% ($n = 18$), and 39.6% ($n = 19$), respectively. In the first year of life, 7 infants (12.3%) visited a doctor at least three times for putative RTIs; this number increased to 13 children (22.8%) in the second year of life and decreased thereafter to 5 children (9.4%) in the third year of life. General population characteristics are presented in Table 2.

TABLE 2. Population characteristics[a]

Population characteristic	Value[b]
Gestational age (wk)	40.2 (1.39)
Birth wt (g)	3,677 (489)
Gender female	28 (49.1%)
Positive for colonization at:	
1.5 mo	7 (17.5%)
6 mo	14 (28.6%)
14 mo	18 (36.0%)
24 mo	19 (39.6%)
36 mo	8 (19.5%)
Frequent RTIs (>3 times)	
1st yr	7 (12.3%)
2nd yr	13 (22.8%)
3rd yr	5 (9.4%)

[a] Data are given for the 57 children included in the study. Data are missing for colonization status at 1.5 months ($n = 17$), at 6 months ($n = 8$), at 14 months ($n = 7$), at 24 months ($n = 9$), at 36 months ($n = 16$) and for RTIs in 2nd ($n = 1$) and 3rd year of life ($n = 4$).

[b] Values are given as means (standard deviation) or absolute numbers (%).

Dynamics of antipneumococcal antibodies. The levels of IgG, IgM, and IgA directed against pneumococcal proteins showed a dynamic process over the first 2 years of life (Fig. 1). There was extensive variability in serum responsiveness over time for each pneumococcal protein. Overall, IgG levels against pneumococcal proteins tended to be high in cord blood, but these levels decreased significantly in the first 6 months of life. This holds true for all antipneumococcal antibodies except for anti-PsaA, anti-SP0189, anti-SP1633, and anti-SP1651 antibodies. The latter anti-*S. pneumoniae* antibodies were low at birth and showed neither a significant increase nor a notable decrease. Apparently, these proteins are poorly immunogenic. A significant increase was observed after the first 6 months for all proteins except for Eno, Hyl, PspA, and the *S. pneumoniae* proteins. Low values were obtained for IgA and IgM in the cord blood samples, increasing significantly in the first 2 years of life (*P* values, <0.001 for all proteins except for IgM against PspC).

Maternal antipneumococcal antibodies. For 54 infants, cord blood samples for analyses of maternal antipneumococcal antibodies were available. Antipneumococcal IgA and IgM levels in cord blood were low because maternal IgA and IgM are not transported across the placenta. Hence, we studied maternal IgG levels only in relation to infant colonization and infection. Maternal IgG levels in cord blood were on average higher in children with higher colonization prevalence in the first year of life. Elevated levels of maternal anti-BVH-3, anti-NanA, and anti-SP1651 IgG were significantly associated with enhanced child colonization rates at 1.5 months (BVH-3, $P = 0.003$) and 14 months (BVH-3, $P = 0.049$, and NanA, $P = 0.047$). IgG levels against BVH-3 were also significantly increased in children frequently colonized with *S. pneumoniae* in the first year of life ($P = 0.003$). This indicates that these antibodies are not able to protect the child against colonization. In contrast, these antibodies seem to facilitate colonization. There were no maternal IgG antibodies found to provide protection against colonization. Moreover, we did not observe a protective effect of maternal IgG antibody levels and RTIs in the first year of life.

Antipneumococcal antibodies and colonization. To study whether antibodies in the child protect against future coloni-

