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Short communication

Salivary antibody responses to ten-valent pneumococcal conjugate vaccination following two different immunization schedules in a healthy birth cohort

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ABSTRACT

Pneumococcal conjugate vaccines reduce pneumococcal colonization via serotype-specific immunoglobulin G (IgG) at mucosal surfaces. The infant immunization schedule with the ten-valent pneumococcal conjugate vaccine (PCV10) changed from a 3 + 1 schedule (2–3-4–11 months) to a 2 + 1 schedule (2–4–11 months) in The Netherlands in 2013. We compared anti-pneumococcal IgG concentrations in saliva between the schedules. IgG was measured using a fluorescent bead-based multiplex immunoassay at the ages of 6 (post-primary) and 12 (post-booster) months in 51 infants receiving the 3 + 1 schedule and 68 infants receiving the 2 + 1 schedule. Post-primary IgG geometric mean concentrations (GMCs) were comparable between schedules for all vaccine serotypes. Post-booster IgG GMCs were significantly lower after the 2 + 1 schedule for serotypes 4 ($p = 0.035$), 7F ($p = 0.048$) and 23F ($p = 0.0056$). This study shows small differences in mucosal IgG responses between a 3 + 1 and a 2 + 1 PCV10 schedule. Future studies should establish correlates of protection against pneumococcal colonization for mucosal antibodies.

1. Introduction

Pneumococcal conjugate vaccination in children has drastically reduced the burden of disease caused by *Streptococcus pneumoniae,* ranging from respiratory infections like acute otitis media and pneumonia to invasive disease like septicaemia and meningitis [1,2]. Apart from direct protection, a reduction in nasopharyngeal pneumococcal colonization is the second pillar of success of conjugate vaccines [3]. Nasopharyngeal colonization can be considered both a prerequisite for infection as well as the source of community spread [4,5]. The pneumococcal conjugate vaccine does not only induce long-term immunoglobulin type G (IgG) in serum, but also at mucosal surfaces [6], which were shown to correlate well with serum levels [7]. Although the exact mechanisms by which pneumococcal acquisition at the nasal mucosa is prevented following pneumococcal conjugate vaccination remain unclear, mucosal IgG has been suggested as a protective agent [8,9].

The Netherlands introduced the 7-valent pneumococcal conjugate vaccine (PCV7) in the national immunization program (NIP) in 2006, and switched to the 10-valent vaccine (PCV10, Synflorix) in 2011. Initially, immunization was advised in a primary series of 3 vaccines at the ages of 2, 3 and 4 months followed by a booster dose at the age of 11 months (3 + 1 schedule). In November 2013, the schedule was adapted by dropping the 3-month dose (2 + 1 schedule). This decision was based on non-inferiority data from a trial investigating the impact of timing and number of doses for the 13-valent vaccine [10]. However, less is known for the 10-valent vaccine, which covers fewer serotypes, and differs in carrier proteins and immunogenicity [11,12], and in particular, the impact on mucosal IgG concentrations has not been compared between different immunization schedules. Therefore, we aimed to compare post-primary and post-booster serotype-specific anti-pneumococcal IgG concentrations in saliva following vaccination with PCV10 in a birth cohort of infants who received either the

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3 + 1 (2–3-4–11 months) or the 2 + 1 (2–4–11 months) immunization schedule.

2. Methods

2.1. Study population and sample collection

Saliva was available from 119 healthy, full-term infants born between December 2012 and June 2014, who participated in the Microbiome Utrecht Infant Study [13]. Follow-up until the age of 12 months was complete for 117 (98.3%) infants (Fig. 1). According to the Dutch NIP, 51 infants born before September 2013 received PCV10 in a 3 + 1 schedule with vaccinations at 2, 3, 4 and 11 months of age, and 68 infants born from September 2013 received a 2 + 1 schedule with vaccinations at 2, 4 and 11 months of age. Mothers were not vaccinated with PCV10. Saliva was collected from infants during home visits at 1 month of age (baseline), after finishing the primary series at 6 months of age (post-primary), and after the booster dose at 12 months of age (post-booster). Saliva was collected by placing an absorbent sponge (S10 Oracol, Malvern Medical Developments Ltd., Worcester, UK) in the cheek pouch and under the tongue for 1 minute, so that the sponge became saturated with saliva, and was immediately transferred to a tube containing EDTA (BD Vacutainer, New Jersey, USA) plus protease inhibitor (Roche, Basel, Switzerland). Samples were transported on dry ice and stored at −80 °C awaiting laboratory analyses. Ethical approval was granted by the Dutch national ethics committee (METC Noord-Holland, M012-015, NTR3986), and parental informed consent was obtained from all participants. The study was conducted in accordance with the European Statements for Good Clinical Practice.

