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Higher levels of mucosal antibody to pneumococcal vaccine candidate proteins are associated with reduced acute otitis media caused by *Streptococcus pneumoniae* in young children

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Mucosal immunity has a crucial role in controlling human respiratory tract infections. This study characterizes the naturally acquired mucosal antibody levels to three *Streptococcus pneumoniae* (*Spn*) protein antigens, pneumococcal histidine triad protein D (PhtD), pneumococcal choline binding protein A (PcpA), and pneumolysin (Ply), and assesses the association of the mucosal antibody levels with occurrence of acute otitis media (AOM) caused by *Spn.* Both nasopharyngeal (NP) immunoglobulin G (IgG) and IgA levels to all three proteins slightly decreased in children from 6 to 9 months of age and then gradually increased through 24 months of age. *Spn* NP colonization was associated with higher mucosal antibody levels to all three proteins. However, children with *Spn* AOM had 5–8-fold lower IgG and 3–6-fold lower IgA levels to the three proteins than children without AOM but asymptomatically colonized with *Spn*. Antigen-specific antibody levels in the middle ear fluid (MEF) were correlated with antibody levels in the NP. Children with AOM caused by *Spn* had lower antibody levels in both the MEF and NP than children with AOM caused by other pathogens. These results indicate that higher naturally acquired mucosal antibody levels to PhtD, PcpA and Ply are associated with reduced AOM caused by *Spn*.

INTRODUCTION

Streptococcus pneumoniae (*Spn*) is responsible for a large spectrum of infectious diseases in children and adults such as sepsis, pneumonia, meningitis, sinusitis, and acute otitis media (AOM).[1–3] To date, 94 distinct serotypes have been documented based on capsular composition.[3,4] Current licensed 23-valent pneumococcal polysaccharide vaccine (PPV-23) and 7-, 10-, and 13-valent pneumococcal conjugate vaccines (PCV-7, -10, -13) significantly reduce invasive pneumococcal diseases,[1,5] but coverage is limited to the vaccine serotypes. After introduction of PCV-7, and more recently PCV-13, there has been a decrease in carriage caused by vaccine serotypes but an emergence of non-vaccine replacement serotypes.[1,3,6–8] Furthermore, current polysaccharide-based vaccines are less effective in non-invasive pneumoniae and AOM.[9,10] In addition, PPV-23 is currently recommended in the elderly and high-risk adults. It is poorly immunogenic in children, especially <2 years of age.[11] Because of these shortcomings, protein-based vaccine candidates have been sought to replace or complement current polysaccharide-based vaccines.[9,12,13]

A number of pneumococcal protein antigens have been studied as vaccine candidates against pneumococcal infection, and multiple proteins have shown sufficient promise to enter human clinical trials.[12,13] Monovalent protein vaccine candidates of PhtD (pneumococcal histidine triad protein D),[14] PcpA (pneumococcal choline binding protein A),[15] PspA (pneumococcal surface protein A),[12] and dPly (detoxified pneumolysin),[16] bivalent protein vaccine candidates PhtD/PcpA,[15] PspA/PsaA (pneumococcal surface adhesin A),[12] PhtA/PhtB,[12] and PhtD/Ply alone or along with PCV-7, (ref 12) and trivalent protein vaccine candidates PhtD/PcpA/PlyD1 and [12] PhtD/Ply with protein D of *Haemophilus influenzae*[13] were or are in human clinical trials. Our laboratory has been studying three pneumococcal proteins—PhtD, PcpA, and

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PlyD1 (genetic detoxified pneumolysin), which are the components of a candidate vaccine in a trial sponsored by Sanofi Pasteur. PhtD is a divalent cation-regulated surface protein, shown to elicit protection against nasopharyngeal (NP) colonization, and is highly conserved across pneumococcal serotypes.[17] PcpA is another divalent cation-regulated surface protein that has a major role in pneumococcal adherence.[15] Both PhtD and PcpA have been shown to be involved in adherence of *Spn* to human NP epithelial cells *in vitro*.[18,19] Ply is a cholesterol-dependent secreted cytolysin, which is a key virulence factor contributing to bacterial pathogenesis at both early and late stages of infection.[16] In mice studies, immunization with PhtD, PcpA, and PlyD1 have been shown to elicit protection.[20–24] In our previous studies, we have detected specific antibodies to PhtD, PcpA, and PlyD1 antibodies in children from natural exposure to *Spn* in sera after NP colonization and AOM.[25] However, upon further analysis, no correlation between serum antibody titers to these proteins and protection of occurrence of *Spn* AOM could be identified (unpublished data). We therefore hypothesize that mucosal immunity has a critical role in the control of pneumococcal mucosal diseases, such as AOM, sinusitis, and non-bacteremic pneumonia.