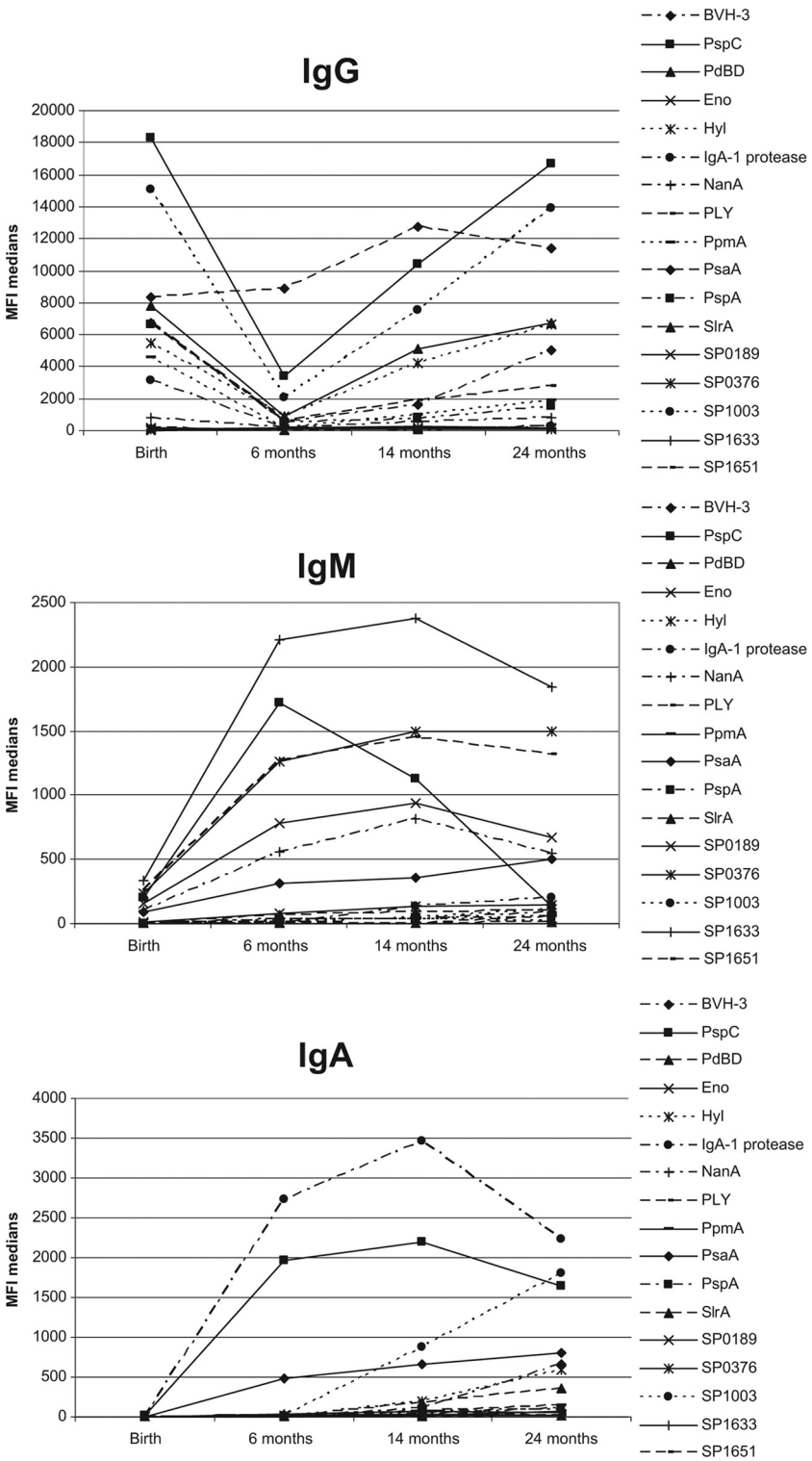

FIG. 1. Dynamics of IgG, IgM, and IgA levels in the first 2 years of life. Median MFI values, averaged for all children (n = 57), are presented by age (1.5, 6, 14, and 24 months). High levels of placentally transferred IgG are observed at 1.5 months and decrease in the first 6 months of life. After 6 months, an increase in the IgG levels is observed. Low levels of IgM and IgA in serum were observed after birth, but these clearly increased during the first year of life.

