# Antibody response to *Streptococcus pneumoniae* proteins PhtD, LytB, PcpA, PhtE and Ply after nasopharyngeal colonization and acute otitis media in children

Michael E. Pichichero,[1,*] Ravinder Kaur,[1] Janet R. Casey,[2] Qingfu Xu,[1] Anthony Almudevar[3] and Martina Ochs[4]

[1]Rochester General Hospital Research Institute; Rochester, NY USA; [2]Legacy Pediatrics; Rochester, NY USA; [3]Department of Biostatistics; University of Rochester School of Medicine; Rochester, NY USA; [4]Sanofi pasteur; Toronto, ON Canada

We prospectively compared serum antibody levels of five *Streptococcus pneumoniae* (*Spn*) proteins: PcpA PhtD, PhtE Ply and LytB associated with nasopharyngeal (NP) colonization and acute otitis media (AOM) infection in a cohort of 6–30 mo old children. Antigen-specific antibody titers were determined by ELISA. A total of 731 visits among 168 children were studied. There were 301 *Spn* NP colonization episodes documented in 109 (65%) children and 42 *Spn* AOM episodes in 34 (20%) children. IgG antibody titers to the 5 proteins were significantly different among children over time (p < 0.001), with a rank order as follows: PcpA > PhtE = PhtD > Ply > LytB Characterization of IgG and IgM acute and convalescent serum antibody levels after *Spn* AOM infection showed the kinetics of the response differed among children, with the same rank order of antibody levels over time. Individual data showed that some children responded to AOM with an antibody increase to one or more of these *Spn* proteins but some children failed to respond. We conclude that antibody levels to *Spn* proteins PcpA PhtD, PhtE, Ply and LytB, all rise over time in children age 6 to 30 mo following natural exposure to *Spn* after NP colonization and AOM; however, there were significant differences in quantity of antibody elicited among these potential vaccine antigens.

## Introduction

*Streptococcus pneumoniae* (*Spn*) is a frequent cause of episodic and recurrent acute otitis media (AOM) in children in the United States.[1-3] AOM and all respiratory bacterial infections begin pathogenesis with nasopharynx (NP) colonization. However, colonization is mostly asymptomatic; only when the condition of the host is altered, *Spn* may invade the middle ear, causing AOM.

A vaccine against *Spn* based on the organism's proteins is an area of current research as it is anticipated that new serotypes will likely emerge under the selection pressure of vaccination with pneumococcal polysaccharide conjugates.[4] Several *Spn* proteins have been eliminated as vaccine candidates due to surface epitope heterogeneity, variable expression or other characteristics. Desirable *Spn* candidate antigens should be conserved among strains and immunogenic in children and adults. In the work reported here, we studied five *Spn* protein candidates: PhtD, the detoxified pneumolysin derivative, PlyD1, and a truncated form of LytB, PcpA and PhtE. PhtD and PhtE are pneumococcal histidine triad (Pht) proteins D and E with possible complement binding/degrading/interaction activity.[5] LytB is a pneumococcal choline binding protein that is a cell wall hydrolase[6] and PcpA is a choline binding surface protein that elicits protection against pneumococcal infection in an animal model.[7,8] Pneumolysin (Ply) has a wide range of cytotoxic and inhibitory effects on host tissue and immune cells.[9] In this study, we used the pneumolysin derivative, PlyD1, which has three point mutations that do not interfere with anti-pneumolysin antibody responses.