Although *Spn* NP colonization is a necessary prerequisite for infections to develop, carriage is mostly asymptomatic.[10] However, when the condition of the host is altered, such as by an upper respiratory viral infection, *Spn* may cause AOM.[26] Unfortunately, the human mucosal immune response against pneumococci[10] and to pneumococcal proteins after natural *Spn* exposure and AOM is poorly understood. In the present study, we characterized the induced mucosal antibody levels in the NP to PhtD, PcpA, and PlyD1 and assessed the association of these antibody responses with the occurrence of natural *Spn* AOM infections in children aged 6–24 months. In addition, in a previous study, we found middle ear fluid (MEF) antibody in humans originates predominantly from sera and NP secretions.[27] Here we assessed the correlation of antibody levels in NP secretions with MEF.

RESULTS

Study cohort

This analysis involved a total of 424 NP and 152 MEF samples collected during 234 health and 208 AOM visits from 176 children between the ages of 6 and 24 months. One hundred and thirty-three (76%) children had both health and AOM visits and 43 (24%) children had only AOM visits. The characteristics of the children are shown in **Table 1**. As our group has previously shown age-related differences in serum antibody response to the studied antigens,[25,28] the NP and MEF samples for this study were age matched.

Table 1 Characteristics of children ($n = 176$)

	Number (%)
Female	80 (45%)
Male	96 (55%)
Breast feed	55 (31%)
Formula	67 (38%)
Both	54 (31%)
Smoke exposure	13 (7%)
Non-smoke exposure	163 (93%)
Daycare	70 (40%)
Non-daycare	106 (60%)
PCV-7 up-to-date	176 (100%)

Abbreviation: PCV-7, 7-valent pneumococcal conjugate vaccine.

Natural acquisition of mucosal antibody in the NP over time

First, we evaluated the natural acquisition of NP mucosal antibody in 233 NP samples from 55 children who had at least 4 sequential health visits during the study period. **Figure 1** shows the geometric means (GM) of antibody levels to PhtD, PcpA, and PlyD1 in the NP of children aged 6, 9, 12, 15, 18, and 24 months. Both IgG and IgA antibodies to all three *Spn* proteins decreased from 6 to 9 months and then increased steadily over time with a peak at 18–24 months of age. The NP antibody levels were associated with age for both IgG and IgA.

Mucosal antibody levels in the NP of *Spn*-colonized vs. non-colonized children

Next, we sought to determine differences in detectable antibodies in nasal secretions in *Spn*-colonized vs. non-colonized children. It is well established that *Spn* colonization in children occurs in the first 6 months of life.[29] Therefore, we anticipated that detectable mucosal antibodies would be present at the first sampling of our study cohort at 6 months of age. Our hypothesis was that detectable *Spn* colonization would result in higher mucosal IgG and IgA antibody levels to the pneumococcal proteins. We compared IgG and IgA levels to PhtD, PcpA, and PlyD1 in NP samples from children who had *Spn* detected in their NP secretions at the time of sampling vs. those who were *Spn* culture-negative. Children with *Spn* had significantly higher GM of specific IgG and IgA antibody in the NP than *Spn* culture-negative children (**Figure 2**). There was a fivefold higher GM of IgG anti-PhtD (4.39 vs. 0.87 ng μg^{-1}, $P < 0.0001$), a fivefold higher GM of IgG anti-PcpA (5.93 vs. 1.11 ng μg^{-1}, $P < 0.0001$), and a twofold higher GM of IgG anti-PlyD1 (1.20 vs. 0.54 ng μg^{-1}, $P = 0.001$). The NP IgA levels to PhtD, PcpA, and PlyD1 were higher by twofold (11.38 vs. 4.67 ng μg^{-1}, $P < 0.0001$), twofold (5.49 vs. 2.33 ng μg^{-1}, $P < 0.0001$), and fourfold (0.75 vs. 0.19 ng μg^{-1}, $P = 0.0003$) in children colonized with *Spn* vs. non-colonized, respectively.

Higher mucosal GM of antibody levels in the NP are associated with reduced AOM caused by *Spn*

We next investigated whether there is an association of mucosal antibody levels with development of *Spn* AOM. We compared antibody levels with the pneumococcal proteins in the NP between children with *Spn* AOM and healthy children asymptomatically colonized with *Spn*. We found that children

