Memory B cell response to a PCV-13 booster in 3.5 year old children primed with either PCV-7 or PCV-13

Running title: B cell response to a preschool PCV-13 booster

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Abbreviations used in this article

ASC, antibody secreting cells; BCR, B cell receptor, B_{MEM}, memory B cells; GC, germinal center; LLD, lower limit of detection; MenC, group C meningococcus; OPA, opsonophagocytic activity; PBMCs, peripheral blood mononuclear cells; PBS, phosphate buffered saline; PCV-13, 13-valent pneumococcal conjugate vaccine; PCV-7, 7-valent pneumococcal conjugate vaccine; PC_L, long-lived plasma cells; PCVs, pneumococcal conjugate vaccines

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Abstract

Pneumococcal protein-polysaccharide conjugate vaccines provide direct protection against *Streptococcus pneumoniae* through the induction of persistent anti-polysaccharide antibodies, and by priming for a rapid secondary antibody response. Memory B cells (B_{MEM}) generated during an initial immune response are responsible for both the more rapid and quantitatively greater secondary antibody response and are also thought to contribute to the ongoing production of plasma cells providing long-term antibody persistence. We recruited 3.5-year-old children who had participated in a previous clinical trial comparing infant immunization with either a 7-valent (PCV-7) or a 13-valent pneumococcal conjugate vaccine (PCV-13) to investigate whether prior priming with pneumococcal antigens influences B_{MEM} responses. Blood was taken before and 1 month after a PCV-13 booster. B_{MEM} were quantified using a cultured ELISpot assay for pneumococcal serotypes 1, 3, 4, 14, 19A, 23F, and with diphtheria and tetanus toxoid as controls, and then correlated with serotype-specific IgG concentrations and opsonophagocytic activity (OPA) titers. In total, blood samples from 62 participants were available for analysis. Serotype-specific B_{MEM} frequencies were generally low at baseline (before boost) although for serotypes 14 and 3, they were significantly higher in children primed with PCV-13 than PCV-7 primed children. Following the PCV-13 booster, B_{MEM} frequencies increased and were not different between the groups for all serotypes. A strong inverse correlation was found between antibody concentrations and OPA titers at baseline and B_{MEM} following booster vaccination for serotype 3 but not for other serotypes suggesting that, for this serotype, pre-existing serotype-specific antibodies may inhibit B_{MEM} formation in response to vaccination.

(252 words)

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Introduction

Streptococcus pneumoniae is a significant cause of mortality and morbidity in both children and older adults [1,2]. Effective pneumococcal vaccines have been generated through conjugation of pneumococcal serotype-specific polysaccharides to carrier proteins. These vaccines are highly immunogenic in very young children and induce immune memory. Vaccine-induced protection against pneumococcal infection is mediated and maintained by serotype-specific functional antibodies, which may be generated by long-lived plasma cells (PC_L) [3] and can facilitate elimination of invading pathogens. During an initial immune response to vaccination with a thymus-dependent antigen, not only plasma cells but also antigen-specific memory B cells (B_{MEM}) are generated. B_{MEM} may contribute to the maintenance of long-term antibody persistence through ongoing turnover and differentiation into plasma cells by antigen-specific and non-antigen-specific mechanisms [4,5]. Furthermore, these cells remain in a resting state in secondary lymphoid organs and can be reactivated upon re-exposure to antigen resulting in a rapid and marked secondary antibody response [6]. The rapid secondary response that is the hallmark of immunological memory may also be an important mechanism of protection, particularly after antibody levels have waned [7,8].

Although the inhibitory effects of maternal antibody on a primary schedule of infant immunization are well described [9], there is more limited information about the relationship between pre- and post-vaccination antibody (or cellular immunity) during a secondary response in previously immunized individuals. A further matter that is not well studied represents the functional impact and boostability of antigen-specific immune responses that are generated through natural exposure. We studied the B_{MEM} response to a booster dose of a 13-valent pneumococcal conjugate vaccine in pre-school children who had been previously primed with one of two pneumococcal conjugate vaccines containing 7 or 13 serotypes (PCV-7 or PCV-13). By correlating antibody levels to B_{MEM} frequencies before and after vaccination we aimed to assess the effect of pre-existing immunity on the response to a booster dose of vaccine containing both previously encountered and novel polysaccharide antigens.

Methods

Participants, design and study objectives

A follow-on, open label, phase IV clinical trial was conducted from May to December 2010 in four sites across the United Kingdom involving 108 healthy children as described elsewhere [10]. Briefly, study participants who had completed a previous infant trial [11], in which they had been randomized to receive either PCV-7 or PCV-13 at 2, 4 and 12 months of age, were recruited at around 3.5 years of age and given a PCV-13 booster (Prevenar 13®, Pfizer Inc., Pearl River, USA). Blood was taken before and 1 month after vaccination.

Here we report on participants who were enrolled at one of 2 sites where additional blood was taken for cultured B_{MEM} ELISpot assay. Laboratory staff remained blinded to vaccine group allocation. The study was approved by the Oxfordshire Research Ethics Committee C (Reference Number 10/H0606/09). This report describes the B_{MEM} responses of the recruited infants following the PCV-13 booster. The primary objective of the study, the persistence of serotype-specific antibody at pre-school age, has been reported previously [10].