For *Spn* vaccine development it is useful to know whether a target antigen is immunogenic in the human host in the age time frame when vaccination is anticipated to be given. This study sought an answer to that question by measurement of antibodies elicited in young children after natural *Spn* exposure such as after asymptomatic NP colonization and after a local infection such as AOM. To our knowledge, this is the first study to prospectively compare the natural antibodies elicited to 5 *Spn* proteins simultaneously in a cohort of children 6–30 mo of age during NP colonization and AOM. The comparisons of interest we report here include: (1) Changes in the levels of PhtD, LytB, PcpA, PhtE and Ply-specific IgG antibodies in children as they increased in age
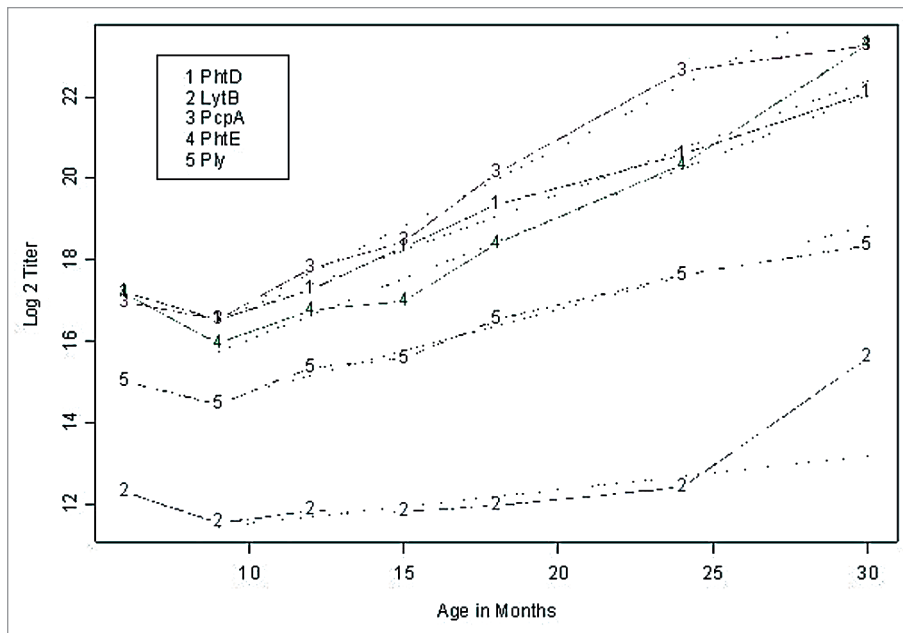
**Figure 1.** Serum IgG antibody levels to *Spn* proteins PhtD, LytB, PcpA, PhtE and Ply in healthy children increased with age. Natural acquisiton of serum IgG antibodies to *Spn* proteins PhtD, LytB, PcpA, PhtE and Ply. Plots of the geometric mean titers (end point titer) during 7 sampling visits at 6, 9, 12, 15, 18, 24 and 30 mo of age. The y axis is changed to log2 scale. The numbers of sera included at each time point were 107, 88, 65, 61, 55, 44 and 6. A linear regression fit from 9 to 30 mo is superimposed.

from 6 to 30 mo of age; (2) Changes in antibody levels following detected colonization of the NP with *Spn* and AOM infection by *Spn*; (3) Characterization of IgG and IgM in acute and convalescent serum following *Spn* AOM; and (4) Variations in individual antibody repertoire and responses in the AOM vaccine target age of children.

## Results

**NP colonization and AOM events.** A total of 731 visits among 168 children were studied. There were 301 *Spn* NP colonization episodes documented in 109 (65%) children and 42 *Spn* AOM episodes in 34 (20%) children. Because the study design called for NP/OP sampling at 7 specific times separated by 3 to 6 mo, some *Spn* colonization events were not detected by culture but most likely occurred as reflected in significant rises in specific antibody to one or more of the *Spn* antigens studied.

