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## No evidence for impaired humoral immunity to pneumococcal proteins in Australian Aboriginal children with otitis media



Ruth B. Thornton<sup>a, b, \*</sup>, Lea-Ann S. Kirkham<sup>a, b</sup>, Karli J. Corscadden<sup>a, b</sup>,  
Harvey L. Coates<sup>a, b, c</sup>, Shyan Vijayasekaran<sup>a, b, c</sup>, Jessica Hillwood<sup>a</sup>, Sophie Toster<sup>a</sup>,  
Phillipa Edminston<sup>a</sup>, Guicheng Zhang<sup>a, d</sup>, Anthony Keil<sup>e</sup>, Peter C. Richmond<sup>a, b, c</sup>

<sup>a</sup> School of Paediatrics and Child Health, The University of Western Australia, GPO Box D184, Perth, Western Australia, 6840, Australia

<sup>b</sup> Telethon Kids Institute, The University of Western Australia, 100 Roberts Road, Subiaco, Western Australia, 6008, Australia

<sup>c</sup> Department of Otorhinolaryngology, Princess Margaret Hospital for Children, Roberts Road, Subiaco, Western Australia, 6008, Australia

<sup>d</sup> School of Public Health, Curtin University, GPO Box U1987, Perth, Western Australia, 6845, Australia

<sup>e</sup> PathWest Laboratory Medicine WA, Princess Margaret Hospital for Children, Roberts Road, Subiaco, Western Australia, 6008, Australia

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## ABSTRACT

**Background:** The Australian Aboriginal population experiences disproportionately high rates of otitis media (OM). *Streptococcus pneumoniae* is one of the main pathogens responsible for OM and currently no vaccine offering cross strain protection exists. Vaccines consisting of conserved antigens to *S. pneumoniae* may reduce the burden of OM in high-risk populations; however no data exists examining naturally acquired antibody in Aboriginal children with OM.

**Methods:** Serum and salivary IgA and IgG were measured against the *S. pneumoniae* antigens PspA1 and 2, CbpA and Ply in a cross sectional study of 183 children, including 36 non-Aboriginal healthy control children and 70 Aboriginal children and 77 non-Aboriginal children undergoing surgery for OM using a multiplex bead assay.

**Results:** Significant differences were observed between the 3 groups for serum anti-PspA1 IgA, anti-CbpA and anti-Ply IgG and for all salivary antibodies assessed. Aboriginal children with a history of OM had significantly higher antibody titres than non-Aboriginal healthy children with no history of OM and non-Aboriginal children with a history of OM for several proteins in serum and saliva. Non-Aboriginal children with a history of OM had significantly higher salivary anti-PspA1 IgG than healthy children, while all other titres were comparable between the groups.

**Conclusions:** Conserved vaccine candidate proteins from *S. pneumoniae* induce serum and salivary antibody responses in Aboriginal and non-Aboriginal children with a history of OM. Aboriginal children do not have an impaired antibody response to the antigens measured from *S. pneumoniae* and they may represent vaccine candidates in Indigenous populations.

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\* Corresponding author. University of Western Australia, School of Paediatrics and Child Health, Princess Margaret Hospital for Children, GPO Box D184, Perth, Western Australia, 6840, Australia.

E-mail addresses: [ruth.thornton@uwa.edu.au](mailto:ruth.thornton@uwa.edu.au) (R.B. Thornton), [lea-ann.kirkham@uwa.edu.au](mailto:lea-ann.kirkham@uwa.edu.au) (L.-A.S. Kirkham), [karli.corscadden@uwa.edu.au](mailto:karli.corscadden@uwa.edu.au) (K.J. Corscadden), [harvey.coates@uwa.edu.au](mailto:harvey.coates@uwa.edu.au) (H.L. Coates), [shyan\\_vijayasekaran@me.com](mailto:shyan_vijayasekaran@me.com) (S. Vijayasekaran), [20352947@student.uwa.edu.au](mailto:20352947@student.uwa.edu.au) (J. Hillwood), [20348782@student.uwa.edu.au](mailto:20348782@student.uwa.edu.au) (S. Toster), [20348263@student.uwa.edu.au](mailto:20348263@student.uwa.edu.au) (P. Edminston), [guicheng.zhang@uwa.edu.au](mailto:guicheng.zhang@uwa.edu.au) (G. Zhang), [Anthony.Keil@health.wa.gov.au](mailto:Anthony.Keil@health.wa.gov.au) (A. Keil), [peter.richmond@uwa.edu.au](mailto:peter.richmond@uwa.edu.au) (P.C. Richmond).

