Otitis-prone children produce functional antibodies to pneumolysin and pneumococcal polysaccharides

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ABSTRACT

Background: The pneumococcus is a major otitis media (OM) pathogen but data are conflicting on whether otitis-prone children have impaired humoral immunity to pneumococcal antigens. We and others have shown that otitis-prone and healthy children have similar antibody titres to pneumococcal proteins and polysaccharides (vaccine and non-vaccine type), however the quality of antibodies from otitis-prone children has not been investigated. Antibody function, rather than titre, is considered to better correlate with protection from pneumococcal disease. Therefore, we compared the capacity of antibodies from otitis-prone (cases) and healthy children (controls) to neutralise pneumolysin, the pneumococcal toxin currently in development as a vaccine antigen, and to opsonise pneumococcal vaccine and non-vaccine serotypes.

Methods: The pneumolysin neutralising assay was conducted on cholesterol-depleted complement-inactivated sera from 165 cases and 61 controls. Multiplex opsonophagocytosis assay (MOPA) was conducted on sera from 20 cases and 20 controls. Neutralising and opsonising titres were calculated with antigen-specific IgG titres to determine antibody potency for pneumolysin, pneumococcal conjugate vaccine (PCV) polysaccharides and non-PCV polysaccharides.

Results: There was no significant difference in antibody potency between cases and controls for the antigens tested. Anti-pneumolysin neutralising titres increased with number of episodes of acute OM but antibody potency did not. Pneumolysin antibody potency was lower in children colonised with pneumococci when compared with non-carriers, and this was significant in the otitis-prone group, $p<0.05$.

Conclusions: Production of functional anti-pneumococcal antibodies in otitis-prone children demonstrates that they respond to current PCV and are likely to respond to pneumolysin-based vaccines as effectively as healthy children.

1. INTRODUCTION

Otitis media (OM), middle ear infection, is responsible for the greatest number of general practitioner visits, antibiotic prescriptions, and surgical procedures for children in industrialised countries (1). *Streptococcus pneumoniae* (the pneumococcus) is a major OM pathogen (1). Current pneumococcal conjugate vaccines (PCVs) are composed of capsule polysaccharides from up to 13 of the 95 immunologically-distinct pneumococcal serotypes. PCVs have significantly reduced OM caused by the serotypes included in the vaccine (2, 3) but the overall reduction in OM has been negligible due to replacement disease with non-vaccine serotypes and other bacterial species (3-5). To address the limitations of serotype specific vaccines including the issue of replacement disease, research efforts are focusing on development of pneumococcal vaccines that confer species-wide protection, either by using whole-cell formulations or multicomponent recombinant pneumococcal proteins (6-11).

An attractive vaccine candidate is the highly conserved pneumococcal toxin pneumolysin (Ply). Immunisation of animals with native or non-toxic derivatives of Ply elicits production of neutralising antibodies that confer serotype-independent protection from pneumococcal pneumonia and bacteraemia (12-15). Recent clinical trials with Ply-based vaccines have demonstrated that they are safe (16, 17) and elicit high circulating titres of neutralising anti-Ply antibodies in humans (16). Ply-induced protection against OM in humans remains to be demonstrated for these vaccines but fusion of Choline binding protein A (CbpA) peptides to a Ply toxoid has been shown to enhance protection against pneumococcal OM in mice (11). The role of Ply in pneumococcal OM is not fully understood but direct instillation of Ply into the cochlea of guinea pigs damages the inner and outer hair cells (18), suggesting that Ply may contribute to permanent hearing loss that can occur in severe cases of pneumococcal OM. Ply is involved in early biofilm development (19), a key feature of OM pathogenesis

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(20) that contributes to recurrence of infections and bacterial resistance to antibiotic treatment. Together these data indicate that Ply-containing vaccines may have the potential to reduce the burden of pneumococcal OM.