**Natural acquisition of serum antibody to proteins (PhtD, LytB, PcpA, PhtE and Ply) over time. Figure 1** shows the geometric mean end point titers of serum antibodies to PhtD, LytB, PcpA, PhtE and Ply at 6, 9, 12, 15, 18, 24 and 30 mo of age corresponding to visits 1–7 for the study cohort. A decrease between 6 to 9 mo is evident, whereas a linear gradient is observed between 9 to 30 mo. Estimated slopes (log 2 titer/month) were 0.28 (SE = 0.02), 0.08 (SE = 0.02), 0.38 (SE = 0.03), 0.30 (SE = 0.02), 0.20 (SE = 0.02) for PhtD, LytB, PcpA, PhtE and Ply, respectively. At 6 mo, no significant difference was detected between PhtD, PcpA and PhtE antibody levels, while significant differences

($p < 0.05$) were detected between Ply and LytB, and between these and the remaining 3 proteins. A similar conclusion holds at 9 mo. At 15 mo and higher, all pairs of antibody levels to the specific proteins are significantly different ($p < 0.05$). Overall, IgG antibody titers to the five proteins were significantly different among the study children: PcpA > PhtD = PhtE > Ply > LytB ($p < 0.001$).

**Comparison of serum antibody levels to PhtD, LytB, PcpA, PhtE and Ply in children *Spn* NP colonized vs. non-colonized children, at various ages.** We compared the level of antibody to PhtD, LytB, PcpA, PhtE and Ply in 21, 21, 18, 23, 15 and 24 children who were culture-positive for *Spn* at age 6, 9, 15, 18 and 24–30 mo respectively with 71, 62, 45, 39, 34 and 27 children of the same age who were not culture-positive. The patterns were significantly different for the 5 proteins (**Fig. 2**). For PhtD, a significant difference was shown at age 9, 12 and 15 mo. For LytB, no significant difference was seen between those with detected colonization and those without detected colonization at any age. For PcpA, the differences between children with detected colonization and those without was significantly different at all the ages (p value < 0.001). For PhtE, a significant difference was identified between children with detected colonization and those without detected colonization at 9 mo of age. For Ply, a significant difference was identified between children with detected colonization and those without detected colonization at age 9, 12 and 15 mo. Variation in antibody quantity to each of the studied proteins was noted in both colonized and non-colonized groups and contributed to the absence of significant differences among groups for some time points. Overall, NP colonization was associated with variably higher IgG antibody levels to PcpA > PhtE > PhtD > Ply > LytB ($p < 0.001$).

**Paired acute and convalescent serum IgG, IgM and IgA antibody levels to *Spn* proteins in children with AOM.** The geometric mean titers of IgG, IgM and IgA antibody at acute and convalescence sera of AOM children is shown in **Table 1**. In **Figure 3** individual responses of children to all five proteins before and after an AOM are shown. When we examined the individual responses for the 11 paired sera available we observed that 5 (45.5%) of 11 children showed a rising IgM and to a lesser extent IgG antibody level, suggesting a primary antibody response. Four (36.4%) of 11 children showed a rising IgG and to a lesser extent IgM antibody level suggesting a secondary antibody response. One (9%) of 11 children had no change in antibody level between acute and convalescent sera suggesting the absence of an antibody response. Serum IgA antibody levels were largely unchanged comparing acute to convalescent sera for all 5 proteins. No protein had a significant difference in the frequency

of rises in IgA among the children. Among these 11 children, 5 children have AOM caused by serotype 19A, 3 of serotype 11, one of serotype 4, one child of serotype 21 or 39 and one of both serotype 15 and 19A. We did not observe any correlation of between serotype and antibody titers to the studied *Spn* proteins.

## Discussion

Progress in the development of a serotype-independent, protein-based *Spn* vaccine to prevent AOM is hampered by gaps in knowledge of the immune response to vaccine candidates. Here we have characterized the serum antibody response to 5 *Spn* proteins mounted by children who experienced *Spn* NP colonization and AOM. We found a rise in the serum levels of PhtD, LytB, PcpA, PhtE and Ply-specific IgG antibodies in children as they increased in age from 6 to 30 mo of age. The rank order of antibody concentration was highest to PcpA followed by PhtE, then PhtD, then Ply and last LytB. Changes in serum antibody levels following detected NP colonization without progression to AOM and NP colonization associated with AOM caused by *Spn* were of a similar magnitude and rank order. Examination of specific IgG and IgM antibody responses with AOM showed that some children experienced a primary response, others a secondary response and others an absent response (no change in acute vs. convalescence antibody titer) to the studied *Spn* proteins in this age group of children.