### 1. Introduction

Australian Aboriginal children suffer from disproportionately high rates of otitis media (OM) in comparison with their non-Aboriginal counterparts [1]. Some of the highest reported rates of OM in the world are in those Aboriginal children living in remote areas of Australia, where more than 90% of children have evidence of OM, and up to 40% of children are reported to have tympanic membrane perforation at any given time [2–4]. The World Health Organization has stated that regions with a prevalence of tympanic membrane perforations of 4% or more have a significant public health problem, requiring urgent attention [5]. OM in Australian

Aboriginal children often occurs within weeks of birth and is more prolonged and severe than in non-Aboriginal children [6–8]. Infections will often persist throughout childhood and the middle ear rarely normalizes [9]. The high rates of OM experienced by Aboriginal children are associated with moderate to severe hearing loss and long-term complications [10] that impact on speech, language development and education, and may result in serious sequelae such as cholesteatoma, meningitis and permanent hearing loss [11–13].

Despite the introduction of pneumococcal conjugate vaccination for all Australian Aboriginal children in 2001, *Streptococcus pneumoniae* is still a common OM pathogen [4]. High levels of antibiotic resistance have been reported in pneumococcal isolates from Aboriginal children and most serotypes identified are not covered by any of the currently licensed vaccines [4,14]. Protein-based vaccines may offer serotype-independent protection against the pneumococcus and are the subject of intense research. Several pneumococcal proteins are in early clinical trials to assess whether they confer protection against pneumococcal carriage and transmission or mucosal and systemic diseases [15]. Anti-protein antibodies are important (along with cellular immune responses) in protection from the pneumococcus by either blocking pathogenic mechanisms or through opsonisation of the bacteria [15]. Three pneumococcal proteins that are currently under investigation as vaccine candidates are pneumococcal surface protein A (PspA), pneumolysin (Ply), and choline binding protein A (CbpA) [15–17]. There is a paucity of data assessing the development of natural immunity to pneumococcal proteins in children, particularly in Indigenous populations that are at high risk of pneumococcal disease.

Otitis-prone children have been described as having impaired humoral immune responses to the major otopathogens, leading to recurrent infections [18,19]. However, data from our laboratory and others found that children with a history of chronic or recurrent OM have similar antigen-specific antibody responses to pneumococcal proteins and polysaccharides, including those in the pneumococcal conjugate vaccine when compared to healthy children [20–26]. It is unknown if the differences observed in these studies reflect differences in assays, study design or the populations studied. Similar IgG titres to pneumococcal polysaccharide and conjugate vaccines have been described in Aboriginal and non-Aboriginal populations [27], suggesting no impairment in polysaccharide responses in Aboriginal populations. Aboriginal babies were found to have higher cord-blood IgG titres (to 6 of the 12 pneumococcal serotypes tested) and higher avidity (for 4 of the 8 serotypes tested) than Gambian infants who have similar rates of early pneumococcal colonisation without the high rates of OM [28]. No data exists on the levels of naturally acquired pneumococcal protein antibodies in Australian Aboriginal children and this is important in considering potential protein vaccine studies. This study investigated the naturally acquired antibody titres to potential pneumococcal vaccine antigens in Aboriginal children undergoing surgery for OM and compared these to age-matched non-Aboriginal children with OM and healthy controls. It is important to measure baseline immunity to vaccine candidates, particularly in high-risk populations, to determine the suitability of each antigen for inclusion in a vaccine.

## 2. Methods

### 2.1. Patient populations

#### 2.1.1. Otitis media cases

Children aged between 1 and 15 years were recruited at time of admission for tympanoplasty or ventilation tube insertion through public hospitals in urban and remote West Australian hospitals

during 2003–2008. Written informed consent was obtained from parents or guardians. Clinical data was collected using parental questionnaires and medical records. Children were included if they met clinical criteria to undergo ventilation tube insertion for either otitis media with effusion (OME) or recurrent acute otitis media (rAOM) or tympanoplasty for chronic suppurative OM (CSOM). Diagnosis was recorded at the time of surgery. OME was defined as the presence of a middle ear effusion (MEE) without symptoms or signs of suppurative infection [29] for longer than 3 months. rAOM was defined as at least 3 acute OM presentations within a 6 month period or 4 presentations in a 12 month period in between which clinical symptoms resolved. CSOM was defined as having persistent discharge from the middle ear for more than 6 weeks through a perforation of the tympanic membrane [29], however discharge was resolved prior to surgery. Children were identified as Aboriginal or non-Aboriginal based on parental report.

Approval for this study was obtained from the Princess Margaret Hospital for Children (831EP), Armadale-Kelmscott Hospital and Osborne Park Hospital Ethics Committees, the Western Australian Aboriginal Health Information and Ethics Committee and Aboriginal Community Controlled Health Organisations in the Kimberley and Eastern Goldfields regions.