Pneumococcal carriage and acute OM induces local and systemic production of anti-Ply and anti-capsule antibodies in children within the first years of life (21-28). It has been suggested that children with recurrent episodes of OM (otitis-prone) have impaired naturally-acquired and vaccine-induced antibody responses to pneumococcal antigens with reports of lower anti-Ply IgG (21) and anti-capsule IgG (23), IgG2 and IgA titres (23). In contrast, we and others have observed similar or even higher titres of anti-Ply IgG (25, 28) and anti-capsule polysaccharide IgG, IgG2, IgA (29-32) in serum from otitis-prone children when compared to non-otitis-prone children. Previous studies into humoral immunity in otitis-prone children assess antibody titre rather than function, but high titres of anti-pneumococcal polysaccharide antibodies do not necessarily correlate with antibody function (33, 34) or protection from disease (35). We hypothesised that although otitis-prone children may produce similar serum IgG titres to pneumococcal vaccine and non-vaccine antigens, the functionality of these antibodies may be reduced and thus responsible for susceptibility to recurrent OM. We used the Ply neutralising assay (36) and the multiplexed quadruple-serotype opsonophagocytic killing assay (MOPA4) (37) to compare the functional properties of anti-Ply and anti-capsule antibodies in children with and without a history of recurrent acute OM (rAOM). Investigation into antibody quality in otitis-prone children is important for evaluating current vaccine strategies and the suitability of a Ply-based vaccine to reduce the burden of pneumococcal OM.

2. MATERIALS AND METHODS

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2.1 Recruitment of the study cohort and sample collection

Recruitment of children to this cross-sectional study (the GROMIT study) has been previously described (38). Briefly, recruitment was from 2007 to 2009 and children were aged 6 to 36 months. Cases were recruited at the time of surgery and were defined as children with a history of at least 3 episodes of AOM in 6 months or 4 episodes in 12 months and requiring insertion of ventilation tubes. Children with no significant history of OM and who were undergoing general surgery for non-infectious reasons were recruited as healthy age-matched controls. None of the children had signs of acute infection at the time of sample collection. Children with diagnosed immunodeficiency, cystic fibrosis, immotile cilia syndrome, craniofacial abnormalities, and chromosomal or genetic syndromes were excluded. Data on ear disease and host- and environmental risk factors were collected by parental questionnaires and from medical records. Nasopharyngeal swabs and serum samples were collected from all cases and controls as previously described (38). The study was approved by the Princess Margaret Hospital for Children Human Research Ethics Committee, Perth, Western Australia, and by the institutional boards of the private hospitals where recruitment took place.

2.2 Measurement of anti-Ply IgG and pneumococcal polysaccharide IgG titres

Serum anti-Ply IgG antibody titres were measured with a bead-based immunoassay as previously described (28). Serum anti-polysaccharide IgG titres to PCV7 vaccine serotypes 4, 6B, 14 and 23F and non-PCV7 vaccine serotypes 1, 5, 7F and 19A were also measured using a multiplex bead-based immunoassay as previously described (29).

2.3 Cholesterol depletion and complement inactivation of serum

Free cholesterol, which blocks the cytolytic activity of Ply, was depleted from sera by adding 10% v/v of a cholesterol depletion working solution (10g/L dextran sulphate/0.5M $MgCl_2$ in distilled water) (39) (All Sigma-Aldrich). Samples were then vortexed, incubated for 15 min at room temperature (RT), then centrifuged at 1500g for 30 min at RT. The cholesterol-free serum (supernatant) was transferred to a fresh tube and incubated at 56°C for 30 min to inactivate serum complement. Cholesterol depletion was confirmed on a subset of serum samples (n = 10) using the VITROS CHOL slides system (Ortho Clinical Diagnostics, Rochester, NY, USA).