2.2. Laboratory methods

Concentrations of serotype-specific anti-pneumococcal IgG in saliva were measured using a fluorescent bead-based multiplex immunoassay (MIA), which had been validated internally for use in saliva and was previously published [7,14]. In short, 10 sets of carboxylated microspheres (Luminex, Austin, TX) were coated with the pneumococcal polysaccharide antigens 1, 4, 5, 6B, 7F, 9V, 14, 18C, 19F and 23F (ATCC, Manassas, VA). Antigens were linked to Poly-L-lysine, and the complex was bound to the microspheres in a reaction using EDC with sulpho-NHS. The in-house reference serum IVIG (Sanquin, Amsterdam, The Netherlands) with previously assigned concentrations of IgG, determined by calibration with international standard serum, was used in serial dilutions as standard reference [15]. Saliva samples were thawed and centrifuged, and supernatants were diluted 1:2 and 1:10 using phosphate buffered saline (PBS; pH = 7.2) with 5% antibody-depleted human serum (Valley Biomedical, Winchester, VA) and with 15 μg/ml multi cell wall polysaccharide (Statens Serum Insititut, Copenhagen, Denmark). From each dilution, 25 μl was mixed with an equal volume of beads. R-phycoerythrin conjugated goat anti-human IgG solution diluted 1:200 (Jackson ImmunoResearch, West