TABLE 3. Levels of antipneumococcal antibodies following pneumococcal colonization[a]

Colonization moment	Antibody detected at[b]:								
	6 mo			14 mo			24 mo		
	IgG	IgM	IgA	IgG	IgM	IgA	IgG	IgM	IgA
1.5 mo	BVH-3, NanA, PpmA, PsaA, SlrA	PspC, Eno, NanA, SP0189, SP0376	PsaA	SP0189			NA	NA	NA
6 mo	BVH-3, Hyl, PpsA, SP1003	PsaA	Hyl, PsaA, PspA, SP1003	PspC, Eno	PspC[c], SlrA	BVH-3, NanA, PsaA, SP1003	NA	NA	NA
14 mo	NA	NA	NA	BVH-3, PspC, PdbD, NanA, PLY[c], PspA, SP1003	PspC[c]	PspC, PdbD, NanA, PLY, PspA, SP1003	NanA	PLY[c]	

[a] Antipneumococcal antibodies in this table were significantly ($P < 0.05$) increased until 1 year after colonization.
[b] The antibody levels at 6 months might be obscured by the presence of maternal antibodies. NA, not applicable, either because the determinant occurs after the outcome (colonization at 14 months and antibody levels at 6 months) or because of a long time span between the determinant and the outcome (colonization at 1.5 and 6 months and antibody levels at 24 months).
[c] This antibody was at a significantly decreased level following colonization (P value < 0.05).

zation with *S. pneumoniae*, we focused on antibody levels at 14 and 24 months since the samples at 6 months may still contain maternal antibodies. At the age of 24 months, IgG levels against PspC were significantly increased in children who were noncolonized at the age of 36 months compared to the IgG levels in children colonized at this same age. No other protective associations between protein-specific antipneumococcal antibody levels and colonization were observed.

However, pneumococcal colonization does induce an antibody response close to the time nearest to the colonization moment, as can be seen in Table 3. This may be due to colonization itself or to clinical or subclinical infections.

Colonization at 1.5 months induces both an IgG and an IgM response against several pneumococcal proteins at 6 months and an IgA response against PsaA. IgG levels at 6 months are elevated in the colonized children compared to the children who were noncolonized at 1.5 months (median MFI values for colonized versus noncolonized children, respectively: for BVH-3, 942 versus 392; NanA, 287 versus 112; PpmA, 1,076 versus 180; PsaA, 13,000 versus 6,406; SlrA, 176 versus 16). At 14 months, only the IgG level against SP0189 was significantly elevated in children earlier colonized with the pneumococcus at 1.5 months (Table 3). Colonization at 6 months is correlated with elevated levels of IgG and IgA against several pneumococcal proteins at the time of colonization and later at 14 months but is barely correlated to IgM levels. Children who were colonized at 14 months had higher levels of IgG and IgA to several pneumococcal proteins at 14 months than noncolonized children. Few differences between colonized and noncolonized children at 14 months were noted in the antibody levels at 24 months (Table 3).

Children with frequent colonization in the first 14 months (at least twice) barely showed differences in antibody levels at 24 months compared to the children with no colonization in the first 14 months. Only the level of IgG against PpmA antigens at 24 months was elevated in children with frequent colonization in the first 14 months (data not shown). This may be due to a long time span between colonization and antibody level at 24 months.

Antipneumococcal antibodies and RTIs. Besides colonization, we studied the correlation between systemic antibody levels and the number of doctor visits for RTIs. Table 4 shows all correlations between levels of specific antipneumococcal antibodies and RTIs in the third year of life with P values below 0.08.

Increased levels of IgG against BVH-3, NanA, and SP1003 at 6 months, IgG directed against NanA, PpmA, PsaA, SlrA, SP0189, and SP1002 at 14 months, and IgG against SlrA at 24 months were observed in children with a lack of doctor visits for RTIs in the 3rd year of life compared to children with at least three visits (Table 4 and Fig. 2). The same significant effect was observed for increased levels of IgA against NanA and SP0376 at 6 months and against SP1003 at 14 months. IgM levels at 6 months were not associated with RTIs in the 3rd year of life. At 14 months, however, IgM levels against IgA1 protease and BVH-3 were significantly increased in children with a lack of doctor visits for RTIs in the 3rd year (Table 4).