2.4 Ply neutralisation assay

The ability of cholesterol-depleted complement-inactivated sera to neutralise the cytolytic activity of Ply was measured as previously described (12). Briefly, cholesterol-depleted complement inactivated serum samples were serially diluted 1:2 in phosphate-buffered saline (PBS, Sigma) in a U-bottom 96-well plate. The haemolytic activity of the recombinant Ply was measured (14) and 50µl of 80 Haemolytic Units (HU)/ml of Ply added to each well to give 4HU/well. The plate was incubated at 37°C for 15min to allow anti-Ply antibodies to bind to and potentially neutralise Ply. A 1% washed human red blood cell solution in PBS was added to reach a final concentration of 0.25% in each well. The plate was incubated at 37°C for 30min and centrifuged for 5min at 200g at RT to facilitate precipitation of intact erythrocytes. The endpoint of the assay (neutralising titre, NT) was taken as the reciprocal of the serum dilution at which complete inactivation of 4HU of Ply was observed (no haemolysis). An adult reference serum, a negative haemolysis control (saline) and a positive haemolysis control (4HU Ply) were run on each plate.

To confirm that the observed neutralising activity was antibody-mediated, IgG was depleted from the adult reference serum using Protein G sepharose columns (Invitrogen, Victoria, Australia) and serum neutralising titres were compared before and after IgG depletion. IgG depletion of the serum was confirmed by nephelometry (data not shown).

The potency of the anti-Ply antibodies present was calculated as the ratio of neutralising titre to anti-Ply IgG antibody concentration and gives a measure of the quality of anti-Ply antibodies in serum. Potency has previously been used to describe the quality of anti-polysaccharide responses in PCV vaccinated adults (40).

2.5 Multiplexed quadruple-serotype opsonophagocytic killing assay (MOPA4)

MOPA was conducted as previously described (37), using eight antibiotic resistant pneumococcal serotypes (serotypes 1, 4, 5, 6B, 7F, 14, 19A and 23F) that were kindly provided by Professor Moon Nahm. Each serotype was resistant to one of four antibiotics (optochin, streptomycin, spectinomycin and trimethoprim) and susceptible to the other three. A detailed protocol is available at www.vaccine.uab.edu. MOPA was conducted on a subset of sera from 20 children with a history of rAOM (cases) and 20 healthy controls due to limitations of available sample volumes and the impact of antibiotic use on MOPA. To assess potential differences in natural versus vaccine-elicited responses in otitis prone children when compared to healthy controls, MOPA was conducted on 4 selected PCV7 serotypes and 4 non-PCV7 serotypes. Opsonisation titres (OT) were expressed as the serum dilution that kills 50% of the specific pneumococcal serotype. Serum samples were used at a 1:2 dilution in opsonisation buffer B and then underwent 3-fold dilutions to reach a final dilution of 1:26,000. Samples with a titre below the detection limit of 2 were assigned a value of 1 for data analysis.

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The potency of the anti-polysaccharide antibodies was calculated as the ratio of opsonisation titre to anti-polysaccharide IgG antibody titre for each pneumococcal serotype tested (40). This gives a measure of the quality of opsonising antibodies in each serum sample.

2.6 Statistical analyses

Host and environmental risk factors were compared between cases and controls using Student's *t* tests for continuous variables (age) and Pearson Chi-square analyses (p-value asymptotic significant 2-sided) for categorical variables (gender, day-care attendance, PCV7 vaccination status, current antibiotic use, AOM category and pneumococcal carriage). Spearman's rank correlation coefficient was used to determine the correlation between anti-Ply IgG titres and the neutralising titre. A general linear regression model (on LOG-transformed data and adjusting for confounders of age, gender and day-care attendance) was used to analyse differences in anti-pneumococcal (anti-Ply and serotype specific anti-polysaccharide IgG) antibody levels between otitis-prone and non-otitis-prone children. The IBM SPSS Statistics 22 for Windows software package (IBM, New York, USA) was used for all statistical analyses and data were plotted using GraphPad Prism 6 (GraphPad Software Inc, California, USA).