#### 2.1.2. Healthy control cohort

Children aged between 1 and 15 years were recruited through the Vaccine Trials Group at Princess Margaret Hospital for Children, Perth, Australia between 2007 and 2009. Children were eligible if they were healthy, had no history of obstructive sleep disorder, no history of pneumonia, had less than 2 ear infections in the past 12 months, or less than 3 ear infections ever, no history of chronic or recurrent sinusitis or rhinitis and no history of previous ear, nose or throat surgery. Written informed consent was obtained from parents or guardians. Clinical data was collected using parental questionnaires and from medical records. Approval for this study was obtained from the Princess Margaret Hospital for Children Ethics Committee (1385EP).

Exclusion criteria for both cohorts included any chromosomal or craniofacial disorders, known immunodeficiency or receiving immunosuppressive therapy.

### 2.2. Sample collection

Saliva was collected from children prior to surgery for the surgical group or at the clinic visit for the healthy controls using jumbo swabs (Multi-gate, Villawood, Australia) which were placed in 15ml tubes (Sarstedt, Nümbrecht, Germany) for storage at  $-80^{\circ}\text{C}$ . Prior to analysis, swabs were thawed and centrifuged at 3200 g for 15 min at  $4^{\circ}\text{C}$ , saliva was collected and aliquoted. Blood was collected in a serum clot tube (Vacuette<sup>®</sup> Greiner Bio-one, Frickenhausen, Germany) and centrifuged for 10 min at 3200 g and serum aliquoted. All aliquoted samples were stored at  $-80^{\circ}\text{C}$  until analysis. For the cases, if MEE was present following an anterior-inferior myringotomy incision it was collected utilizing a 2ml syringe and blunt drawing up needle. MEE were transported on ice within 4 h of collection for culture and PCR.

### 2.3. Bacteriological culture

MEE samples were cultured using standard techniques. Primary inoculations of MEE samples were made on blood agar, cysteine lactose electrolyte deficient agar, Filde's agar and colistin nalidixic acid (colistin sulphate 7.5 mg/L and nalidixic acid 5 mg/L) blood agar plates. Plates were incubated at  $35^{\circ}\text{C}$  in 5%  $\text{CO}_2$  and inspected for growth at 24 and 48 h. In addition, blood agar plates with vancomycin (2.5 mg/L) and nalidixic acid (10 mg/L) were used for

culture of anaerobes and incubated anaerobically at 35 °C in 5% CO<sub>2</sub> with inspection at 48 h and 7 days. All predominant bacteria were recorded including normal nasopharyngeal flora.

#### 2.4. Pneumolysin PCR

Genomic DNA was extracted from the MEE specimens using Roche Total Nucleic Acid Extraction kits (Roche Diagnostics) and following the manufacturer's instructions. Real time PCR to detect the *ply* gene was performed on the extracted DNA as previously described [30].

#### 2.5. Measurement of IgG and IgA against *S. pneumoniae* proteins using a multiplex bead assay

*S. pneumoniae* proteins PspA1, PspA2, CbpA and Ply were expressed and purified, and IgG and IgA antibody titres were measured by bead-based immunoassay as described previously for IgG [20]. Briefly, purified recombinant proteins were covalently conjugated to activated fluorescent carboxylated microspheres using a two-step carbodiimide reaction. Pooled sera from adult volunteers were used as the reference sera, and inter-assay variability was determined using quality control sera. Test serum samples were diluted 1:100 (PspA1) or 1:250 (PspA2, CbpA and Ply) and saliva samples were diluted at 1:10 in phosphate buffered saline (PBS) containing 2% newborn bovine serum and 0.05% Tween20 (all Sigma-Aldrich). Twenty five microliters of diluted sample was incubated with a mixture of 25 µl of the protein coated microspheres for 30 min at room temperature. Plates were washed with PBS containing 0.05% Tween20 and 100 µl of R-phycoerythrin conjugated anti-human IgG or IgA were added (Jackson ImmunoResearch Laboratories, Pennsylvania, USA), before incubation for 30 min at room temperature. Plates were again washed in PBS containing 0.05% Tween20 prior to analysis. Fluorescence of 100 beads within each bead region was measured on the BioPlex<sup>®</sup>200 system (Bio-Rad). Mean fluorescence data was collected in real-time and analysed using Bio-plex Manager 5.0 software. Data in arbitrary units (AU)/ml were generated from a 5-PL standard curve of mean fluorescence intensity against the in-house reference sera and plotted as geometric mean concentration (GMC) with 95% confidence intervals (CI). Out of range values were repeated using appropriate higher or lower serum dilutions.