Laboratory measurements

Serum which had been separated within 24 hours of sampling and stored at -80 °C, was tested for IgG antibody concentrations and opsonophagocytic activity (OPA) titers against PCV-13 serotypes at Pfizer Vaccine Research, Pearl River, USA, as already published [10]. The cultured ELISpot assay to detect antigen-specific B_{MEM} was performed as previously described [12,13]. Briefly, peripheral blood mononuclear cells (PBMCs) were isolated from fresh whole blood using Lymphoprep™ (Axis-Shield Diagnostics, UK) density gradient centrifugation and after a further wash step were cultured at a concentration of $2x10^5$ cells per well in a mixture of *S. aureus* Cowan strain (1:5000, Calbiochem, UK), CpG ODN 2006 (1.8 μg/ml, Source Bioscience, UK), and pokeweed mitogen (83 ng/ml, Sigma, UK) for 5-6 days at 37 °C, 5% CO_2 and 95% humidity. Cells were then harvested, washed and seeded at $2x10^5$ viable cells per well onto a 96-well plate with a PVDF-membrane (Millipore, UK), pre-coated with purified pneumococcal polysaccharides (LGC Promochem, Teddington, UK) conjugated to methylated human serum albumin (National Institute for Biological Standards and Control, South Mimms, UK).

Pneumococcal serotypes were chosen in order to reflect a) serotypes contained in both PCV-7 and PCV-13 (serotypes 4, 14, 23F) or unique to PCV-13 (serotypes 1, 3, 19A) and b) serotypes with low (1, 4),

intermediate (3, 14), and high (19A, 23F) expected colonization prevalence in the unimmunized population [14,15]. Control wells were pre-coated with diphtheria or tetanus toxoid (Statens Serum Institut, Copenhagen, Denmark), or phosphate buffered saline (PBS). Following overnight incubation, plates were washed before incubating the wells with a goat anti-human IgG conjugated to alkaline phosphatase (Calbiochem, UK) to detect bound antibody. The antibody was visualized using the alkaline phosphatase substrate kit (Bio-Rad, UK) and the reaction stopped with distilled water.

Plates were then dried overnight in a drying-oven before being read using an automated ELISpot reader with pre-defined settings (AID ELR03, AID Diagnostika, Strassberg, Germany). Spot numbers were manually corrected to exclude artifacts identified as spots by the machine. Antigen-specific spot counts were calculated as the mean of 4 wells minus the mean spot count from PBS control wells and expressed as B_{MEM} frequencies per million cultured PBMCs. Values below the lower limit of detection (LLD) of the assay and zero counts were replaced with by a value half the LLD (0.625).

Statistical analysis

Data were $\log_{10}$-transformed prior to analysis to obtain normality. Geometric means with their 95% confidence intervals are presented in the graphs. Groups were compared using independent samples t-tests using $\log_{10}$-transformed data with Satterthwaite's correction if variances were found to be unequal. Paired data were compared using paired t-tests. To compare B_{MEM} frequencies with total and functional antibody data, Pearson correlation coefficients were calculated on $\log_{10}$-transformed data. As many secondary endpoint comparisons were conducted in this study, no single result was considered in isolation. No adjustment of any p-values to account for multiplicity was made. Data were analyzed using SAS version 9.2 (SAS Institute Inc., Cary, NC, USA) and R version 3.1.0 [16]; plots were generated using the ggplot2 package for R [17].

Results

Samples were available for B_{MEM} assays from 62 out of the 76 study participants recruited at the 2 selected study sites (n = 35 and n = 27 in the PCV-13 and PCV-7 group, respectively). Missing data resulted from insufficient availability of blood to perform the B_{MEM} ELISpot assay in addition to serum analysis. There were no significant differences in the demographic (sex, age, ethnicity) or study

variables between study groups (Table I). These data were representative of data from the total study population [10].

Memory B cells determined by cultured B cell ELISpot assay

In total 98 blood samples from 62 participants were analyzed by the B_{MEM} ELISpot assay. As a positive control, B_{MEM} frequencies specific for diphtheria toxoid (representing the carrier protein CRM_{197}) were found to be significantly higher 1 month after the booster, whereas B_{MEM} frequencies specific for tetanus toxoid, which served as a negative control because it was not included in the booster vaccine, remained unchanged (Fig. 1).

At baseline, B_{MEM} frequencies specific for PCV-7/13 shared serotypes (4, 14, 23F) were generally low but significantly higher for serotype 14 in children primed with PCV-13 compared with those primed with PCV-7 (Table II and Fig. 1). After the booster vaccination at 40 months of age, frequency of B_{MEM} increased greatly for serotypes 4 and 23F whereas a smaller increase was seen for serotype 14, especially in the PCV-13 group. No significant differences of the post-booster frequencies were seen between the groups for all serotypes.