3. RESULTS

3.1 Study population

Table 1 details host and environmental risk factors for children in this study. Age, gender and day-care attendance were adjusted for in all analyses as we had previously found these to be confounding factors for pneumococcal polysaccharide and protein IgG titres in this cohort (28, 29). Ply IgG antibody titre and neutralising titre was measured in serum from 165 cases

and 61 controls. The mean age of cases was higher than the healthy controls (21.2 months versus 18.7 months, p=0.03). Although males are more likely to be otitis-prone than females, there was no difference in gender distribution in this cohort (62% of cases and 72% of controls were male, p=0.15). Otitis-prone children were significantly more likely to attended a day-care facility for ≥4hrs/week than healthy controls (62% versus 32%; p<0.001). Almost all children were fully PCV7-vaccinated (99% of cases and 97% of controls). Antibiotic use was similar, with 22% of cases and 20% of controls taking antibiotics at the time of surgery (p=0.77). Otitis-prone children in the Ply study had experienced an average of 7.3 AOM episodes, with 33% of children having had 3-4 episodes, 18% 5-7 episodes, 35% with 8-9 episodes and 15% having experienced more than 10 AOM episodes. Significantly more cases were colonised with *S. pneumoniae* at the time of sample collection than controls (42% versus 26%; p = 0.03).

MOPA was conducted on a subset of sera from 20 cases and 20 controls. No differences were observed in risk factors between the cases and controls (Table 1). Antibiotic use and PCV7 vaccination affect the opsonophagocytosis assay, therefore children were excluded if they had received antibiotics within the month prior to surgery and all children were fully PCV7 vaccinated. Otitis-prone children in the MOPA study experienced an average of 5.6 AOM episodes, with 50% of children having had 3-4 episodes, 10% 5-7 episodes, 25% with 8-9 episodes and 10% having experienced more than 10 AOM episodes. There was no significant difference in the number of cases and controls that were colonised with *S. pneumoniae* at the time of sample collection (50% versus 35%, p=0.34).

3.2 The potency of anti-Ply neutralising antibodies is similar between otitis-prone and healthy children

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229 The geometric mean Ply IgG titre (Figure 1A), neutralising titre (Figure 1B) and antibody
230 potency (Figure 1C) was similar between otitis-prone and healthy children (p=0.394, p=0.592
231 and p=0.069 respectively). There was a strong and significant correlation between Ply IgG
232 titre and neutralising titre in all cohorts (r=0.842, p<0.001). This significant positive
233 correlation remained when cases and controls were assessed separately (r=0.888 and r=0.728;
234 p<0.001) and also for those colonised or not colonised with *S. pneumoniae* (r=0.883 and
235 r=0.817, p<0.001); Figure 2. Colonising serotypes were identified for all children in this
236 study that had S. pneumoniae isolated from their nasopharyngeal swab. There were mostly
237 non-vaccine serotypes with the most common serotypes isolated from the cases being 19A
238 (n=23), 11A (n=9), 6A (n=5) and 15B (n=5), whilst the most common colonising serotypes in
239 the controls were 19A (n=5), 6A (n=4) and 23B (n=2). The colonising serotype did not
240 influence the potency of Ply antibodies but this would be expected as Ply is conserved and
241 expressed by virtually all serotypes.
242
243 *3.3 The number of AOM episodes experienced does not affect anti-Ply antibody potency*
244 Increasing number of AOM episodes resulted in an overall trend towards higher anti-Ply IgG
245 titres (Figure 3A) and neutralising titres (Figure 3B) but overall there was no difference in
246 anti-Ply antibody potency (Figure 3C), regardless of number of AOM episodes. When raw
247 data was corrected for confounding factors including age, which differed between the cases in
248 the different #AOM categories, children experiencing 10+ AOM episodes had a downward
249 but not significant trend in geometric mean anti-Ply IgG titre (207 AU/mL) compared with
250 children experiencing 8-9 AOM episodes (260 AU/mL) and similar anti-Ply IgG titres in
251 children experiencing 5-7 AOM episodes (209 AU/mL). There was a significant increase in
252 adjusted geometric mean neutralising titre of serum from children with 8-9 episodes of AOM

compared with 3-4 episodes (*p<0.05) but this did not remain when antibody potency was calculated.