DISCUSSION

Our study demonstrates that several antipneumococcal protein antibodies are induced upon colonization, possibly due to clinical or subclinical infection during colonization, and that some of these specific antibody levels are also associated with reduced number of doctor visits for RTIs. Because of the change in pneumococcal serotypes causing colonization and infection following the implementation of the polysaccharide-based vaccine, novel protein-based vaccines are needed for prevention of pneumococcal infections. However, data on antibodies against pneumococcal virulence proteins in relation to human colonization and infection are lacking. Our observations are relevant in the context of future pneumococcal protein vaccine development.

We describe that IgG antibodies against BVH-3, NanA, PpmA, PsaA, SlrA, SP0189, and SP1003 are increased in children who suffered fewer respiratory infections in the third year of life, suggesting that these antibodies are either protective or markers of other protective agents. This is in line with data presented by Bogaert et al., who also found anti-PpmA IgG antibodies to significantly protect against RTIs (4).

We did not find evidence for protection against pneumococcal colonization in young children by any of our antipneumo-

TABLE 4. Correlation between protein-specific IgG, IgM, and IgA antibody levels and RTIs in children

Time of measurement and antibody	MFI level in children with indicated no. of RTIs in 3rd year of life[a]		
	Never (n = 29)	≥3 times (n = 5)	P value
6 mo			
IgG			
BVH-3	779 (42–1,931)	133 (99–168)	0.050[b]
SP1003	2,138 (344–15,291)	826 (448–1,205)	0.050[b]
NanA	173 (38–654)	40 (37–43)	0.038[b]
IgA			
Eno	10 (0–217)	1 (0–2)	0.063
IgA1 protease	2,859 (846–6,454)	1,190 (1,061–1,319)	0.064
NanA	21 (0–146)	0 (0–0)	0.022[b]
SP0376	18 (1–329)	3 (2–3)	0.049[b]
SP1003	11 (0–1,102)	0 (0–0)	0.061
14 mo			
IgG			
NanA	701 (46–3,019)	252 (9–865)	0.045[b]
PpmA	985 (0–12,962)	0 (0–296)	0.015[b]
PsaA	13,531 (833–16,944)	4,091 (769–11,533)	0.021[b]
SlrA	58 (0–2,550)	9 (3–20)	0.032[b]
SP0189	127 (31–1,512)	58 (22–96)	0.030[b]
SP1003	9,823 (53–18,717)	522 (27–7,307)	0.021[b]
IgM			
IgA1 protease	202 (0–2,690)	0 (0–97)	0.026[b]
BVH-3	67 (0–1,187)	0 (0–9)	0.041[b]
IgA			
PpmA	14 (0–1,454)	0 (0–62)	0.074
SP1003	870 (1–7,807)	298 (0–571)	0.021[b]
24 mo			
IgG			
PpmA	2,047 (0–8,113)	518 (0–1,101)	0.065
SlrA	335 (15–3,975)	53 (34–74)	0.049[b]
SP1003	14,161 (2,018–18,743)	8,007 (81–11,630)	0.075
IgM			
PspA	40 (0–1,817)	154 (98–234)	0.066[c]
IgA			
SlrA	20 (0–979)	0 (72–712)	0.059

[a] Values are median MFI levels (5 to 95% range), reflecting antigen-specific IgG, IgM, or IgA levels. All correlations with a P value of <0.08 are shown.
[b] P value < 0.05.
[c] Increased levels occurred in children with frequent RTIs.

coccal antibodies (except for IgG levels against PspC), which is in line with experimental studies conducted in mice (36).

However, colonization does induce a humoral immune response against several pneumococcal proteins, some of which are also associated with a lack of doctor visits for RTIs. This suggests a potential protective role of colonization against RTIs in the long run. Pneumococcal colonization may increase the risk of clinical or subclinical pneumococcal infection, inducing an immune response which protects against RTIs in the long run.