For serotypes included only in PCV-13 (1, 3, 19A), baseline B_{MEM} frequencies were low and not different between the groups for serotypes 1 and 19A. Serotype 3-specific B_{MEM} frequencies were generally even lower than other serotypes but significantly higher in the PCV-13 group compared with children previously primed with PCV-7. Post-booster B_{MEM} frequencies were greatly increased for serotype 1 and moderately for serotypes 3 and 19A and were not significantly different between the groups (Table II and Fig. 1).

Correlation between B_{MEM} frequencies and antibody

No significant correlation was found between B_{MEM} frequencies at baseline (40 mo) and IgG levels or OPA titers both before and following the PCV-13 booster for any of the serotypes apart from a significant but low correlation between B_{MEM} and IgG concentrations at baseline for serotype 1 (Fig. 2). Apart from serotype 3 (described below), there were no or only weak correlations between post-booster B_{MEM} frequencies and IgG concentrations or OPA titers at both visit time points (Fig. 2).

For serotype 3, post-booster B_{MEM} frequencies were significantly and negatively correlated with the amount of pre-existing antibody at baseline. This was true for both the PCV-13 primed group (data not

shown) as well as using data from both groups (Fig. 2 and Fig. 3). In addition, a significant and moderate negative correlation was found between B_{MEM} frequencies and IgG concentrations both measured after the PCV-13 booster, which again was unique to serotype 3 and not seen with any other serotype (Fig. 2 and Fig. 3).

When evaluating serotype-specific antibody responses by calculating geometric mean ratios post- vs. pre-booster, there were large differences between pneumococcal serotypes (Fig. 4). In general, rarely carried serotypes showed higher ratios because of relatively low pre-booster antibody levels. Antibody responses to serotype 3 were particularly low compared with other serotypes (Figs. S1 and S2).

Discussion

In this study investigating the B_{MEM} response to a PCV-13 booster in pre-school children, serotype-specific B_{MEM} were detectable in most participants when measured 2.5 years after infant PCV-7 or PCV-13 immunization. Post-booster B_{MEM} responses to all serotypes were comparable in both groups irrespective of the type of vaccine given in infancy (Fig 1). Further, we have shown for the first time that the level of pre-existing serotype 3-specific antibody is negatively correlated with, and appears to directly impair the B cell memory response to a booster dose of PCV-13 containing serotype 3 glycoconjugate (Fig. 2 and Fig. 3).

The fact that pre-booster B_{MEM} were detectable for serotypes exclusive to PCV-13 in children who had not been previously vaccinated with these antigens suggests that carriage not only induces antibody responses [18,19] (Figs. S1 and S2) but also the formation of B_{MEM}. However, both pre- and post-booster B_{MEM} frequencies were variable between serotypes providing further data to the known differences in the epidemiological, physical and immunological properties of individual pneumococcal serotypes [20–23]. For the shared serotype 14, significantly lower B_{MEM} frequencies were seen in the PCV-7 group at baseline. This finding is unexpected as both vaccines have previously been shown to induce similar antibody responses to the common serotypes [10] and it may be due to variation caused by the smaller number of samples available for analysis of this serotype. Another explanation might be that the higher serotype 14 polysaccharide content in PCV-13 (2.2 µg vs. 2.0 µg) results in the recruitment of more B cells and therefore higher peripheral B_{MEM} frequencies although it is unclear why this would not apply to B_{MEM} directed against serotypes 4 and 23F.

Post-booster B_{MEM} frequencies for shared serotypes were not different between the groups implying that the 2 vaccines are similar in their capacity to prime the immune system, which is in line with the antibody data of the same study (Figs. S1 and S2) [10]. However, for serotypes exclusive to PCV-13 (1, 3, 19A), pre- and post-booster B_{MEM} frequencies were also comparable between the groups except for serotype 3, which showed significantly lower baseline B_{MEM} frequencies in children with previous PCV-7 vaccination compared with PCV-13 recipients (Fig. 1). These findings are partly in contrast to the antibody results that show group differences in pre-booster (and sometimes post-booster) antibody responses (Figs. S1 and S2) and may suggest that B_{MEM} are as effectively generated through carriage as through vaccination. It may also be that sample numbers were not sufficiently high enough for detecting group differences. For example, although no significant group differences were found for post-booster B_{MEM} frequencies for serotype 1, geometric mean frequencies were broadly different between the PCV-7 group and the PCV-13 group (24 vs. 36 IgG ASC per million PBMCs) (Fig. 1). Yet another explanation for similar post-booster B_{MEM} frequencies irrespective of the priming vaccine may be that, in toddlers, glycoconjugates might drive naïve B cells to go through the germinal center, activating pre-existing memory B cells to a lesser extent. In line with this idea, no differences in capsular C meningococcal (MenC)-specific memory B cells were found following a booster in 12-month-old children previously primed with 1 or 2 doses of a MenC conjugate vaccine and unprimed children [24] supporting the view that in the post-infant age group the B_{MEM} frequency seems independent of priming. Due to the design of the study, potential differences in the kinetics of the memory B cell response between the groups could not been addressed. It is possible that children who had been primed with PCV-13 experience a faster increase in B_{MEM} specific for serotypes 1, 3 and 19A when challenged with a further dose of vaccine than do children who had not been primed with a vaccine containing these serotypes. Previous studies suggested a difference in kinetics for B_{MEM} between primed and unprimed individuals although 1 month post-vaccination should capture B_{MEM} irrespective of priming [25].