#### 2.6. Total protein assay

To standardise salivary antibody levels across the cohort, total protein was measured using the Micro BCA Protein Assay Kit (Thermo Scientific), following the manufacturer's instructions. Briefly, saliva samples were diluted 1:30 in a 96 well plate with phosphate buffered saline. The working solution (25 parts BCA reagent MA, 24 parts reagent MB and 1 part reagent MC) was added and incubated at 37 °C for 120 min. Plates were cooled to room temperature and the absorbance measured at 595nm. Blanks were subtracted and protein concentration was calculated according to the protein standard curve in mg/ml. Saliva was standardised as AU per mg of total protein in the saliva.

#### 2.7. Statistical analyses

Host and environmental risk factors were compared between Aboriginal and non-Aboriginal children with a history of OM and healthy control children using Student's t tests for continuous variables (age) and Pearson Chi-square analysis (p-value asymptomatic significant 2-sided) for categorical variables (gender and day-care attendance). Differences in frequencies of diagnoses,

surgery and samples below the assay detection limit between the Aboriginal children and non-Aboriginal children were assessed using a Chi-square analysis. Serum antibody titres were expressed as Arbitrary units/mL (AU/mL) against a reference human serum pool and reported as adjusted antigen-specific geometric mean concentrations (GMCs) with 95% confidence interval (CI), adjusting for confounding variables as appropriate. GMCs were calculated using adjusted and logged antibody concentrations. Salivary antibody titres were calculated as for serum and then adjusted to correct for differences in salivary stimulation (ie by food or drink etc) and time of collection by correcting to 1 mg/ml of total protein in the sample. To compare differences in anti-pneumococcal (PspA1, PspA2, CbpA and Ply) antibody levels between Aboriginal and non-Aboriginal children with a history of OM and healthy control children univariate analysis using a general linear regression model adjusting for age and day-care attendance was used. To determine differences in the antibody titres based on Ply PCR positive MEE, independent t-tests were used. Paired samples t-tests were used to determine differences in ratios of IgA to IgG in serum and saliva. ANOVA with post hoc Tukey was used to assess difference in mean antibody titres between age categories for each group. IBM SPSS Statistics 20 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

One hundred and eighty three children were recruited, including 36 healthy children who were all non-Aboriginal as well as 70 Aboriginal and 77 non-Aboriginal children undergoing surgery for OM. None of the healthy control children had any significant history of respiratory infection including OM, 74% of the non-Aboriginal cases and 63% of Aboriginal cases were undergoing surgery for ventilation tube insertion (Table 1) and 21% and 31% for myringoplasty respectively (Table 1). The majority of non-Aboriginal cases had a current diagnosis for surgery of chronic OME (79%) and/or rAOM (47%) (Table 1). In Aboriginal children, chronic OME was the most common diagnosis (61%) followed by CSOM (34%) (Table 1). One non-Aboriginal child was undergoing a mastoidectomy for mastoiditis. One Aboriginal child and 2 non-Aboriginal children were diagnosed with cholesteatoma.

Eleven of 56 Aboriginal children and 10 of 73 non-Aboriginal children with a history of OM had received Prevenar<sup>®</sup> (data missing from 14 to 4 children respectively), 12 of the 36 healthy control children had received Prevenar<sup>®</sup>. Children were only reported as having up to date immunisations if the parents responded yes to the question "are your child's immunisations up to date?" and/or if it was recorded on the Australian Childhood Immunisation Registry (ACIR). No significant difference in immunisation status was observed between the groups (p = 0.148) (Table 1).

#### 3.2. Culture and PCR for *S. pneumoniae* from middle ear effusions

One hundred and six MEE from 74 children were cultured, from these only 2 MEE from 1 non-Aboriginal child was positive for *S. pneumoniae*, 5 MEE from 5 children (3 Aboriginal and 2 non-Aboriginal) were positive for nontypeable *Haemophilus influenzae* and 5 MEE from 3 children (2 Aboriginal and 1 non-Aboriginal) were positive for *Moraxella catarrhalis*. Pneumolysin PCR was performed on genomic DNA isolated from 110 MEE from 75 children. Fourteen (12.7%) MEE from 10 (13.3%) children (2 Aboriginal and 8 non-Aboriginal) were PCR-positive for pneumolysin indicating the