Antibody responses in serum have been studied previously following NP colonization in children for *Spn* protein antigens PhtD, CbpA, LytC, PsaA, PspA and Ply.[12-14] In those studies, similar to ours, the gradual acquisition of serum antibody in children over time was observed, *Spn* exposure resulted in higher serum antibody titers and rises in antibody titers had different kinetics for different antigens studied. For example, Holmlund et al.[12] studied the serum antibody concentrations to PspA, PsaA, Ply, PhtD, CbpA and LytC in pregnant women, cord blood samples and young children from 6 weeks to 10 mo of age. The children had NP cultures obtained at the time of the serum samples to detect *Spn* colonization events. They found similar GMCs for all proteins in the cord blood and maternal samples. In the young children, the GMCs for PhtD, CbpA, LytC, PspA and Ply decreased until 18 weeks of age and started to increase after that age suggesting that the infant's own antibody production started to rise at 4 to 5 mo of age. The increase in antibody by the young children did not approach the levels of the adult mothers by the age of 10 mo. Unlike the other pneumococcal protein antigens, the GMCs of anti-PsaA increased significantly as the children aged and reached the GMC of the mothers by 3.5 mo of age. The increase in antibody concentration in young infants was found to be associated with *Spn* NP colonization events but followed different kinetics depending upon the *Spn* antigen, similar to our study.

In a study with similar design to ours, Simell et al. evaluated the development of serum anti-CbpA and -PhtD in young children at 6, 12, 18 and 24 mo of age in relation to *Spn* exposure and obtained MEF at the time of AOM to determine etiology. Serum samples from mothers were obtained at the infant's 2-mo visit. They found the GMCs of serum anti-CbpA and -PhtD
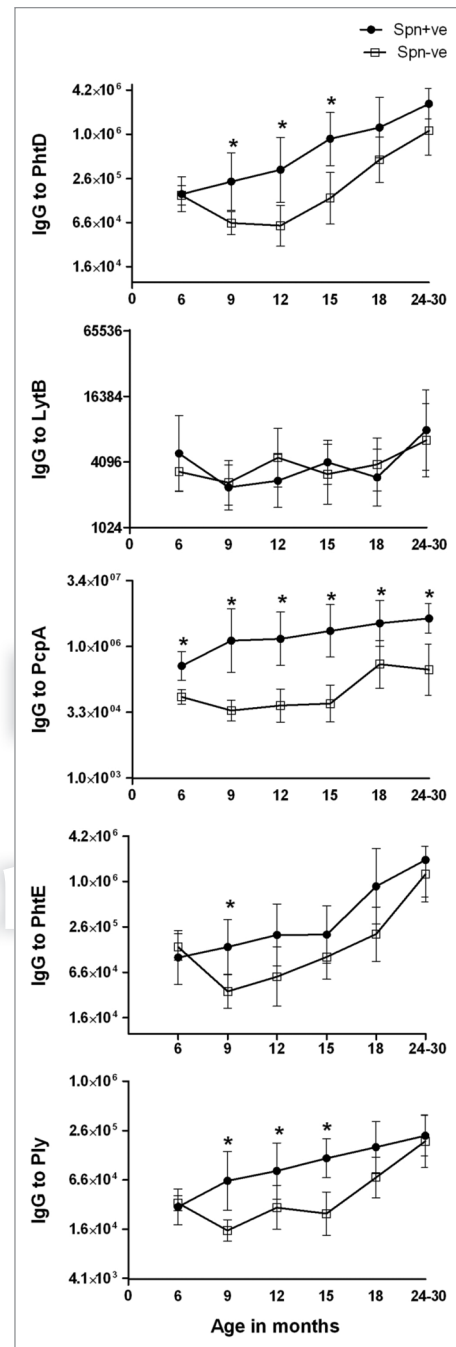