As one of the most striking aspects of the present study, we found the level of pre-existing serotype 3-specific antibody to be negatively correlated with the B cell memory response to a booster dose of PCV-13 containing serotype 3 glycoconjugate (Fig. 2 and Fig. 3). The immune response to serotype 3 pneumococcal polysaccharide is known to be different from other serotypes both *in vitro* [13,22] and *in vivo*, resulting in reduced booster responses [26] and associated with a lack of protection against acute

otitis media in a large efficacy trial [27,28]. Although antibody responses against serotype 3 were generally found to be well above the presumed protective thresholds in previous immunogenicity studies [11,29], they were still lower than other serotypes (Figs. 4, S1 and S2). However, this is the first report demonstrating a correlation between production of B_{MEM} and pre-existing antibody, unique to serotype 3, in this study. The reason for this distinct immunological relationship remains unclear, but might have an impact on antibody persistence. In a previous study investigating the booster response to a MenC conjugate vaccine in 12 month old children a strong correlation between MenC-specific antibody and B_{MEM} following a primary course of vaccination at 5 months of age and the persistence of MenC-specific antibody levels 7 months later was found indicating that the strength of the initial immune response may determine antibody persistence [30]. Hence, in the current study, the fact that less serotype 3-specific B_{MEM} were generated in individuals with higher baseline antibody levels may suggest that the immune response after receipt of the booster is driven towards extrafollicular generation of short-lived ASCs rather than PC_L being able to maintain adequate antibody concentration in the long term. This finding may also be a contributing factor to the limited vaccine effectiveness associated with a high estimated correlate of protection for serotype 3 invasive disease observed in UK surveillance data [31]. Given the fact that booster responses are particularly attenuated, it is unlikely that further vaccine doses would lead to an improvement of the immune response against serotype 3. Whether fewer priming doses such as in a 1+1 vaccine schedule, which has been shown to improve booster MenC-specific antibody responses [32], might enhance protection remains unclear.

The relationship between baseline immunity and booster responses is not well understood and animal studies have revealed conflicting results indicating that the antibody response may either be enhanced or suppressed by pre-existing circulating antibody (reviewed in [33]). Proposed mechanisms for a possible enhanced antibody response included (i) a more efficient uptake of antigen by antigen-presenting cells and subsequent stimulation of T cells when antigen is bound to IgG, and (ii) a lowering of the threshold for B cell activation antibody-mediated enhancement of complement. Conversely, antibody responses may be suppressed by pre-existing antibody through (i) elimination of antibody-antigen complexes before B cell activation can take place, (ii) epitope masking so that antigen is not recognized by B cells, and (iii) co-crosslinking of the B cell receptor leading to inhibition of B cell activation [33]. In the present study, participants with higher baseline antibody levels responded less

well to the PCV-13 booster compared with individuals with low levels of pre-existing antibody when baseline antibody and fold-changes between pre- and post-booster antibody were compared (Figs. S3 and S4). Similar findings have been reported for other polysaccharide-containing vaccines such as MenC [34]. However, compared with other serotypes, the overall increase in antibody against serotype 3 was low as expressed by geometric mean ratios (Fig. 4) despite relatively low baseline levels [10].

In conclusion, following a pre-school PCV-13 booster, we found a strong B_{MEM} response to all tested pneumococcal serotypes irrespective of whether these children had previously been vaccinated with these antigens indicating that pre-school vaccination might be sufficient to generate a strong and long-lasting immune response. The strong inverse correlation that was only found between pre-existing serotype 3-specific antibody and the generation of B_{MEM} formation following PCV-13 booster vaccination suggests a unique immunological behavior of this serotype potentially resulting in an inhibition of the B_{MEM} formation in response to vaccination.

Disclosures

A.J.P. and M.D.S. act as chief or principal investigators for clinical trials conducted by the University of Oxford, sponsored by vaccine manufacturers, but receive no personal payments from them. M.D.S. has participated in advisory boards and industry sponsored symposia for vaccine manufacturers, but receives no personal payments for this work. M.D.S. and J.T. have received financial assistance from vaccine manufacturers to attend scientific conferences. A.F. undertakes clinical research, consultancy and speaking engagements on behalf of all the major vaccine manufacturers including Pfizer and GSK who market PCVs. All associated income is paid to his employers the University of Bristol and University Hospitals Bristol NHS Foundation Trust.

The other authors have no financial conflicts of interest.

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Authorship contributions

Study concept and design: J.T., E.A.C., D.F.K., M.D.S., and A.J.P. Acquisition of data: J.T., A.T., E.A.C., B.M.A., and E.C. Statistical analysis: M.V. and J.T. Interpretation of the data: J.T., M.V., and A.J.P. Drafting of the manuscript: J.T. Critical revision of the manuscript for important intellectual content: J.T., A.T., E.A.C., M.V., B.M.A., E.C., A.F., M.D.S., and A.J.P.