**Table 1**  
Study population.

	Healthy children	Aboriginal cases	Non-Aboriginal cases	P -value	
Number	36	70	77	NA	
Mean age years (range)	8.3 (1.6–14.4)	6.7 (1.7–12.7)	5.0 (1.1–13.6)	0.000	
Male	13 (36%)	39 (56%)	39 (51%)	0.199	
Have attended day-care	23 (64%)	28 (40%)	43 (56%)	0.290	
History of CSOM	0 (0%)	26 (37%)	8 (10%)	0.005	
Current surgery	NA	44 (63%)	57 (74%)	0.200	
VTI	Myringotomy	4 (6%)	4 (5%)	0.349	
	Myringoplasty <sup>a</sup>	NA	22 (31%)	16 (21%)	0.098
	Adenoidectomy	NA	1 (1%)	10 (13%)	0.015
	OME	NA	43 (61%)	61 (79%)	0.046
Principal diagnosis/es	rAOM	NA	21 (30%)	36 (47%)	0.020
	CSOM	NA	24 (34%)	6 (8%)	0.000
Immunisations up to date	36 (100%)	52 (74%)	69 (90%)	0.148	
Children who have received Prevenar <sup>b</sup>	33%	20%	14%	0.093	
On antibiotics at enrolment <sup>c</sup>	0%	3%	12%	0.021	

<sup>a</sup> Some myringoplasties were performed due to failure of the tympanic membrane healing post-VTI.

<sup>b</sup> Healthy n = 36, Aboriginal cases n = 56, non-Aboriginal cases n = 73.

<sup>c</sup> Healthy n = 36, Aboriginal cases n = 65, non-Aboriginal cases n = 75.

presence of *S. pneumoniae*. Pneumococcal carriage could not be assessed as nasopharyngeal swabs were not collected.

### 3.3. Age and day-care attendance effected pneumococcal protein antibody titres and were treated as confounding factors

Confounding factors associated with differences in antibody levels between the different groups were assessed between Aboriginal cases, non-Aboriginal cases and healthy controls. Age had the greatest effect on antibody titres against all antigens in the serum and saliva ( $p \leq 0.05$ ), whilst day-care attendance (current or previous) only appeared to influence anti-Ply IgA antibody titres ( $p = 0.011$ ). Gender and current antibiotic use were demonstrated to have no effect on antibody titres to any of the antigens tested (data not shown). All antibody titres reported were therefore adjusted for age and having ever attended day-care.

### 3.4. Aboriginal children with OM have higher or comparable serum IgG and IgA titres to pneumococcal proteins when compared with both non-Aboriginal children with OM and healthy controls

There was no evidence that Aboriginal children had lower serum IgG (Fig. 1A) or IgA (Fig. 1B) antibodies to any of the pneumococcal proteins tested. Serum antibody levels were significantly higher in Aboriginal children with OM than in healthy control children for anti-PspA1 IgA, anti-CbpA IgG and anti-Ply IgG ( $p = 0.001$ ,  $p = 0.012$  and  $p = 0.004$  respectively) (Fig. 1). Aboriginal children with OM also had significantly higher serum anti-CbpA IgA ( $p = 0.047$ ), and anti-PspA2 IgG, CbpA IgG and Ply IgG antibody levels than non-Aboriginal children with OM ( $p = 0.03$ ,  $p = 0.002$  and  $p = 0.012$  respectively). No significant differences in serum IgG and IgA antibody titres for any of the pneumococcal proteins were observed between non-Aboriginal children with a history of OM and healthy control children (Fig. 1A and B). Almost all serum samples had detectable pneumococcal protein-specific IgG and IgA antibodies. No serum samples had titres below the limit of detection in the Aboriginal cases, 1 non-Aboriginal case had IgA below the limit of detection for PspA2, CbpA and Ply and one healthy control child had serum IgA below the limit of detection for PspA1 (data not shown).