**Figure 2.** Comparison of serum IgG antibody GMTs to *Spn* proteins PhtD, LytB, PcpA, PhtE and Ply in NP colonized (•*Spn*⁺ᵛᵉ) and non-colonized (□ *Spn*⁻ᵛᵉ) healthy children from 6 mo to 30 mo of age. To better represent the data, y axis is changed to log2 scale. Mann-Whitney test was used to compare the colonized vs. non-colonized children at particular visits for all the proteins. The *sign indicates that the difference in colonized vs. uncolonized children were statistically significant: for PhtD at visit 9, 12 and 15 mo with p < 0.001; for PcpA at all the visits (p < 0.001), for PhtE at 9 mo with p < 0.05 and for Ply at 9, 12 and 15 mo with p < 0.005.

decreased between 6 and 12 mo of age and started to increase thereafter. Despite the increase in serum antibody the levels in 24 mo olds were well below that of the adult mothers. Again Simell

**Table 1.** Comparison of serum IgG, IgM and IgA antibody to *Spn* proteins PhtD, LytB, PcpA, PhtE and Ply in the acute and convalescence stage of AOM

| | | Acute | | Convalescence | |
|---|---|---|---|---|---|
| | | Geometric mean | 95% Confidence interval | Geometric mean | 95% Confidence interval |
| **PhtD** | IgG | $4.90 \times 10^5$ | $1.46 \times 10^5$–$1.64 \times 10^6$ | $5.39 \times 10^5$ | $1.41 \times 10^5$–$2.06 \times 10^6$ |
| | IgM | 1694 | 551–5212 | 1198 | 287–5002 |
| | IgA | 788 | 199–3129 | 430 | 83–2224 |
| **LytB** | IgG | 3705 | 1177–11669 | 3941 | 1089–14268 |
| | IgM | 1001 | 311–3221 | 820 | 188–3570 |
| | IgA | 15 | 7–32 | 15 | 9–23 |
| **PcpA** | IgG | $1.04 \times 10^6$ | $1.19 \times 10^5$–$9.11 \times 10^6$ | $2.31 \times 10^6$ | $3.61 \times 10^5$–$1.48 \times 10^7$ |
| | IgM | 1320 | 424–4113 | 1110 | 270–4563 |
| | IgA | 853 | 167–4368 | 1460 | 418–5097 |
| **PhtE** | IgG | $7.22 \times 10^5$ | $1.11 \times 10^5$–$4.72 \times 10^6$ | $1.16 \times 10^6$ | 74746–$1.79 \times 10^7$ |
| | IgM | 923 | 286–2977 | 910 | 204–4064 |
| | IgA | 80 | 18–356 | 68 | 13–1346 |
| **Ply** | IgG | 85378 | 32961–$2.21 \times 10^5$ | $1.08 \times 10^5$ | 42055–$2.78 \times 10^5$ |
| | IgM | 1260 | 425–3738 | 949 | 224–4017 |
| | IgA | 88 | 35–220 | 58 | 18–190 |

The data are represented as geometric mean titers with lower and upper 95% confidence intervals. No significant difference was found between acute and convalescence titers of IgG, IgM and IgA antibody for the 5 Spn proteins.

et al. showed that the development of GMCs to *Spn* antigens was strongly associated with NP or MEF cultures positive for *Spn*. Children with prior cultures positive for *Spn* had significantly higher GMCs as compared with children without prior cultures positive for *Spn*. In addition to the above findings, Simell et al. looked at the GMCs for CbpA and PhtD at 12 and 18 mo of age and the relationship to the subsequent development of *Spn* AOM in the following 6 mo. Children with higher serum anti-CbpA showed a trend toward lower rates of *Spn* AOM during the subsequent 6 mo.