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FIGURE LEGENDS

Figure 1 **Geometric mean frequencies (along with 95% confidence interval) of B_{MEM} specific for pneumococcal serotypes as well as diphtheria and tetanus toxoid.** The 3 serotypes in the first row (annotation above the graphs indicate the serotype) are common to both PCV-7 and PCV-13 and the 3 serotypes in the second row are only included in PCV-13. Diphtheria toxoid was used as a positive control reflecting B_{MEM} frequencies specific for the carrier protein CRM_{197} contained in PCV-13 whereas tetanus toxoid was chosen as a negative control as not an ingredient of PCV-13. Before the booster, B_{MEM} frequencies were significantly lower in the PCV-7 group for serotype 14 and serotype 3. Post-booster B_{MEM} frequencies were higher for all serotypes than pre-booster with no significant differences between the groups. Diphtheria toxoid-specific B_{MEM} significantly increase following the PCV-13 booster in both groups, whereas tetanus toxoid-specific B_{MEM} frequencies remained unchanged between the two time points.

Groups were compared using independent samples t-tests using log_{10}-transformed data with Satterthwaite's correction for unequal variances and stars indicate the associated p-value (*** <.001; ** <.01; * <.05). ASC, antibody secreting cells; CI, confidence interval; Dip, diphtheria toxoid; GMF, geometric mean frequency; IgG, immunoglobulin G; PBMCs, peripheral blood mononuclear cells; Tet, tetanus toxoid

Figure 2 **Serotype-specific correlations between antibody (IgG and OPA) at each study time point separately and B_{MEM} frequencies at baseline (40 mo) or 1 month following the PCV-13 booster (40 mo).** From all the correlations that were calculated between IgG antibody concentrations, OPA titers and B_{MEM} frequencies only serotype 3 stands out with a consistent and significant negative correlation between antibody (IgG and OPA) at baseline and B_{MEM} frequencies following the PCV-13 booster. Each row shows the results of correlations performed for one serotype. The top 3 serotypes are common to both PCV-7 and PCV-13 and the 3 bottom serotypes are only included in PCV-13. Shown are Pearson correlation coefficients of correlations including data from both groups with red color and blue indicating negative and positive values, respectively. The size of the bubbles indicates the associated p-value.

Figure 3 **Correlations between serotype 3 specific antibody at baseline and frequencies of B_{MEM} generated one month following a PCV-13 booster for both vaccine groups combined.** An inverse correlation is shown for B_{MEM} frequencies post vaccination compared with both serotype 3-specific IgG antibody concentration (left panel) and OPA titers (right panel). Both axes are on a log scale; n indicates the number of paired measurements, r the Pearson correlation coefficient, and p the associated p-value.

Figure 4 **Geometric mean ratios (GMR) for IgG and OPA for serotypes common to PCV-7 and PCV-13 (4, 14, and 23F) and for serotypes only included in PCV-13 (1, 3, 19A) for both groups combined.** GMR are variable between serotypes and different for IgG concentrations and OPA titers.