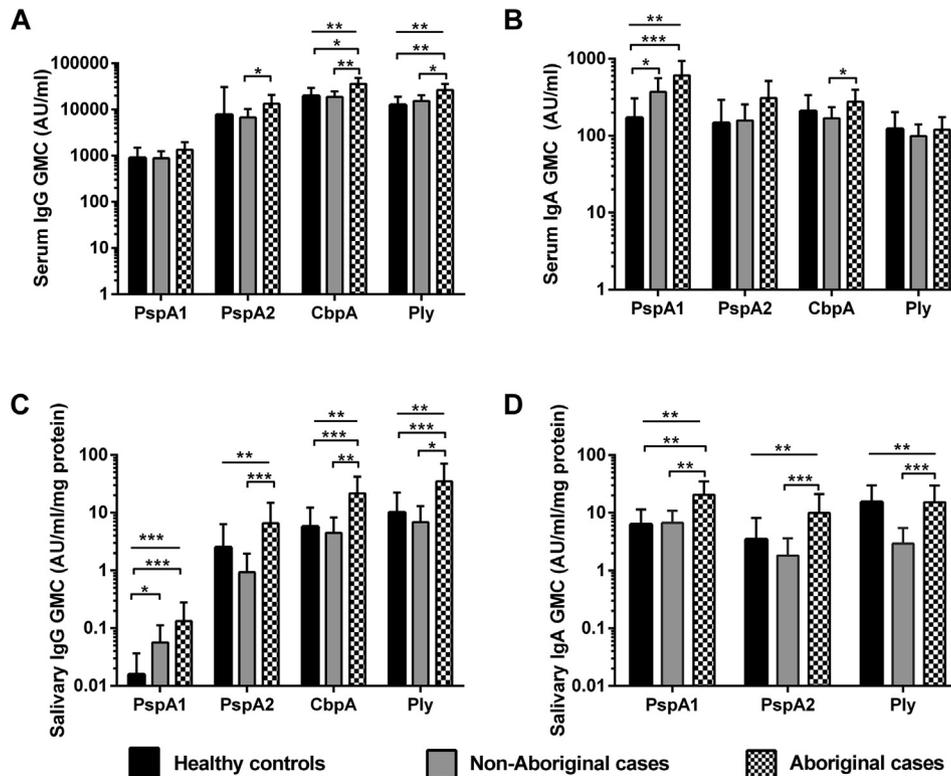
To determine differences in antibody titres in the optimal age group for vaccination (0–3 years) versus the older age groups (3–6 years and over 6 years) data were categorised and mean antibody titres compared using ANOVA. Aboriginal children with OM had

significantly lower antibody titres in the 0–3 year category compared to the 2 older categories (14994 vs 23116 and 59856 AUs;  $p = 0.001$ ) for Ply IgG, but not for any other antibody. Non-Aboriginal children with OM aged 0–3 years had significantly lower antibody titres for anti-Ply IgA and IgG, and anti-PspA1 IgG than the older age categories (38 vs 171 and 376 AUs;  $p = 0.041$ ; 3373 vs 17093 and 67473 AUs;  $p = 0.001$ ; and 341 vs 1376 and 3058 AUs;  $p < 0.001$  respectively). No differences were seen for any other antibody in the non-Aboriginal children with OM nor for the healthy controls.

### 3.5. Aboriginal children with OM have higher or comparable salivary IgA and IgG titres to pneumococcal proteins when compared with non-Aboriginal children with OM and healthy controls

Differences were observed between the 3 groups for all salivary IgA and IgG antibodies measured (Fig. 1C and D). Significantly higher titres of salivary anti-PspA1 IgA ( $p = 0.004$ ), and salivary anti-PspA1, CbpA and Ply IgG ( $p < 0.001$ ,  $p = 0.001$  and  $p = 0.001$  respectively) were observed in Aboriginal cases compared with healthy controls (Fig. 1). Aboriginal children with OM also had significantly higher salivary antibody titres than non-Aboriginal children with OM for anti-PspA1, PspA2 and Ply IgA ( $p = 0.003$ ,  $p = 0.001$  and  $p < 0.001$  respectively) (Fig. 1 D) and anti-PspA2, CbpA and Ply IgG ( $p = 0.001$ ,  $p = 0.01$  and  $p = 0.022$ ) (Fig. 1C). Only the salivary anti-PspA1 IgG titres were significantly higher in non-Aboriginal children with a history of OM when compared to control children ( $p = 0.025$ ).

Differences in the number of responders were compared by assessing frequencies of salivary samples with antibody titres within the limit of detection for each group. Differences between all of the groups were observed (Table 2): CbpA IgG,  $p = 0.004$ ; Ply IgA,  $p = 0.001$ ; Ply IgG,  $p = 0.001$ ; PspA2 IgA,  $p = 0.005$ ; PspA2 IgG,  $p = 0.001$ ; PspA1 IgG, 0.033 except PspA1 IgA ( $p = 0.061$ ). Non-Aboriginal children with OM were less likely to have detectable salivary CbpA IgG ( $p = 0.002$ ), Ply IgA ( $p = 0.001$ ) and IgG ( $p = 0.002$ ), PspA2 IgG ( $p = 0.001$ ) and IgA ( $p = 0.005$ ), and PspA1 IgA ( $p = 0.018$ ) antibodies than the Aboriginal children (Table 2), but this was not observed for PspA1 IgG ( $p = 0.055$ ). More non-Aboriginal children with OM were non-responders than healthy controls for Ply IgA ( $p = 0.042$ ), Ply IgG ( $p = 0.032$ ), PspA2 IgA ( $p = 0.033$ ) and PspA2 IgG ( $p = 0.011$ ). Aboriginal children with a history of OM and healthy controls had similar rates of antibodies