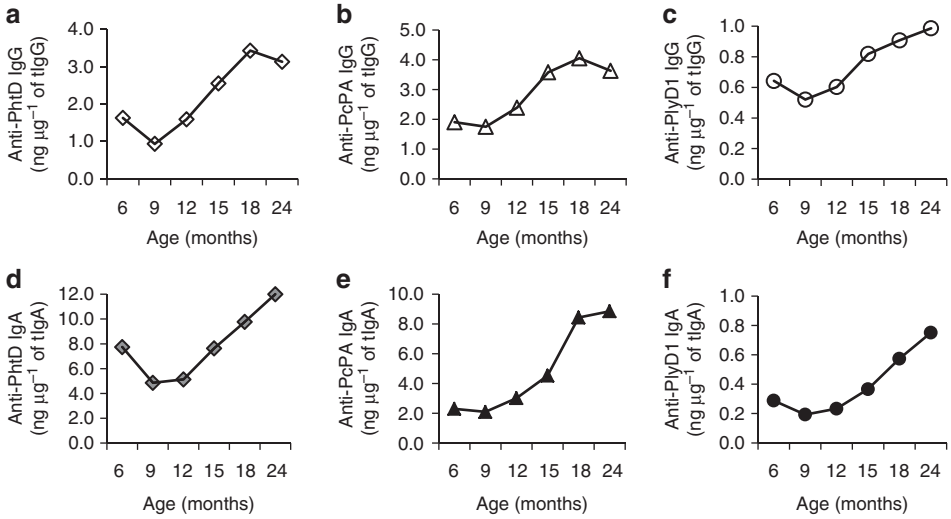

Figure 1 Natural acquisition of nasopharyngeal (NP) mucosal antibody over time. NP samples were collected from children who had at least four regular perspective visits to determine the rates of pneumococcal specific to total immunoglobulin G (IgG) and IgA. Antibody levels were expressed as geometric means of the ratio of specific to total antibody, and association between age and antibody levels was analyzed using repeated-measures logistic regression. $N = 47, 45, 38, 35, 33$, and 34 for 6, 9, 12, 15, 18, and 24 months of age, respectively. (**a**), Anti-PhtD IgG; (**b**), Anti-PcpA IgG; (**c**), Anti-PlyD1 IgG; (**d**), anti-PhtD IgA; (**e**), Anti-PcpA IgA; (**f**), Anti-PlyD1 tIgA; tIgG and tIgA, total amount of IgG and IgA.

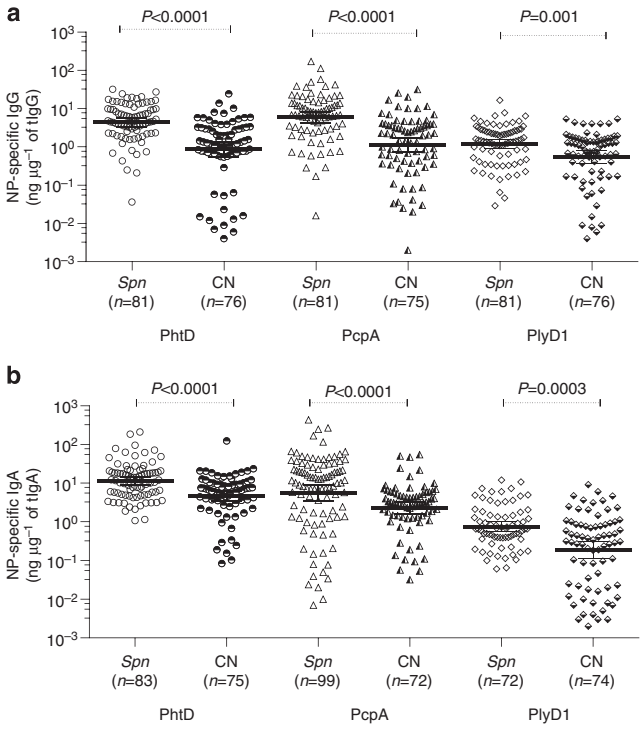

Figure 2 Colonized-children by *Streptococcus pneumoniae* (*Spn*) had higher mucosal geometric means (GM) of antibody levels than non-colonized children. Nasopharyngeal (NP) samples were collected from age-matched healthy children with or without *Spn* colonization. The rates of pneumococcal specific to total immunoglobulin G (IgG) and total IgA were determined by enzyme-linked immunosorbent assay and compared using the non-parametric Mann–Whitney test between culture positive and negative for *Spn*. (**a**), Anti-PhtD, anti-PcpA, and anti-PlyD1 IgG; (**b**), anti-PhtD, anti-PcpA, and anti-PlyD1 IgA; CN, culture negative; tIgG and tIgA, total amount of IgG and IgA. Lines represent GM with 95% confidence intervals.

who developed *Spn* AOM had significantly lower mucosal IgG and IgA levels in their NP to all three studied *Spn* proteins compared with *Spn*-colonized children who did not progress to AOM (**Figure 3a,b**). The NP GM of antibody levels of children with *Spn* AOM were eightfold lower in anti-PhtD IgG (0.56 vs. 4.39 ng μg^{-1}, $P < 0.0001$), fivefold lower in anti-PcpA IgG (1.18 vs. 5.93 ng μg^{-1}, $P < 0.0001$), fourfold lower in anti-PlyD1 IgG (0.29 vs. 1.19 ng μg^{-1}, $P < 0.0001$), fourfold lower in anti-PhtD IgA (2.87 vs. 11.38 ng μg^{-1}, $P < 0.0001$), sixfold lower in anti-PcpA IgA (0.97 vs. 5.49 ng μg^{-1}, $P < 0.0001$), and threefold lower in anti-PlyD1 IgA (0.28 vs. 0.75 ng μg^{-1}, $P = 0.001$) compared with those of healthy children. GM of antibody levels in NP were negatively associated with occurrence of *Spn* AOM in anti-PhtD IgG (odds ratio (OR) = 0.04, 95% confidence interval (CI) 0.02–0.09, $P < 0.0001$), anti-PcpA IgG (OR = 0.06, 95% CI 0.03–0.12, $P < 0.01$), anti-PlyD1 IgG (OR = 0.24, 95% CI 0.15–0.37, $P = 0.0001$), anti-PhtD IgA (OR = 0.03, 95% CI 0.01–0.06, $P < 0.0001$), anti-PcpA IgG (OR = 0.02, 95% CI 0.00–0.07, $P < 0.0001$), and anti-PlyD1 IgG (OR = 0.13 95% CI, 0.07–0.24, $P = 0.0008$).