Our group previously reported that NP colonization was an immunizing event in children relative to *NTHi* outer membrane proteins D, P6 and OMP26.[10,15] When we examined differences between antibody levels in children who had *Spn* NP colonization detected at various time points between 6 and 30 mo of age, we did observe greater increases in serum antibody to PhtD, PcpA, PhtE and Ply but not LytB compared with children who did not have detected *Spn* NP colonization. We interpret those findings to indicate that NP colonization was an immunizing event. However, even among the children who did not have *Spn* NP colonization detected, rises in serum antibody were noted. Probably NP colonization by *Spn* occurred between study visits without detection due to NP sampling frequency. The significant difference in antibody level among detected NP colonized compared with undetected colonized children beginning at age 6 mo for PcpA and for PhtD, PhtE and Ply several months later provides evidence that NP colonization with *Spn* is associated with stimulation of serum antibody to proteins expressed by the bacteria at the target age for an AOM vaccine.

When we examined the isotypes of serum antibody in acute and convalescent sera surrounding an AOM for the subset of children where we had paired serum, we found variable IgG and IgM antibody levels. The repertoire of antibody detected was consistent with primary, secondary and absent immune responses after AOM. In earlier work, Samukawa et al. studied the immune response to *Spn* surface protein A (PspA) in the sera of various age groups in the general population. In the first 2 y of life they found comparable amounts of IgG and IgM serum antibodies to PspA whereas in adults IgG predominated. In contrast, Virolainen et al. evaluated serum antibodies to Ply in children with AOM. Eight of ten children experienced a seroconversion in Ply antibody levels at a median age of 20 mo old but all were of the IgA class only. Rapola et al. studied the serum antibody response to Ply and pneumococal surface adhesion A (PsaA) in children with AOM age 2 mo to 2 y. Antibody levels were compared among three groups: *Spn* NP colonized, *Spn* AOM, and neither colonized nor AOM infections due to *Spn*. At the time of the sampling, children with NP colonization had the highest anti-Ply and PsaA antibody levels, children with a current AOM were next highest, then children with no current but a past history of *Spn* NP colonization or AOM lower. Lowest were those with no current or previous documented history of *Spn* colonization or AOM. Wide variations in antibody levels were measured. Age of the child (as in our study) and pre-existing antibody level were identified as important covariates in predicting an antibody response to NP colonization.

In the course of a prospective study still in progress, we have described serum antibody responses to *NTHi* protein P6, OMP26 and protein D in healthy children after NP colonization and with infrequent (AOM,[10]), the influence of breastfeeding on the frequency of AOM and on serum antibody levels to *NTHi* vaccine candidate protein P6,[15] and serum antibody responses to *NTHi* protein P6, OMP26 and protein D in otitis prone children.[19]

Our study has limitations. The difficulty in obtaining blood from young children repetitively between 6 and 30 mo of age is considerable, particularly when coupled with additional blood drawing for acute and convalescent levels surrounding an infection such as AOM. Therefore, despite best efforts, we did not obtain blood samples from every child at every visit as designed. This created windows of missing data that we addressed as we could statistically. The analysis of acute and convalescent IgG and IgM antibody levels points to the complexity in defining an acute and convalescent time point for AOM infection, and even more so for NP colonization when sampling is spaced by several months as in this study. The proteins we studied are known to be properly folded up to pH 9.0 as determined by biophysical methods and immunization of animals eliciting antibodies that react with intact bacteria in an antibody binding assay with detection by flow cytometry and providing protection against *S. pneumoniae* challenge. However, we cannot be certain that the adsorption of the proteins to a solid phase at pH 9.4 has not affected the structure of the proteins.