3.4 Otitis-prone children colonised with S. pneumoniae have high circulating levels of poor quality anti-Ply antibody

Otitis-prone children colonised with *S. pneumoniae* at the time of sample collection (n = 70) had significantly higher anti-Ply IgG (Figure 4A) and Ply neutralising titres (Figure 4B) but lower antibody potency (Figure 4C) than non-colonised otitis-prone children (n = 95); p<0.01, p=0.01 and p=0.046 respectively. This difference was not observed in healthy children, where anti-Ply antibody titre, neutralising titre and potency was similar between colonised (n = 16) and non-colonised (n = 45) controls (Figure 4). The anti-Ply IgG titre was significantly higher in *S. pneumoniae* colonised cases versus colonised controls (Figure 4A; p=0.01), whereas the neutralising titre was significantly higher in non-colonised controls versus non-colonised cases (Figure 4B, p = 0.01). There was no significant difference in antibody potency between colonised cases and controls or non-colonised cases and controls (Figure 4C).

3.5 The potency of antibodies against pneumococcal capsule polysaccharides is similar between otitis-prone and healthy children

There was no significant difference between cases and controls for anti-capsular polysaccharide IgG concentration (Figure 5A) and opsonisation titre (Figure 5B) for 7 of the 8 pneumococcal polysaccharides tested. Serotype 7F IgG concentration and opsonisation titre was significantly higher in the cases compared with the controls, p=0.03 and p=0.02 respectively. However, when antibody potency was calculated (opsonisation titre/IgG titre),

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there was no significant difference between cases and controls for any of the pneumococcal polysaccharide antibodies (Figure 5C).

Both cases and controls had higher antibody titre, opsonisation titre and antibody potency to vaccine serotypes compared with the non-vaccine serotypes (Figure 5). The geometric mean IgG concentration and opsonising titre for antibodies against all PCV7 serotypes tested was above the conservative correlates of protection of 0.35μg/mL IgG (41) (Figure 5A) and an OPA titre >8 (34) (Figure 5B) for all children, regardless of OM status.

DISCUSSION

This is the first investigation into whether otitis-prone children have deficiencies in their functional antibody responses to pneumococcal antigens. We have shown that otitis-prone children produce neutralising antibodies to the pneumococcal toxin Ply and opsonising antibodies to vaccine and non-vaccine capsule polysaccharides. The potency of these antibodies was comparable to those produced by healthy age-matched controls, indicating that anti-pneumococcal antibody function is not impaired in otitis-prone children. Whether this is true for the stringently-defined otitis-prone child, in which anti-Ply IgG titres are lower than in non-otitis-prone children (22), remains to be determined.

The neutralising antibody assay is the standard test to assess functional antibody responses to vaccines containing Ply. As current Phase II clinical trials are underway with a Ply containing pneumococcal conjugate vaccine (17) and whole cell pneumococcal vaccine [NCT02097472], the anti-Ply neutralising test will become even more relevant for determining immunogenicity of Ply. Whilst numerous studies have assessed neutralising activity in serum following immunisation of animals with Ply (12, 14, 36), this is the first

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report describing Ply neutralising titres in children and only the second report in humans. The first assessment of human anti-Ply neutralising antibodies is from a recent Phase I clinical trial with a Ply toxoid (PlyD1) in adults (16). The trial demonstrated that injection with PlyD1 was tolerated and induced neutralising antibody titres that were 4-fold higher than baseline, thereby validating the use of Ply as a vaccine antigen. Interestingly, the authors found only a weak to moderate association between Ply IgG titre and neutralising titre in the placebo adult sera ($r=0.490$), compared to the strong correlation we observed for the children in our study ($r=0.842$, $p<0.001$). This may suggest that naturally acquired anti-Ply antibodies are more functional and of higher quality in children than in adults. Indeed opsonising activity, but not concentration of anti-capsule antibodies, has been shown to diminish with age (33). It is important to note that Vero cell cytotoxicity rather than inhibition of haemolysis was used in the PlyD1 study, therefore neutralising titres cannot be directly compared between studies.