A similar association was found in the NP GM of antibody levels between children with AOM caused by *Spn* and children with AOM caused by other pathogens. At the onset of AOM, when co-colonization in the NP of *Spn* with other potential pathogens occurs, the children with *Spn* AOM have slightly lower GM of IgG but significantly lower GM of IgA to all three pneumococcal proteins than children with non-*Spn* AOM (**Figure 3c,d**). Compared with children with non-*Spn* AOM, children with *Spn*-AOM had twofold lower GM of anti-PhtD IgG (0.57 vs. 1.10 ng μg^{-1}, $P = 0.08$), twofold lower GM of anti-PcpA IgG (1.18 vs. 2.82 ng μg^{-1}, $P = 0.02$), and twofold lower GM of anti-PlyD1 IgG (0.29 vs. 0.63 ng μg^{-1}, $P = 0.06$). The GM of IgA in the NP of children with *Spn* AOM were fourfold lower in anti-PhtD (2.88 vs. 12.25 ng μg^{-1}, $P < 0.0001$), fivefold lower in anti-PcpA (0.97 vs. 5.21 ng μg^{-1}, $P < 0.0001$) and threefold lower in anti-PlyD1 (0.28 vs. 0.80 ng μg^{-1}, $P = 0.001$).

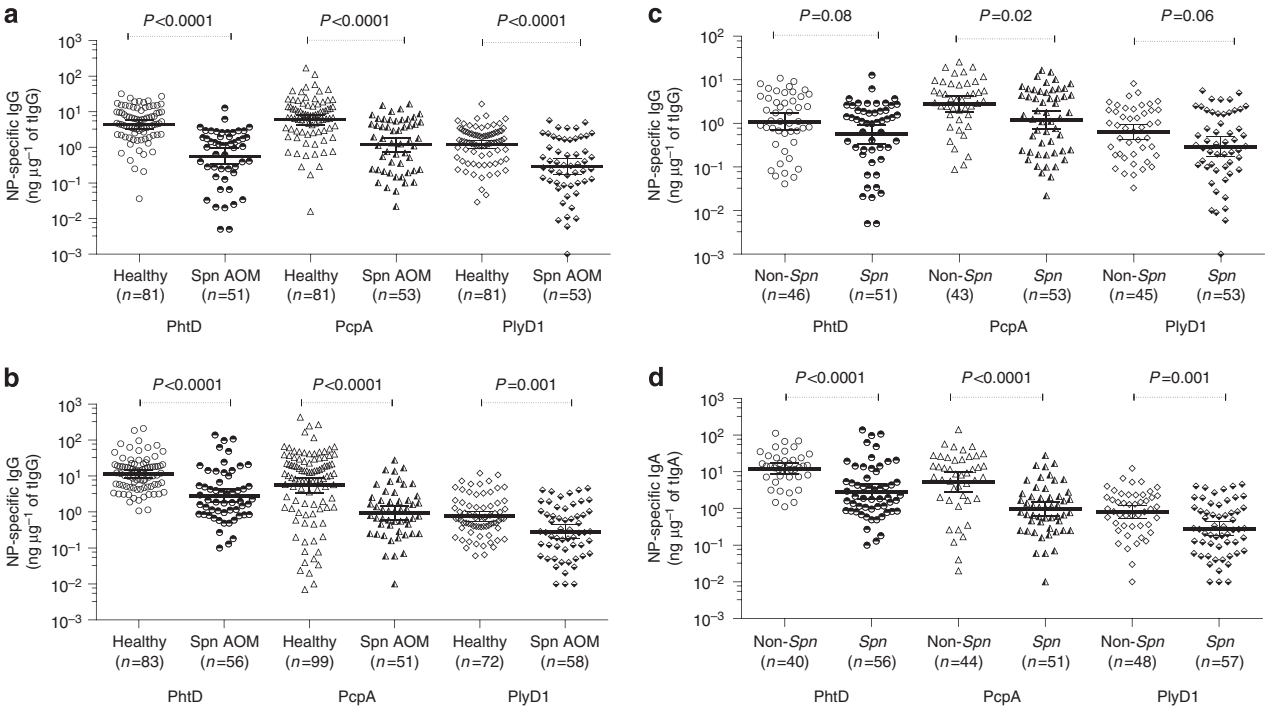

Figure 3 Higher mucosal antibody levels in the nasopharyngeal (NP) are associated with reduced acute otitis media (AOM) caused by *Streptococcus pneumoniae* (*Spn*). NP samples were collected from age-matched healthy *Spn*-colonized asymptomatic children and children with AOM. The levels of pneumococcal specific to total immunoglobulin G (IgG) and IgA (ng μg^{-1}) were compared (**a** and **b**) between children with *Spn* AOM and children with asymptomatic *Spn* colonization and (**c** and **d**) between children with *Spn* AOM and children with non-*Spn* AOM using the Mann–Whitney test. (**a** and **c**): Anti-PhtD, anti-PcpA, and anti-PlyD1 IgG; (**b** and **d**): anti-PhtD, anti-PcpA, and anti-PlyD1 IgA; tIgG and tIgA, total amount of IgG and IgA. Lines represent geometric means with 95% confidence intervals.

Children with *Spn* AOM have lower GM of antibody levels in the MEF than children with non-*Spn* AOM

We also examined the MEF antibody levels to the three studied *Spn* proteins in children with AOM. At the onset of AOM, children with *Spn* AOM, compared with children who experienced AOM caused by other otopathogens, had twofold lower GM of anti-PhtD IgG (0.47 vs. 1.64 ng μg^{-1}, $P = 0.0002$), fivefold lower GM of anti-PcpA IgG (0.63 vs. 3.37 ng μg^{-1}, $P = 0.001$), and threefold lower GM of anti-PlyD1 IgG (0.17 vs. 0.47 ng μg^{-1}, $P = 0.03$) (**Figure 4a**). The MEF IgA GM in *Spn* AOM children were threefold lower in anti-PhtD (1.87 vs. 5.81 ng μg^{-1}, $P = 0.002$), eightfold lower in anti-PcpA (0.82 vs. 6.17 ng μg^{-1}, $P < 0.0001$) and fourfold lower in anti-PlyD1 (0.2 vs. 0.9 ng μg^{-1}, $P < 0.0001$) (**Figure 4b**).