This paper adds to a body of literature evaluating our prospectively enrolled, longitudinal study cohort, with a focus on *Spn* vaccine antigens. For *Spn* we have previously described antibody responses to PcpA PhtD, PhtE Ply and LytB in non-otitis prone vs. otitis prone children.[20] We have shown that PcpA is an adhesin of *Spn* that facilitates adherence to human NP and lung epithelia and that human antibody to PcpA can function to reduce adherence of *Spn* to NP cells.[21] We have clearly established the role of PhtD and PhtE in *Spn* adherence to human upper and lower airway epithelial cells and that human antibody directed to either PhtD or PhtE causes a direct effect of blockage of adhesion of *Spn* to the human epithelial cells.[22] The importance of demonstrating acquisition of serum antibody in a pediatric cohort at an age when vaccination is most likely to occur is paramount to encourage further study. In that regard our observations are strongly supportive of the potential of PcpA, PhtE, PhtD and Ply to be incorporated in pneumococcal vaccines against *Spn* infection in children.
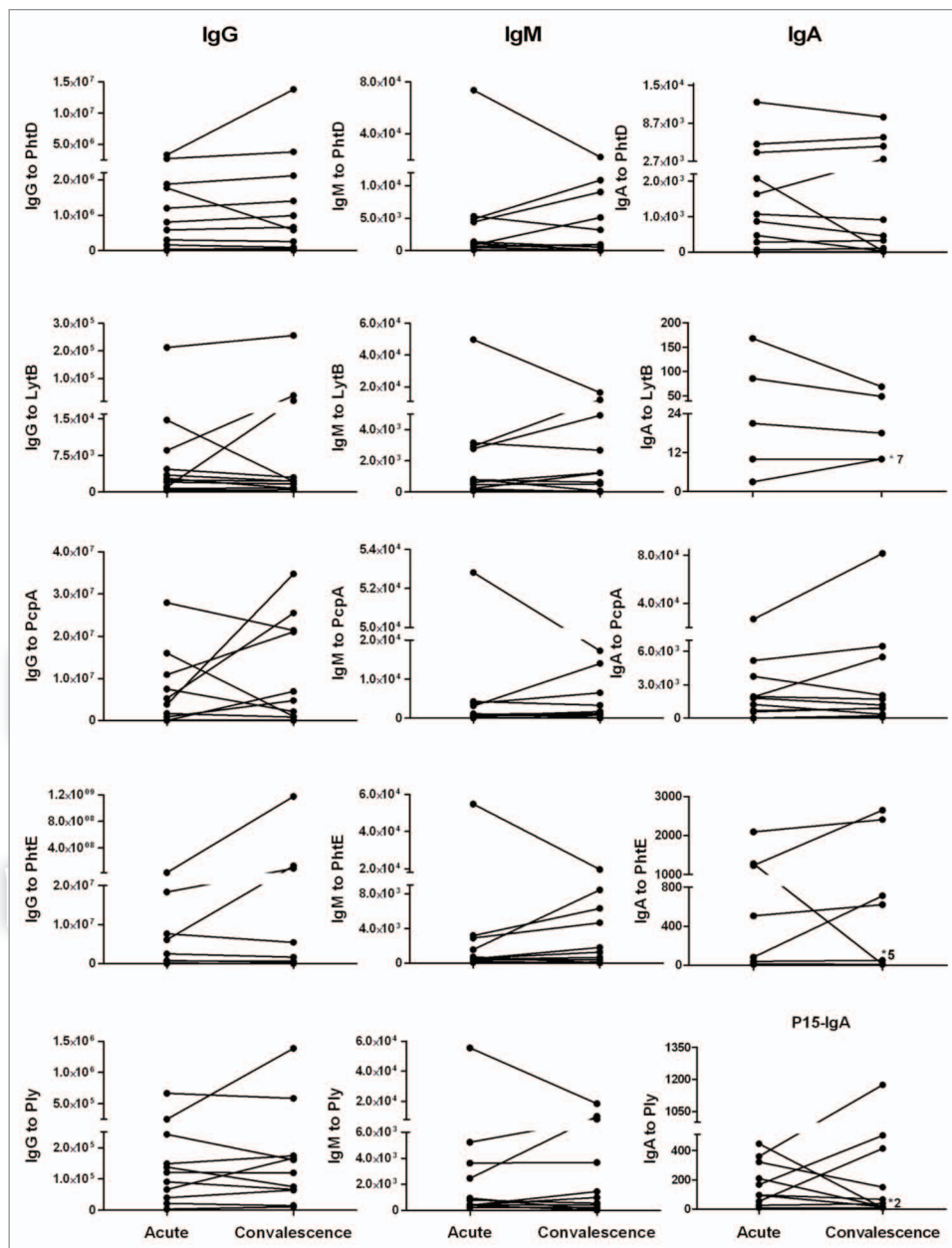