439 **FIGURES**

440 **Figure 1**

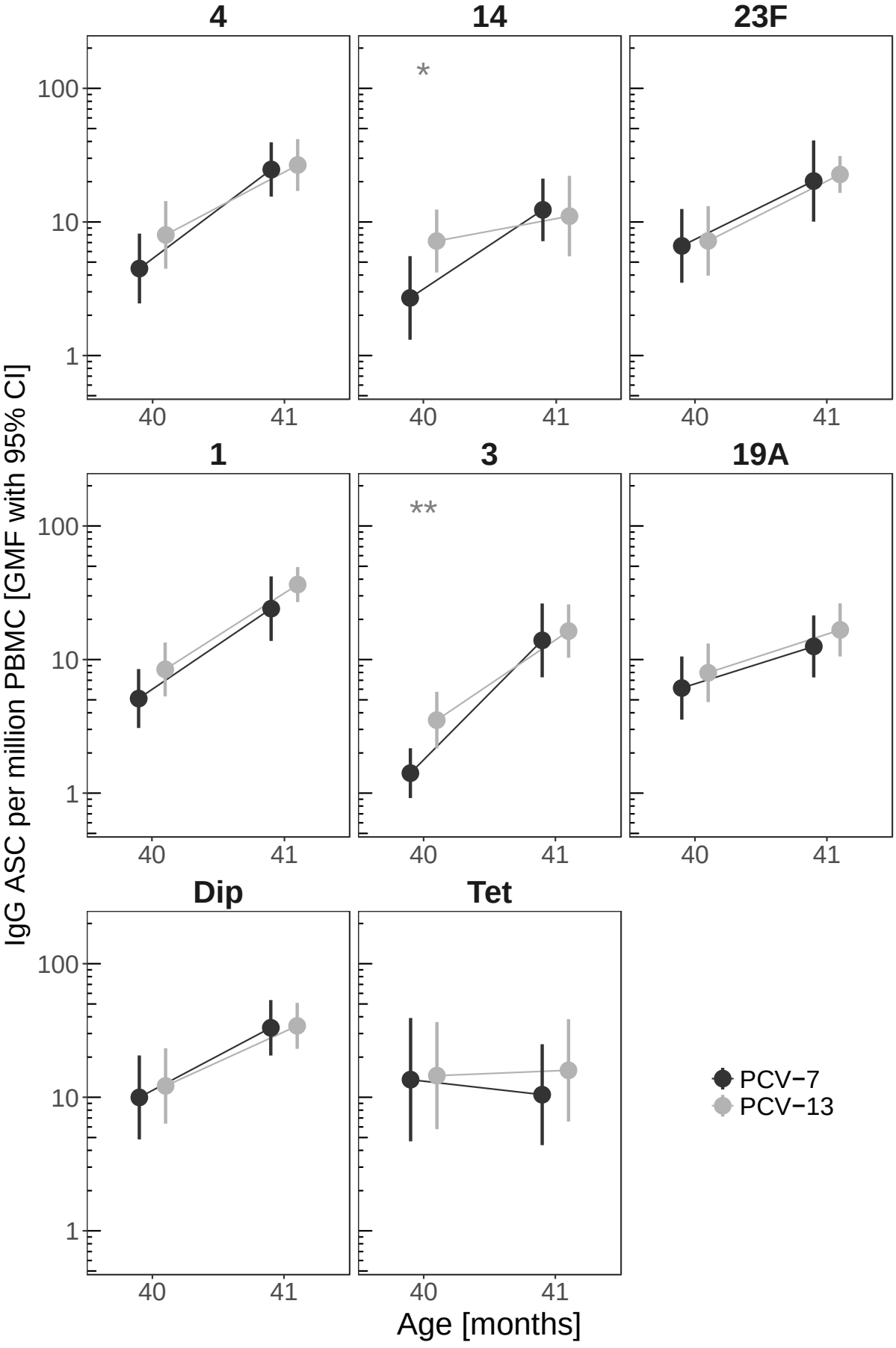

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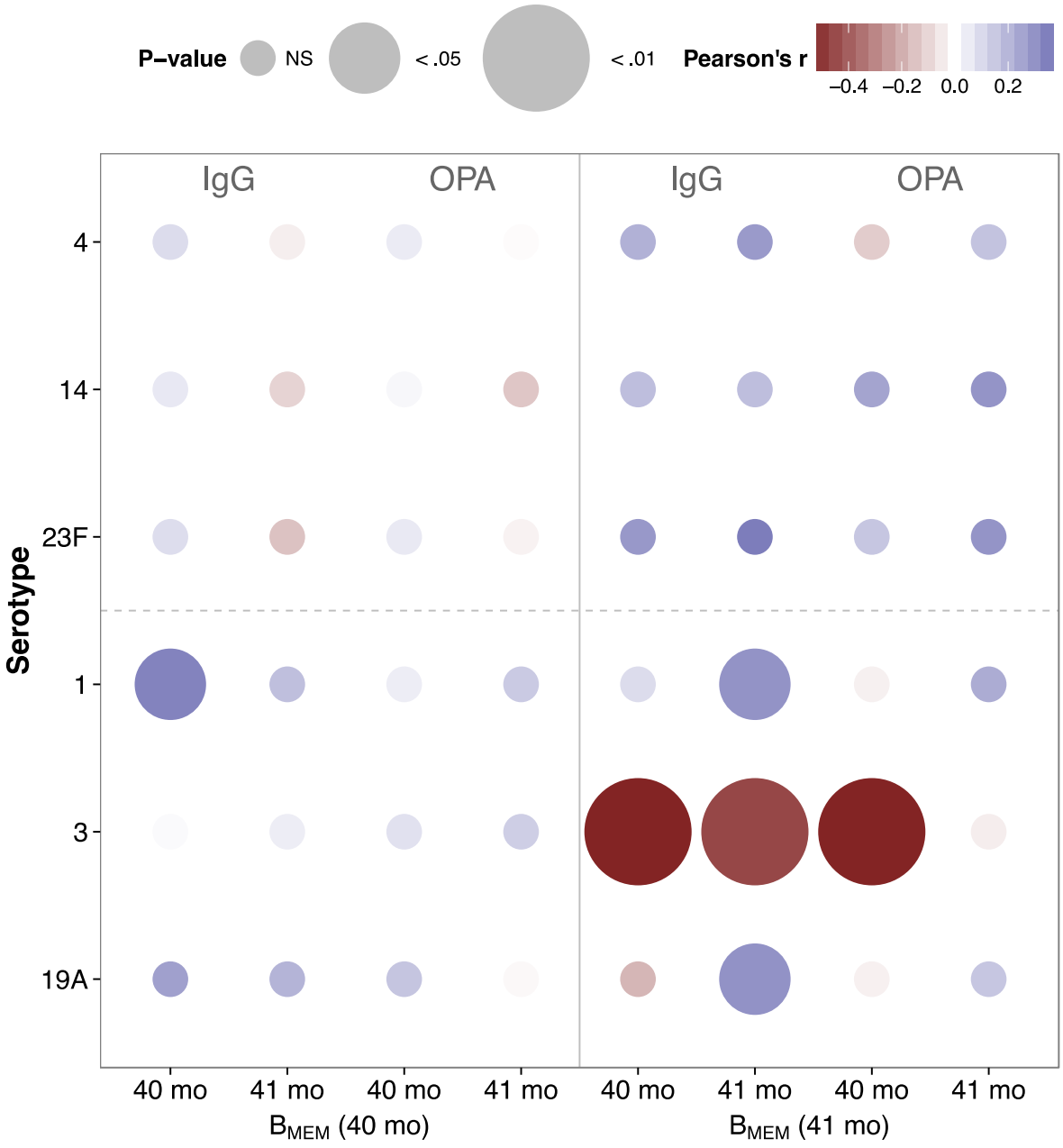
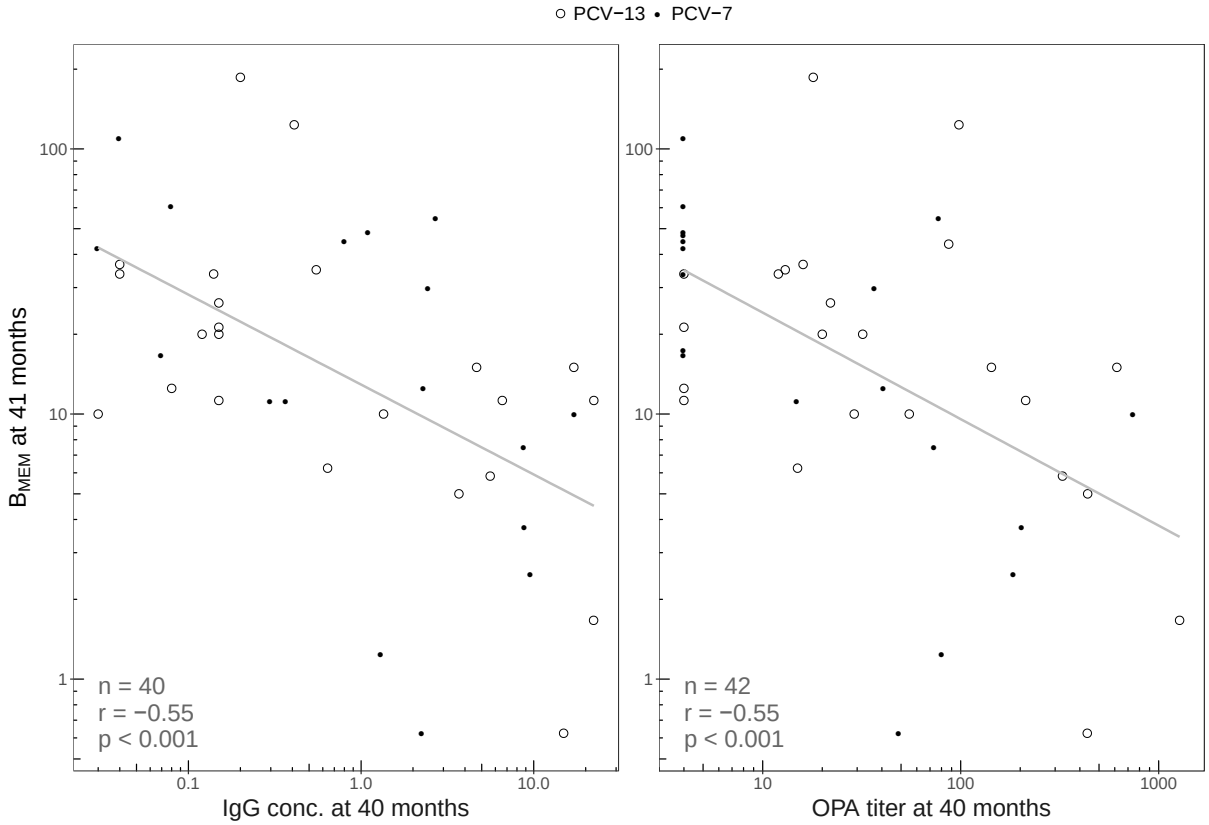
Figure 2


P–value
NS
< .05
< .01
Pearson's r
−0.4
−0.2
0.0
0.2
IgG
OPA
IgG
OPA
Serotype
4
14
23F
1
3
19A
40 mo
41 mo
40 mo
41 mo
40 mo
41 mo
40 mo
41 mo
B_MEM (40 mo)
B_MEM (41 mo)