Correlation between antibody levels in the NP and antibody levels in MEF at AOM

Antibody in NP secretions refluxes from the NP through the Eustachian tube to the middle ear during viral upper respiratory infections. We have previously shown that MEF antibody originates predominantly from sera and NP secretions.[27] Therefore, we selected available paired NP and MEF samples simultaneously collected from the same children to analyze the correlation between antibody levels in the NP and MEF. Because antibodies were found to correlate significantly with age, to control potential spurious latent correlation, antibody levels were adjusted for age. Both IgG and IgA levels to all three *Spn* antigens in the NP were significantly and positively correlated with those in MEF of children at the onset of AOM (all *P*-values < 0.01) (**Figure 5**).

DISCUSSION

Mucosal immunity is thought to have a crucial role in the control of respiratory tract infections.[30] Here we address gaps in the knowledge of immune response mounted by children who experience *Spn* NP colonization and AOM. We characterized the natural acquisition of mucosal antibody to *Spn* candidate vaccine proteins in young children who experience *Spn* colonization and consequent AOM. IgG and IgA antibody levels to PhtD, PcpA, and PlyD1 in nasal secretions increased over time, suggesting that sequential natural exposure to *Spn* results in boosting antibody in mucosal NP secretions. We found that *Spn* colonization of the NP was associated with higher mucosal antibody responses to the vaccine candidate antigens studied vs. non-*Spn* colonized NP samples. The results are consistent with previous observations regarding serum antibody responses to the same antigens.[25,31] Most importantly, we found that children who developed *Spn* AOM had lower levels of IgG and IgA in nasal secretions at the onset of AOM compared with the antibody levels of children who were NP colonized with *Spn* but did not progress to AOM infection. Furthermore, when children developed *Spn* AOM they had lower IgG and IgA levels in their MEF to all three studied proteins compared with children who experienced AOM

caused by other otopathogens. These results suggest an association between higher NP antibody levels to *Spn* proteins and a significantly reduced risk of *Spn* AOM.