**Figure 3.** Individual IgG, IgM and IgA antibody levels to *Spn* proteins PhtD, LytB, PcpA, PhtE and Ply in acute and convalescent sera of children with *Spn* AOM. Note: Some sera pairs titers overlap in IgA and has shown with * and number which indicate how many children has the same titers.

## Methods

**General design.** This report includes data for the time span June, 2006 to December, 2009 from children enrolled in a prospective study supported by the National Institutes of Deafness and Communication Disorders as previously described in reference 10. Healthy children without previous episodes of AOM were enrolled from a middle class, suburban socio-demographic pediatric practice in Rochester, NY (Legacy Pediatrics). Healthy children had serum, NP and oropharyngeal (OP) cultures and NP wash samples obtained seven times, every 3–6 mo, between 6 and

30 mo of age (at age 6, 9, 12, 15, 18, 24 and 30 mo). Since the samples were collected over time, not all children completed their participation at the time when this analysis was performed. In addition, if a child developed symptoms compatible with AOM, they were examined by validated otoscopist pediatricians with pneumatic otoscopy and if middle ear infection was suspected a tympanocentesis was performed to confirm the diagnosis. At the time of the acute AOM diagnosis and three weeks later acute and convalescent serum, NP and OP cultures and NP wash samples were obtained. All children had received pneumococcal conjugate vaccine containing 7 serotypes at age 2, 4, 6 and 15 mo of age. The study was approved by the University of Rochester and Rochester General Hospital Research Subjects Review Board and written informed consent was obtained for participation and all procedures.

**Sample collection.** NP, OP, middle ear fluid (MEF) sampling was as previously described in reference 11.

**Microbiology.** Bacteria were isolated as previously described in reference 11.

**Detection of protein-specific antibodies by ELISA.** Protein-specific antibody titers were determined by ELISA using purified recombinant proteins (provided by sanofi pasteur). The proteins were stored in a neutral pH buffer. Under these conditions, the proteins are properly folded, as evidenced by: (i) Biophysical methods, such as DSC, fluorescence, CD and FTIR and (ii) Immunization of animals elicited antibodies that react with intact bacteria in an antibody binding assay with detection by flow cytometry and provided protection against *S. pneumoniae* challenge. The 96-well Nunc-Immulon-4 plates were coated with 0.25 µg–0.5 µg/ml of individual protein antigens (100 µl/well) in coating buffer (bicarbonate, [pH 9.4]) and incubated overnight at 4°C. After washing the plates were blocked with 3% skim milk at 37°C for 1 h (200 µl per well). After five washes,

100 µl of serum at a starting dilution of 1:100 (in PBS-3% skim milk) was added to the wells and diluted serially 2-fold. The mixture was incubated at room temperature for 1 h followed by the addition of affinity purified goat anti-human IgG, IgM or IgA antibody conjugated to hoarseradish-peroxidase (Bethyl Laboratories, Inc.) as a secondary antibody. The reaction products were developed with TMB Microwell Peroxidase Substrate System (KPL), stopped by the addition of 1.0 molar phosphoric acid and read by an automated ELISA reader using a 450-nm filter. Results are reported as end-point titers. An in-house positive control serum (mixture of human sera) was run on each plate to control for plate-to-plate variability. The inter-test coefficient of variation was ≤ 30%.

**Statistics.** In general, significance levels of comparisons of titer levels was performed using a paired two-sample procedure for pairwise comparisons, or a Friedman's test for multiple treatments, using the subject as blocking variable. In cases where the block design is significantly incomplete, a bootstrap procedure using subjects for replicates was employed. Titer levels were first transformed by a base 2 logarithm.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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