444 **Figure 3**



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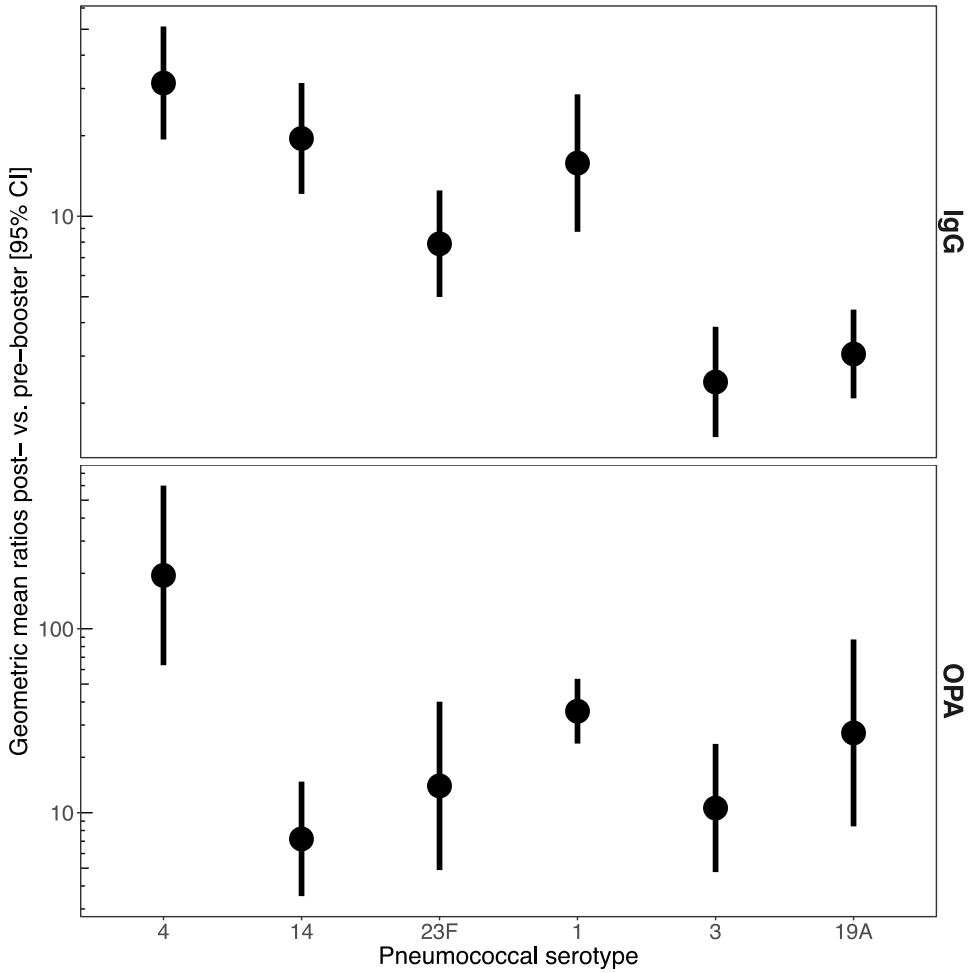
446 **Figure 4**



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448 **TABLES**

Table I Summary of study participants' characteristics

	PCV-13 (N=35)	*PCV-7 (N=27)*
Proportion of female study participants	17 (48%)	15 (56%)
Proportion of White Caucasian study participants	34 (97%)	25 (93%)
Mean age at Visit 1 in years (range)	3.46 (3.16 – 3.75)	3.42 (3.15 – 3.76)
Mean time since 12-month booster to Visit 1 in years (range)	2.45 (2.13 – 2.77)	2.42 (2.15 - 2.76)
Mean time interval between Visit 1 - Visit 2 in days (range)	34.8 (26.5 – 49.4)	34.8 (27.6 - 42.5)

Table II Geometric mean memory B cell count per million cultured PBMCs with 95% confidence intervals

	GMC (95% CI)* ***N (n miss)***			***GMC (95% CI)**** ***N (n miss)***		
Serotype	***PCV-13 40 months***	***PCV-7 40 months***	***P value** 40 months***	***PCV-13 41 months***	***PCV-7 41 months***	***P value** 41 months***
4	7.99 (4.46, 14.32) 20 (15)	4.48 (2.45, 8.18) 19 (8)	0.156	26.66 (17.04, 41.69) 16 (19)	24.69 (15.46, 39.42) 14 (13)	0.802
14	7.19 (4.18, 12.38) 15 (20)	2.7 (1.31, 5.54) 13 (14)	**0.024**	11.05 (5.53, 22.08) 12 (23)	12.31 (7.18, 21.1) 14 (13)	0.788
23F	7.21 (3.96, 13.13) 17 (18)	6.61 (3.5, 12.49) 16 (11)	0.835	22.66 (16.49, 31.14) 14 (21)	20.25 (10.06, 40.76) 14 (13)	0.756*
1†	8.44 (5.31, 13.43) 25 (10)	5.11 (3.07, 8.48) 25 (2)	0.138	36.44 (26.97, 49.24) 27 (8)	24.05 (13.79, 41.96) 21 (6)	0.182*
3†	3.52 (2.16, 5.73) 24 (11)	1.41 (0.92, 2.17) 25 (2)	**0.005**	16.35 (10.32, 25.91) 27 (8)	13.93 (7.37, 26.34) 21 (6)	0.667
19A†	7.96 (4.81, 13.19) 24 (11)	6.12 (3.55, 10.55) 25 (2)	0.469	16.68 (10.55, 26.38) 25 (10)	12.55 (7.35, 21.42) 20 (7)	0.403

** Satterthwaite's method for unequal variances; ** Independent samples t-tests comparing PCV-13 to PCV-7 groups; † Serotypes only present in PCV-13. n miss, number of samples with no available information for this antigen*

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