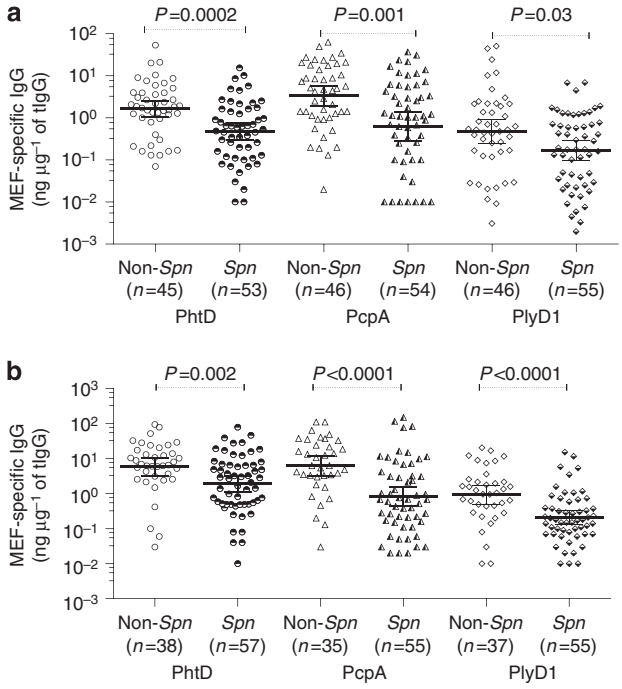

Figure 4 Higher mucosal antibody levels in the middle ear fluid (MEF) are associated with reduced acute otitis media caused by *Streptococcus pneumoniae* (*Spn*). Nasopharyngeal samples were collected from age-matched healthy children. The levels of pneumococcal specific to total immunoglobulin G (IgG) and IgA (ng μg^{-1}) were compared using the Mann–Whitney test. (**a**): Anti-PhtD, anti-PcpA, and anti-PlyD1 IgG; (**b**): anti-PhtD, anti-PcpA, and anti-PlyD1 IgA; tIgG and tIgA, total amount of IgG and IgA. Lines represent geometric means with 95% confidence intervals.

Although numerous studies have shown that serum antibodies rise following pneumococcal carriage,[25,31–34] very limited information is available regarding mucosal antibody responses to respiratory bacterial pathogens in humans, especially infants and children. In large part, this is because reproducible collection of nasal secretions is challenging due to variability in acquiring mucus from the NP. Saliva is another source for study of mucosal immunity, and its collection is easier than NP samples. Zhang *et al.*[35] found that children aged 2–12 years who were culture-positive for *Spn* in their NP had higher IgG but not IgA in serum and saliva to pneumococcal choline-binding protein A (CbpA) and Ply but not PsaA or PspA. However, NP mucosal antibody assays are more reproducible and reliable than saliva antibody levels, because saliva antibody levels are significantly influenced by many variables, such as saliva flow.[36,37] There are no previous reports on mucosal antibody to PhtD, PcpA, and Ply in nasal secretions of young children by natural *Spn* NP colonization. The results of this study, along with our prior findings regarding serum antibody responses to *Spn* NP colonization in the same cohorts of subjects,[25,31] clearly show that *Spn* colonization is an immunizing event for both systemic- and mucosal-acquired immune responses. In addition, PhtD, PcpA, and Ply are highly immunogenic following natural exposure to *Spn*.

The elicitation of serum antibody following NP colonization by potential respiratory pathogens has been well documented in previous studies. Holmlund *et al.*[32] reported an increase in serum antibody concentrations to PsaA and Ply in infants who were colonized with *Spn*. Simell *et al.*[33] showed that children with prior positive NP cultures for *Spn* had significantly higher serum anti-CbpA and anti-PhtD IgG titers. Prevaes *et al.*[34] reported that colonization with *Spn* induced serum IgG against 14 pneumococcal proteins. Verkaik *et al.*[38] found that children aged 6–24 months colonized by *Staphylococcus aureus* had

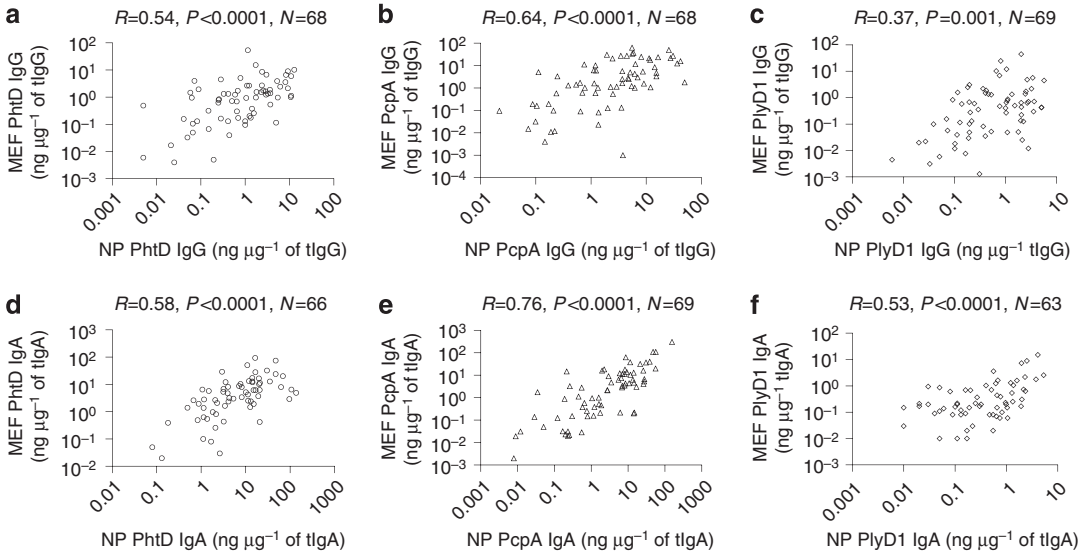

Figure 5 Correlations of antibody levels in the nasopharyngeal (NP) to middle ear fluid (MEF). Paired NP and MEF samples simultaneously collected from the same visit of the same children, and correlation of immunoglobulin G (IgG) and IgA levels in NP to MEF was analyzed using the Spearman's coefficient. (**a**), Anti-PhtD IgG; (**b**), anti-PcpA IgG; (**c**), anti-PlyD1 IgG; (**d**), anti-PhtD IgA; (**e**), anti-PcpA IgA; (**f**), anti-PlyD1 IgA.