**Fig. 1.** Comparison of antibody titres for serum A) IgA and B) IgG, and salivary C) IgA and D) IgG between healthy children, Aboriginal children with a history of otitis media and non-Aboriginal children with a history of otitis media. Adjusted (age and day-care attendance) geometric mean concentration of serum IgA and IgG in Arbitrary Units/mL and salivary IgA and IgG/mL/mg total protein (95% CI) against pneumococcal proteins in healthy controls (black bar), non-Aboriginal children with a history of OM (grey bar) and Aboriginal children with a history of OM (hatched bar). P-value determined using linear regression correcting for age and day-care attendance. PspA 1/2, pneumococcal surface protein A families 1 and 2; CbpA, choline binding protein A; Ply, pneumolysin. \* $p \leq 0.05$ ; \*\* $p \leq 0.01$ ; \*\*\* $p \leq 0.001$ .

**Table 2**

**Saliva samples with detectable antibody according to group.** 50 children in the Aboriginal group, 57 in the non-Aboriginal group, 36 Healthy controls. NA - No salivary IgA is measured for CbpA as it binds the secretory component.

	Aboriginal children		Non-Aboriginal children		Healthy controls	
	IgA	IgG	IgA	IgG	IgA	IgG
<b>PspA1</b>	100% (50)	44% (22)	89% (51)	26% (15)	94% (34)	19% (7)
<b>PspA2</b>	94% (47)	90% (45)	74% (42)	61% (35)	92% (33)	86% (31)
<b>CbpA</b>	NA	100% (50)	NA	82% (47)	NA	94% (34)
<b>Ply</b>	100% (50)	100% (50)	79% (45)	82% (47)	94% (34)	97% (35)

below the limit of detection (Table 2).

### 3.6. Pneumococcal protein IgG and IgA antibodies are produced both at the mucosal surface and systemically

To investigate the site of production of antibody within the saliva sample (mucosal antibody or transudation from the serum), the ratio of IgA to IgG antibody in the serum was compared to that in the saliva. Alterations of the ratio in the saliva when compared to that in the serum, would suggest that antibody may have been locally produced. This could not be performed for CbpA as IgA cannot be measured in the saliva for this antigen due to the binding of secretory component [31,32]. Using paired samples t-tests, all ratios were first assessed as an entire cohort (containing all children within the study) and then by separating and assessing the differences between the groups (healthy controls, non-Aboriginal cases and Aboriginal cases). Differences were observed in the

ratios between serum and saliva for PspA1, PspA2 and Ply ( $p \leq 0.009$ ) suggesting that at least some of the antibody to these proteins was locally produced at the mucosal surface. The site of antibody production was also assessed by performing correlations between serum and salivary IgA (for all proteins except CbpA), and serum and salivary IgG. Antibody to all proteins had significant positive correlations, ranging from weak to strong ( $r = 0.331-0.791$ ;  $p \leq 0.04$ ), both overall and for the individual sub-groups with the exception of CbpA IgG in the Aboriginal cases ( $r = 0.282$ ;  $p = 0.09$ ).

## 4. Discussion

In this cross-sectional study, we examined antibody levels for conserved pneumococcal protein antigens in Aboriginal and non-Aboriginal children undergoing surgery for OM and healthy non-Aboriginal controls. This is the first report of pneumococcal protein antibody titres in any Indigenous population. We found no evidence of impaired pneumococcal antibody titres in Aboriginal children, in fact overall they tended to have higher mucosal and systemic antibody levels for the pneumococcal protein antigens tested compared to both the non-Aboriginal children having surgery for OM and the non-Aboriginal healthy controls (who were both similar). These findings for Aboriginal children are similar to those we reported in young children under the age of 3 years with rAOM who had higher protein antibody levels compared to age-matched healthy controls [20]. These high antibody titres may indeed be protective, given that *S. pneumoniae* detection in the middle ears of these children was low. A limitation of this study is that colonisation could not be assessed as nasopharyngeal swabs