Some studies document that maternal antipolysaccharide antibodies prevent colonization and infection in infants and thus propose active immunization of pregnant women (10, 13). The largest effect of such maternal antibodies would be expected to occur at a young age. We did not find any short-term effects of protection by maternal antipneumococcal virulence protein antibodies against both nasopharyngeal colonization and RTIs in children. Although it is known that maternal IgG can cross epithelial barriers and can reach significant levels at the nasopharyngeal mucosal surface, these specific antibodies are not capable of preventing colonization and infection in youngsters, as was previously described for antipolysaccharide antibodies.

It has been shown that pneumococcal elimination by vaccines may lead to elevated colonization levels for other bacterial species. For example, it was demonstrated that prevention of pneumococcal otitis media was counterbalanced by increasing numbers of cases caused by *Staphylococcus aureus* (32). It is unlikely that antiprotein vaccines against the pneumococcus will be different in this respect. However, in our study, we present antipneumococcal antibodies associated with a decreased RTI rate but not associated with protection against colonization. If colonization with *S. pneumoniae* persisted, while RTIs were prevented, other organisms may remain excluded from colonization.

Some limitations of our study should be discussed. Information about RTIs in the children was obtained through parentally reported questionnaires, which may represent an overreport of complaints; parents with high concerns may report infections that are contrary to a doctor's diagnosis. Furthermore, we do not have specific information on the exact timing of RTIs in the children, and as described in Materials and Methods, a misclassification may have occurred due to the grouping of children by number of RTIs. However, if such a misclassification did occur, it would most likely lead to an underestimation of the effect. Another possible limitation is that we studied antibodies in serum. A suggestion for future studies would be to study IgA levels in saliva, which may play an important role in colonization as well. Moreover, we cannot distinguish whether serum antibodies protect directly or indirectly as part of a more extensive immune response in which protection can be generated by other immunological factors. Alternatively, there may be diffusion to mucosal surfaces or the antibodies may be produced locally following colonization. In other words, the antipneumococcal antibodies may protect directly, they may be markers of alternative responses, or they may result from a combination of these possibilities. In addition, it is possible that the findings presented here simply reflect immune system maturation. Those children with more mature immune systems may have high antibody levels and may remain healthier than those with less mature immune systems. Unfortunately, based on the current study, we are unable to choose one scenario over another. Finally, our study was conducted using only 57 children; the results should be confirmed in a larger sample size.

In conclusion, levels of antibodies against diverse pneumococcal virulence proteins are correlated with a reduced frequency of doctor visits for RTIs in children. This was not due to protection of the specific antibodies against asymptomatic colonization; rather, antibodies resulted from colonization. This study adds to the discussion on improvement of the current preventive strategies against pneumococcal disease. Future studies should aim toward developing protein-based vaccines. In particular, the effect of a combination of several