higher serum IgG and IgA levels to a number of staphylococcal proteins than non-colonized children. Our research group previously reported that colonization with either S*pn* or *H. influenzae* elicited serum IgG and IgA responses to homologous bacterial species.[25,28,31]

The most important observation in this report is the identification of an association between higher mucosal antibody levels in NP and MEF to PhtD, PcpA, and PlyD1 and reduced risk of AOM. This implies the NP mucosal antibody levels to PhtD, PcpA, and Ply may have an important role in preventing *Spn* AOM in young children. Both IgG and IgA antibody levels in MEF are significantly associated with antibody levels detected in nasal secretions. Also of great relevance to vaccine design, we found that antibodies to these three vaccine candidate proteins did not require eradication of *Spn* from the NP to positively impact the occurrence of *Spn* AOM. We hypothesize that protection from AOM occurred by reduction of the effective *Spn* inoculum (bacterial load) in the NP below a pathogenic threshold level. We are initiating a study to investigate this notion.

Our study has limitations. The colonization in our study was defined based on bacterial cultures at a moment in time when samples were collected. We did not determine NP bacterial or viral loads that have been proven to be critical variants influencing host immune responses.[39] The antibody levels in this study were free antibody. The results might be influenced by bacterial load, bacterial agglutination with antibody, and soluble antigens (e.g., pneumolysin) released from *Spn*. The NP is the ecological niche for a variety of commensal microbiota as well as potential respiratory disease causing bacteria and viruses.[40,41] Cross-reactive antigens that are expressed by other non-pneumococcal species (e.g., *Streptococcus mitis*) may influence the detected antibody titers. Capsule is regarded as a critical virulence factor for pathogenesis of pneumococcal infections. We did not measure mucosal anti-capsular titers and thus do not exclude the possibility that *Spn* AOM resulted from lack of capsular antibody to the *Spn* serotypes. In addition, 5% of MEF and 20% of NP samples that had undetectable or very low total antibody levels were excluded in this study. As these samples distributed randomly to each age, colonization, and AOM group, and represented a small fraction of the total samples, the exclusion of those samples had limited influence on the conclusions of this study.

In summary, this is the first report to characterize the natural acquisition of mucosal NP and MEF antibody responses to *Spn* candidate vaccine proteins PhtD, PcpA, and PlyD1 in young children who experience *Spn* colonization and AOM. We found that colonization by *Spn* is positively associated with mucosal antibody levels to PhtD, PcpA, and PlyD1 proteins. Most importantly, we identified an association of higher mucosal antibody levels in the NP to PhtD, PcpA, and PlyD1 with reduced AOM caused by *Spn*. The results imply that NP mucosal antibody against PhtD, PcpA, and Ply are potential markers of anti-pneumococcal immunity and that these three proteins may elicit protection against *Spn* mucosal infections, especially AOM.

METHODS

Study design. This study derives from a 5-year (2006–2011) prospective longitudinal evaluation of immunity to *Spn* and NTHi NP colonization and AOM in young children aged 6–24 months, supported by the US National Institute of Deafness and Communication Disorders. Healthy children without previous episodes of AOM were enrolled at 6 months of age from a middle class, suburban sociodemographic pediatric practice in Rochester, NY (Legacy Pediatrics). NP samples were obtained every 3–6 months prospectively from healthy children aged 6–24 months. When AOM occurred, tympanocentesis was performed to collect MEF and confirm the diagnosis of AOM, as previously described.[42] At the time of an AOM diagnosis, NP and MEF samples were concurrently obtained. All children in this study who developed an AOM had common clinical symptoms of viral upper respiratory infection, such as cough, sore throat, runny nose, nasal congestion, headache, low-grade fever, and sneezing. All of the children received standard vaccinations, including the PCV-7 or PCV-13 (Prevnar, Pfizer Pharmaceuticals, Collegeville, PA), at the appropriate age. The study was approved by the Institutional Review Board of the University of Rochester and Rochester General Hospital, and written informed consent was obtained from parents or guardians of all child subjects.

Sample collection. NP swab samples were obtained by inserting a cotton-tipped wire swab deeply into both nares. NP wash samples were obtained by instilling 1 ml of sterile phosphate buffered saline and aspirating from both nares for antibody measurement. MEF samples for antibody measurement varied in quantity of material obtained from 50 to 250 µl, and the entire sample was added to 1 ml of phosphate buffered saline (pH 7.4). The NP wash samples and MEF samples were centrifuged at 3000 r.p.m. (1100 g) at 4 °C for 10 min, and the supernatants were stored at − 80 °C until use. NP swab samples and MEF samples were for microbiological culture, and NP wash samples and MEF samples were for antibody measurements.