Consideration of the timing of sample collection and number of previous AOM episodes is important when comparing anti-Ply antibody titres and function between otitis-prone and healthy children, as anti-Ply serum IgG titres may diminish during an episode of pneumococcal AOM in naïve children but not in children with multiple AOM episodes (21, 25). Another possibility is that recurrent AOM may induce antigen-specific immune tolerance, as indicated in this study where children experiencing 10+ AOM episodes had lower age-adjusted geometric mean anti-Ply IgG titres than children experiencing 8-9 AOM episodes. Our data indicate that it is also important to know whether children are colonised with *S. pneumoniae* at the time of serum collection, as the anti-Ply antibody titre was higher in otitis-prone children that were colonised with *S. pneumoniae* but the potency of these antibodies was lower in comparison with non-colonised otitis-prone children. This may be

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due to the generation of low-affinity antibodies as part of a primary mucosal response to the colonisation event and prior to avidity maturation. Whether anti-Ply antibodies are protective against pneumococcal OM remains to be determined. It is also possible that pneumococcal colonisation (and disease) induces production of ineffective anti-Ply antibodies, possibly to decoy epitopes, particularly in children experiencing recurrent episodes of pneumococcal AOM. Future studies involving epitope mapping of anti-Ply antibodies from otitis-prone and healthy children are warranted, particularly in the context of using Ply-based vaccines for otitis-prone children.

To validate our finding that anti-Ply antibody function was not impaired in otitis-prone children we also measured the opsonising activity of antibodies to vaccine and non-vaccine pneumococcal polysaccharides in a subset of children using MOPA (34). MOPA is considered to be the gold standard to measure anti-capsule antibody quality and is recommended by the W.H.O. for full evaluation of pneumococcal conjugate vaccine efficacy (42). Demonstration that otitis-prone children in our study produced anti-capsule polysaccharide antibody that was of similar quality to healthy controls, both in response to PCV7 vaccination and from natural acquisition, adds to the increasing evidence that humoral immunity may not be impaired in otitis-prone children. Indeed, evidence is mounting that cell-mediated immune defences and mucosal antibody, rather than circulating antibody, may be important for limiting pneumococcal colonisation, transmission and OM (43-47). The higher opsonising titres observed for serotype 7F IgG in the cases compared to the controls are not able to be explained in this study. Serotype 7F, and the cross-protective serotype 7A, was not isolated from this cohort indicating that this serotype was not circulating at the time of sample collection (38).

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In summary, we have shown that otitis-prone and healthy children have similar antibody potency to pneumococcal vaccine and non-vaccine antigens. This suggests that otitis-prone children respond as well as their healthy counterparts to current PCV immunisation and should also respond well to immunisation with Ply-derived vaccines. Whether protein-based pneumococcal vaccines protect against OM remains to be determined.

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542 **Table 1.** Host and environmental risk factors for otitis-prone and healthy children assessed in

543 the Ply neutralising assay and MOPA.

	Ply Neutralising Assay			MOPA		
	Otitis-prone N=165	Healthy N=61	*p*-Value	Otitis-prone N=20	Healthy N=20	*p*-Value
Mean age in months *(range)*	21.2 *(7.3-36.0)*	18.7 *(7.3-35.0)*	**0.03**	21.0 *(10.9-33.5)*	18.3 *(7.0-35.0)*	0.07
% male	62%	72%	0.15	65%	85%	0.14
At day-care ≥4h/week	62%[a]	32%[b]	**<0.001**	50%	30%	0.20
Fully PCV7 vaccinated	99%	97%	0.30	100%	100%	-
Taking antibiotics	22%[c]	20%[b]	0.77	0	0	-
# AOM episodes						
3-4	33%	-		55%	-	-
5-7	18%	-		10%	-	-
8-9	35%	-		25%	-	-
10+	15%	-		10%	-	-
Pneumococcal carriage	42%	26%	**0.03**	50%	35%	0.34

544 [a]N = 151; [b]N = 59; [c]N = 152.

545

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546 **FIGURE LEGENDS**

547 **Figure 1:** Pneumolysin IgG antibody titres (A), neutralising titres (B) and antibody potency
548 (C) in serum from otitis-prone (open squares) and healthy children (closed circles). Data are
549 presented for each individual child, with the horizontal bar depicting the mean. Statistical
550 analysis was conducted on the geometric mean of LOG-transformed data; correcting for age,
551 gender and day-care attendance. AU, arbitrary units; Ig, immunoglobulin; NT, neutralising
552 titre.