were not collected. Previous studies have found that otitis prone children have lower pneumococcal-specific antibody responses [19]. Furthermore Xu et al. showed that otitis prone children who were colonised with *S. pneumoniae* and went on to develop pneumococcal AOM had lower IgA and IgG mucosal antibody titres to PhtD, CbpA and Ply (in both MEE and nasopharyngeal secretions) at the onset of an *S. pneumoniae* AOM episode than children who were colonised but did not develop pneumococcal AOM [33,34]. These deficiencies in pneumococcal antigen specific mucosal immune responses were shown to correlate with an increased susceptibility to developing OM [34]. Conversely, longitudinal studies of Finnish children found that antibodies to pneumococcal proteins including PspA and Ply, were indicators of prior exposure through carriage and titres did not predict disease susceptibility [21,23,25,26,35]. The children in this study were aged between 1 and 14 years. While most children develop recurrent AOM and chronic OME in the first few years of life, some children have persistent disease and ongoing treatment is required throughout childhood. The older age and high rates of Aboriginal and non-Aboriginal children who were diagnosed with both OME and rAOM reflects the long history of non-resolving disease in this severe OM cohort. In our previous studies, antigen-specific antibodies in younger healthy non-Aboriginal children increased with age [20]. This same age associated increase was not apparent in a sub-analysis of the Aboriginal and non-Aboriginal otitis-prone children during their optimal age for vaccination in this study (0–3 years). A limitation of this sub-analysis is the small number of children aged 0–3 years for each group (10 and 16 respectively). Longitudinal studies of pneumococcal carriage and immunity, particularly in Aboriginal children, are required to determine if antibody to vaccine candidates are indeed protective and/or predictive of a susceptibility to pneumococcal infection.

The presence of mucosal pneumococcal protein antibody suggests that serum IgG crosses into the mucosal space, likely by transudation, while much of the IgA measured at the surface is locally produced. IgG is an important part of the mucosal immunity because unlike IgA it is not susceptible to cleavage by bacterial proteases [36]. In the mouse *S. pneumoniae* transmission model, it is IgG not IgA at the mucosal surface that is responsible for the agglutination and subsequent clearance of the pneumococcus [36]. Currently no data exists demonstrating the functionality of any of the mucosal protein specific antibodies in children with OM (Aboriginal or non-Aboriginal) compared to healthy children. A study conducted by Short et al., has demonstrated in mice that mucosal antibody may actually be detrimental, with IgA mediating the formation of neutrophil extracellular traps in the middle ear, thereby facilitating development of pneumococcal OM [37]. It may be that this enhanced mucosal antibody response combined with host susceptibility to OM enhances the development of chronic ear disease rather than preventing infection. This needs to be further explored in populations at high-risk to mucosal pneumococcal diseases including OM.

A lack of correlation between low antibody titres and enhanced susceptibility to disease appears to be antigen dependent and differs between studies. Recent data has shown that compared to healthy children, those who are otitis prone produce similar levels of antibody to PhtD, CbpA and Ply at a similar rate, however they produce less antibody to PhtE and LytB and at a much lower rate [38]. This antigen specific deficiency may be able to be modified by vaccination, however the most appropriate candidates for both the pathogen and disease process being addressed need to be identified. A recent study has shown that vaccination of human adults with PhtD and CbpA elicited an increase in specific antibody and that these were protective when administered passively into a mouse pneumococcal challenge model in a strain dependent

manner [39]. This suggests that protein vaccination does in fact boost antibody titres to protective levels and may represent an effective prevention mechanism.

No difference in serum or salivary IgA or IgG antibody levels were observed based on the Ply PCR positive MEE results. This likely reflects the small sample size however; previous data has suggested that the antibody titre is highly correlated to nasopharyngeal colonisation [20] rather than middle ear infection.

Previous studies have also suggested that deficiencies may exist in the cellular compartment of the immune response to the common otopathogens with reduced B cell numbers [40], reduced CD4 T cell responses [41] and numbers [42]. Cellular responses to *S. pneumoniae* have not been assessed in this high risk population and this is an area for further research.

## 5. Conclusion

Aboriginal children have high antibody titres to pneumococcal protein antigens in their serum and saliva and it is unlikely that a deficiency in antibody titres to this pathogen is related to their development of higher rates of OM than their non-Aboriginal counterparts. Non-Aboriginal children undergoing surgery for OM have at least as high antibody titres as healthy children with no significant history of any respiratory infection. Thus a deficiency in the ability to mount an antigen specific antibody response to these conserved proteins of *S. pneumoniae* is unlikely to explain their susceptibility to pneumococcal OM. These data suggest that Aboriginal children respond to these *S. pneumoniae* protein antigens and that they represent potential vaccine candidates in Indigenous populations.

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## Conflict of interest

L.S. Kirkham has received institutional funding for studies investigating bacterial pathogens in children with rAOM from GlaxoSmithKline. L.S. Kirkham and R.B. Thornton have received travel funding for educational purposes from GlaxoSmithKline and Pfizer Australia. P.C. Richmond has received institutional funding for epidemiological research from GlaxoSmithKline and CSL and served on advisory boards for CSL and Wyeth Vaccines. All other authors have indicated to have no conflicts of interest.

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