Microbiology. Three potential bacterial pathogens, *Spn*, *H. influenzae*, and *Moraxella catarrhalis*, were isolated and identified according to tests of the eighth edition of the Manual of Clinical Microbiology.[43]

Quantitative enzyme-linked immunosorbent assay (ELISA) to detect pneumococcal antigen-specific IgG and IgA antibodies. PhtD, PcpA, and PlyD1-specific antibody IgG and IgA concentrations in the NP and MEF were determined by quantitative ELISA as previously described[31] with modification. Briefly, 384-well high-binding microplates (Greiner Bio-One, Monroe, NC) were coated with 20 ng of individual purified recombinant proteins in 20 µl of coating buffer (bicarbonate, pH 9.6) at 4 °C overnight and then blocked with 60 µl of phosphate buffered saline containing 4.0% skim milk at 37 °C for 1 h. The samples were twofold serially diluted in 20 µl phosphate buffered saline containing 4.0% skim milk at an initial dilution of 1:5 and incubated at room temperature for 1 h, followed by an incubation with horseradish peroxidase-conjugated anti-human IgG or IgA (Bethyl Laboratories, Montgomery, TX). The reaction products were developed with TMB Microwell Peroxidase Substrate System (KPL, Gaithersburg, MD), stopped by 20 µl of 1.0 M phosphoric acid, and read by an automated ELISA reader (Molecular Devices, Sunnyvale, CA) using a 450 nm filter. A standard curve was generated using a four-parameter logistic-log function and a reference human serum containing known specific antibody concentrations to corresponding individual antigens. Antigen-specific IgG and IgA levels against the three pneumococcal antigens in the reference serum were quantified using Human IgA and IgG ELISA Quantitation Sets (Bethyl Laboratories, Montgomery, TX) as described previously.[31] The assay lower limits of detection were 2.4 ng ml^{-1} for anti-PhtD IgG, 0.18 ng ml^{-1} for anti-PhtD IgA; 2.2 ng ml^{-1} for anti-PcpA IgG, 0.28 ng ml^{-1} for anti-PcpA IgA, 1.0 ng ml^{-1} for anti-PlyD1 IgG, and 0.22 ng ml^{-1} for anti-PlyD1 IgA. An internal control was included in each plate for each antigen and the inter-assay coefficient of variation

was $\leq$ 20% for all antigens and secondary antibody combinations. For the purpose of statistical analysis, undetectable samples were arbitrarily assigned a value equivalent to half the lower limit of detection of corresponding specific antibodies. ELISAs were fully validated according to ICH Guidance and performed in a GLP laboratory.

To correct for differential dilution effects that occurred during the NP wash and MEF sample collection, total IgG and IgA were determined using the Human IgG and IgA ELISA Quantitation Kits (Bethyl Laboratories) according to the manufacturer's protocol. Mucosal antigen-specific IgG and IgA in NP and MEF samples were then corrected according to the total IgG or IgA in the sample. Results were expressed as a ratio of specific IgG to total IgG or specific IgA to total IgA in the same sample ($ng\,\mu g^{-1}$) as described previously.[44,45] Samples with a total IgG or IgA $<0.05\,\mu g\,ml^{-1}$ were excluded, because in preliminary studies we determined that such samples were from children with a difficult or failed sampling process and had undetectable antigen-specific antibodies. In all 5% of MEF and 20% of NP samples were excluded in this study according to this criterion.

Statistics. Statistical analysis was performed with the R Project version 2.13.2 (www.r-project.org/). Antibody levels were expressed as GM with 95% CIs of ratios of specific to total IgG or IgA. The associations between antibody levels and AOM occurrences and ages were analyzed using Generalized Estimating Equations (GEE) to fit a repeated-measures logistic regression models. The AOM ORs between the 95th and 5th percentile antibody level were estimated using GEE models with AR1 subject level correlation and quadratic terms. The paired NP and MEF samples that were collected from the same AOM visits of the same subject were used to analyze the correlation between antibody levels in the NP and MEF. Because antibodies were found to correlate significantly with age, to control for a spurious latent correlation, antibody levels were adjusted for age using a GEE model, assuming a within-subject auto-regressive correlation. Antibody levels were subject to Box–Cox power transformations, and age was log-transformed.[46] CIs and levels of significance for (age-adjusted) antibody correlations were estimated using a bootstrap procedure with subject-level resampling. Antibody levels between age-matched groups (colonization, non-colonization, *Spn* AOM, non-*Spn* AOM groups) were compared using the non-parametric two-tailed Mann–Whitney test with GraphPad Prism 6.0 (La Jolla, CA). $P<0.05$ was considered to indicate statistical significance.

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DISCLOSURE

The authors declare no conflict of interest.

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