553

554 **Figure 2.** Correlation between pneumolysin (Ply) IgG titre (AU/mL) and neutralising titre
555 (NT) for serum from otitis-prone (A) and healthy children (B), and children colonised (C)
556 and not colonised (D) with *S. pneumoniae*. In all groups there was a strong and significant
557 correlation between Ply IgG titre and neutralising titre, $p < 0.0001$. AU, arbitrary units; Ig,
558 immunoglobulin; NPS, nasopharyngeal swab.

559

560 **Figure 3:** Pneumolysin IgG antibody titres (A), neutralising titres (B) and antibody potency
561 (C) in serum from otitis-prone children according to number of AOM episodes experienced.
562 Data are presented for each individual child, with the horizontal bar depicting the mean.
563 Statistical analysis was conducted on the geometric mean of LOG-transformed data;
564 correcting for age, gender and day-care attendance. AU, arbitrary units; Ig, immunoglobulin;
565 NT, neutralising titre.

566

567 **Figure 4:** Pneumolysin antibody IgG titres (A), neutralising titres (B) and antibody potency
568 (C) in serum from otitis-prone (cases) and healthy children (controls) according to
569 nasopharyngeal colonisation with *S. pneumoniae*. Data are presented for each individual
570 child, with the horizontal bar depicting the mean. Statistical analysis was conducted on the

geometric mean of LOG-transformed data; correcting for age, gender and day-care attendance. Closed data points represent children colonised with *S. pneumoniae*, open data points represent non-colonised children. **p<0.001, *p<0.05; AU, arbitrary units; Ig, immunoglobulin; NT, neutralising titre.

Figure 5: Pneumococcal polysaccharide IgG antibody titres (A), opsonisation titres (B) and antibody potency (C) in serum from otitis-prone (open squares) and healthy children (closed circles). Data are presented for each individual child, with the horizontal bar depicting the mean. Statistical analysis was conducted on the geometric mean of LOG-transformed data; correcting for age, gender and day-care attendance. *p < 0.05 when cases are compared with controls for each polysaccharide. Ig, immunoglobulin; NVT, non-vaccine serotype; OT, opsonisation titre; Pn, pneumococcal polysaccharide; VT, vaccine serotype. Grey shading in Figure 5A indicates the IgG level (0.35μg/mL) above which polysaccharide IgG titres are considered to provide protection against pneumococcal disease.

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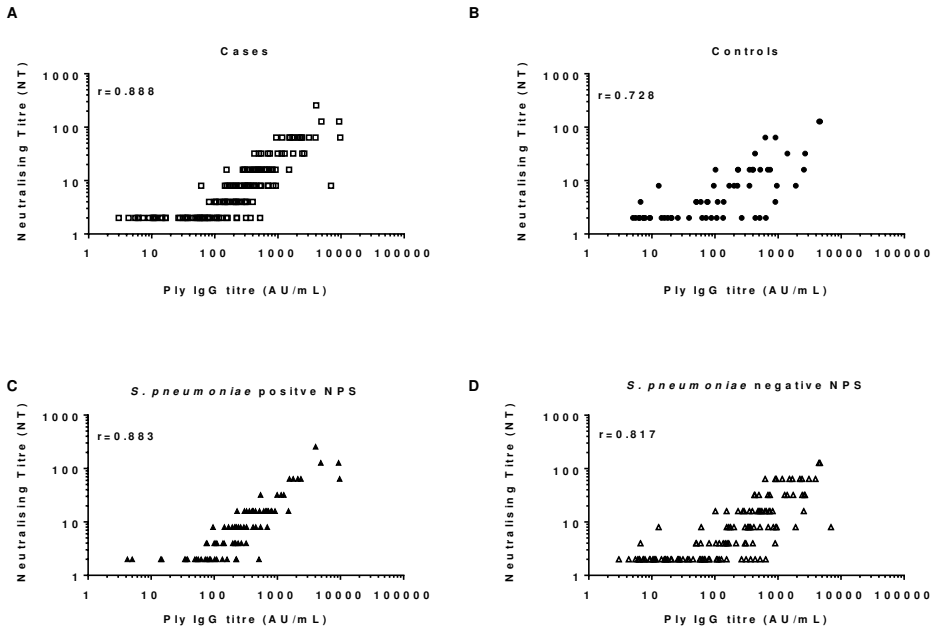