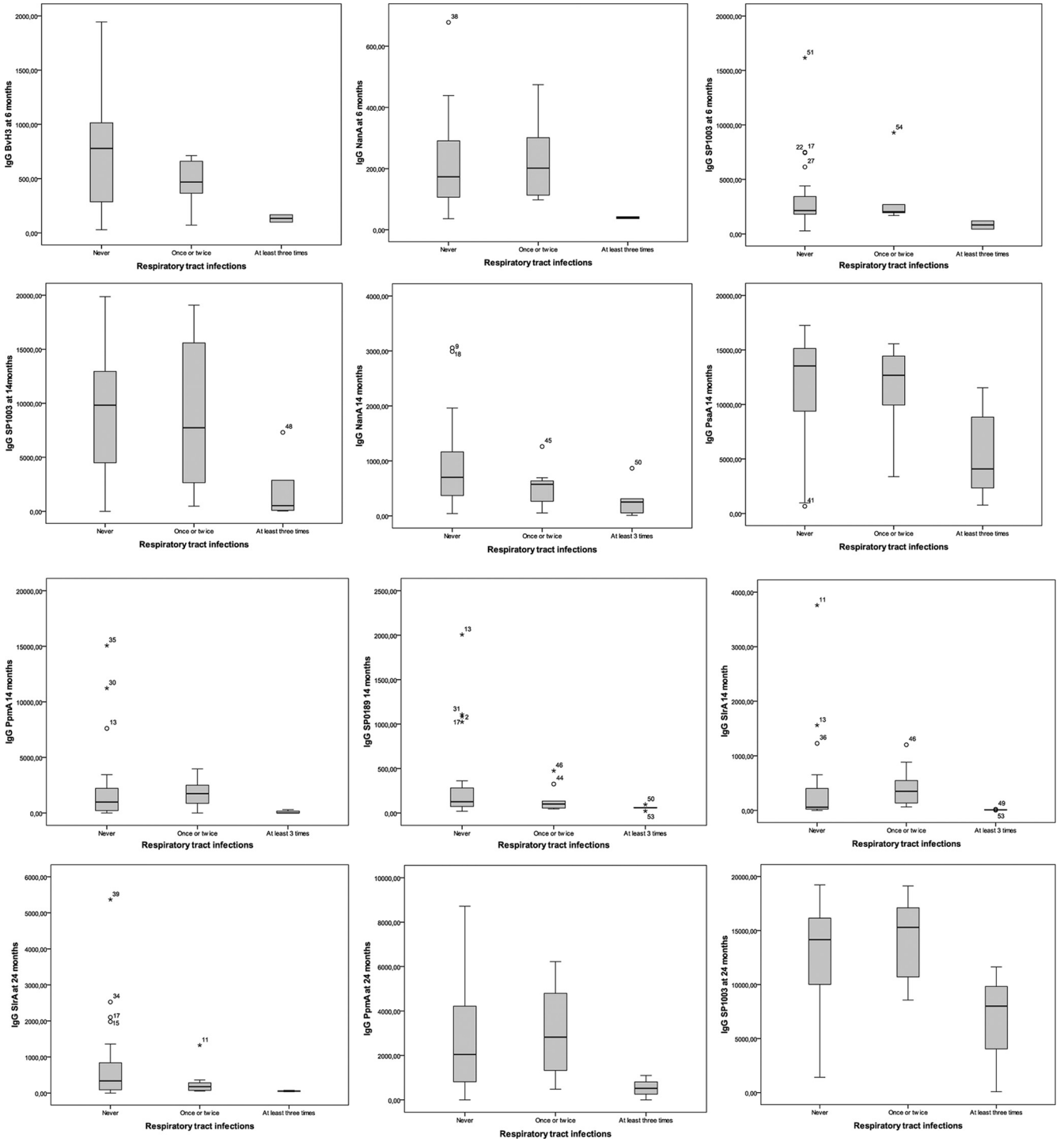

FIG. 2. Association between specific antipneumococcal IgG levels and RTIs in the third year of life. IgG levels at 6, 14, and 24 months are compared with the numbers of doctor visits for RTIs in the 3rd year of life. Higher levels of IgG against certain pneumococcal proteins were correlated with a lack of doctor visits for RTIs (first box in every box plot). The median level of IgG against certain pneumococcal proteins was lower in children with at least 3 doctor visits for RTIs (third box in every box plot). This was statistically significant for all pneumococcal proteins presented in these box plots, except for PpmA and SP1003 at 24 months ($P = 0.065$ and 0.075, respectively). The analyses were conducted for the group of children with at least three doctor visits versus children with a lack of doctor visits. Results for children with one or two doctor visits were included in this figure but not analyzed. *P* values are presented in Table 4. Values are median MFI levels, with an interquartile (25 to 27%) box, a 5 to 95% range, outliers (○), and extreme outliers (∗). Numbers represent child identification numbers.

pneumococcal proteins and their correlate of protection in humans should be studied.

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