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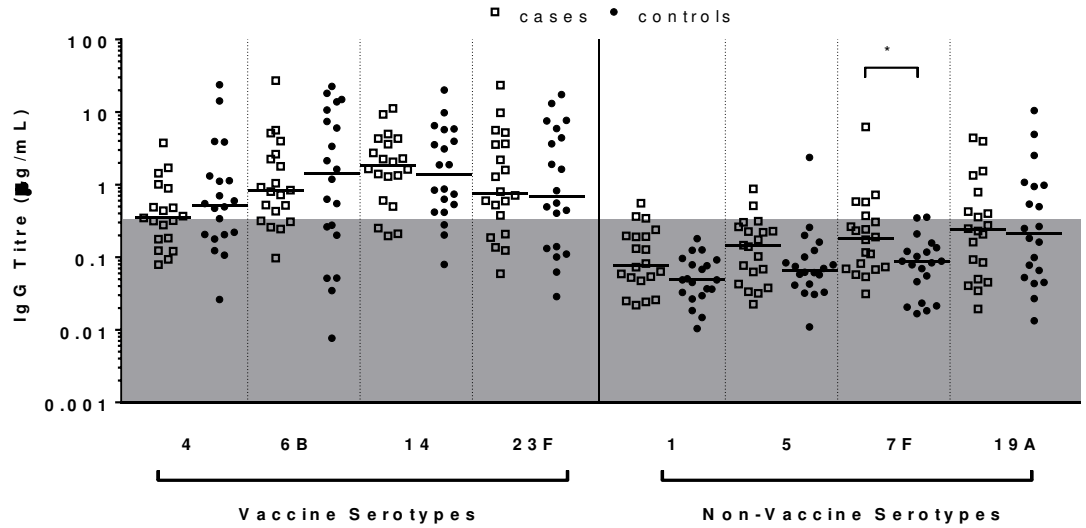
A



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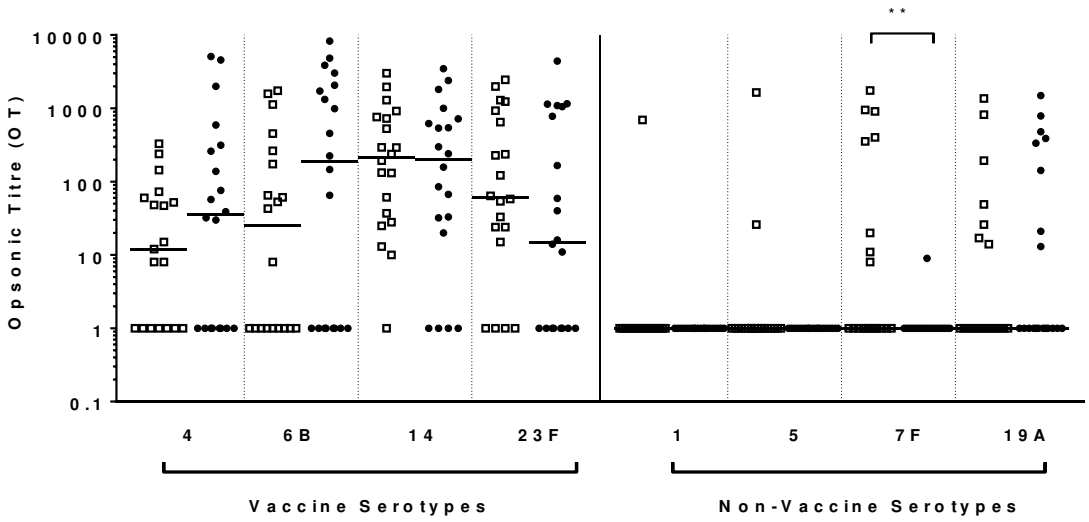

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