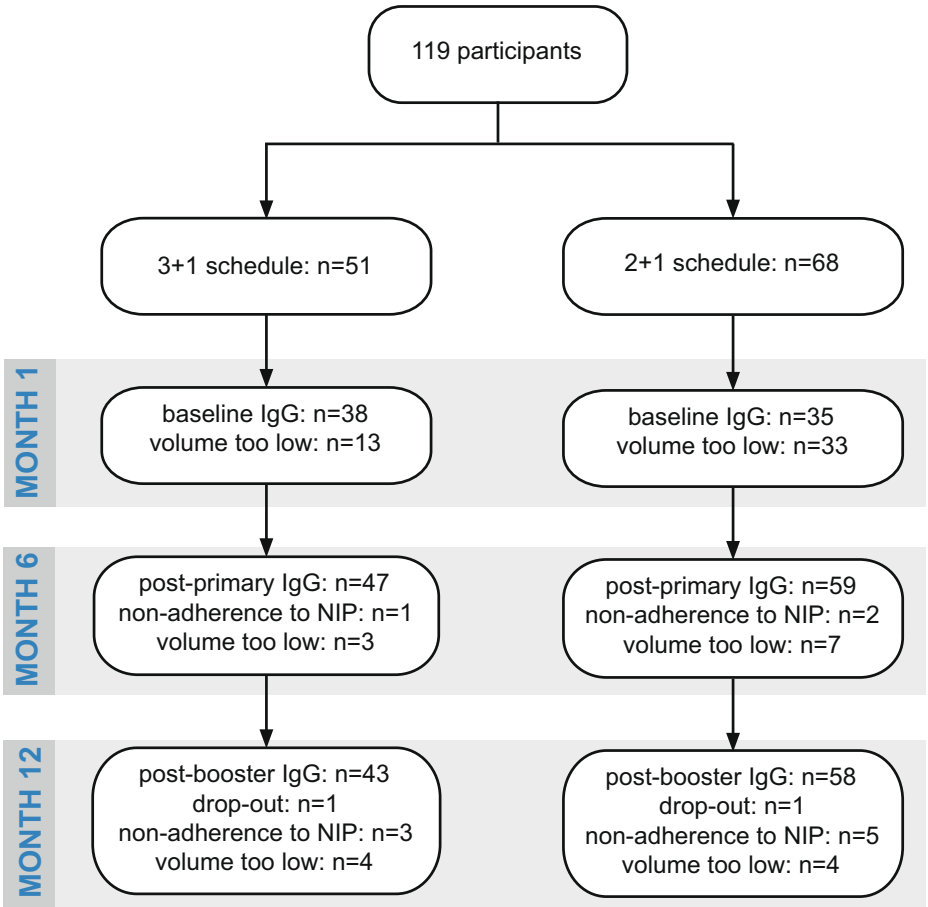

Fig. 1. Flowchart showing number of infants and samples per PCV10 immunization schedule. Numbers of infants in the study and of immunoglobulin G (IgG) measurements at each sampling moment, broken down according to PCV10 immunization schedule (2 + 1 or 3 + 1 schedule). Reasons for missing measurements were a too low sample volume for laboratory analysis, the infant not having received the vaccine before the sampling moment (non-adherence to the national immunization program [NIP]) or the infant dropping out of the study.

Grove, PA) was added to each well. Analysis of the beads was performed on a BioPlex 200 apparatus using the BioPlex software package version 6.2 (Bio-Rad Laboratories, Hercules, CA). Concentrations were determined by averaging results of both dilutions. Results from the two dilutions differed more than twofold (coefficient of variation (CV) greater than 47%) in 23 samples with low IgG concentrations. The 1:10 dilution was considered to be more precise because of less interference from components of the saliva, and therefore, the result of the 1:10 dilution was used when in standard range for samples with a high CV. IgG concentrations were expressed in ng/ml. The lower limit of detection ranged from 0.08 ng/ml for serotype 4 to 0.37 ng/ml for serotype 14. Lower IgG concentrations were set at half the limit.

2.3. Statistical analysis

Serotype-specific IgG antibody concentrations in saliva are reported as geometric mean concentrations (GMCs) with 95% confidence intervals (CI). Fold changes were calculated as the ratio between GMCs. Baseline, post-primary and post-booster IgG concentrations were compared with Kruskal-Wallis tests followed by pairwise comparisons, and p-values were adjusted for 3 multiple comparisons using Bonferroni correction. Differences in post-primary and post-booster salivary serotype-specific IgG concentrations between immunization schedules were first assessed using Wilcoxon rank-sum tests. Multivariable linear regression was used to test independent associations of log2-transformed IgG concentrations with vaccination schedule, month 1 (baseline) log2-transformed IgG concentrations, sex, age in days at time of first vaccination, and time between vaccination and sampling as covariates. The antilog (2^x) of model coefficients with their 95% CIs are presented as geometric mean ratios (GMRs). GMRs indicate the relative increase in salivary IgG concentrations that is related to a 1-unit change in the model covariate. Time between vaccination and sampling was included in the model in a second degree polynomial to reflect the natural kinetics of IgG responses to vaccination. P-values below 0.050 were considered statistically significant, and p-values above 0.050 but below 0.100 were considered relevant trends towards significance. Analyses were performed in R version 4.0.3.

3. Results

Salivary serotype-specific anti-pneumococcal IgG concentrations were measured in 73 baseline saliva samples, 106 post-primary samples (obtained on average 62 days after the last priming dose, range 22–89 days) and 101 post-booster samples (obtained on average 27 days after the booster dose, range 5–64 days) (Fig. 1). IgG concentrations increased over time for all vaccine serotypes (Fig. 2A). After the primary series, salivary serotype-specific IgG GMCs varied between 1.00 ng/ml (95% CI, 0.75–1.34) for serotype 23F and 4.56 ng/ml (95% CI, 3.67–5.65) for serotype 7F, representing a 1.2 to 5.5-fold change compared with pre-immunization concentrations; this increase was significant for serotypes 1, 4, 5, 6B, 7F, 9V and 18C (all Bonferroni-adjusted p-value < 0.050) but not for serotypes 14, 19F, and 23F. After the booster dose, we observed a stronger, significant 3.2 to 9.4-fold increase compared with post-primary salivary IgG GMCs for all vaccine serotypes (all Bonferroni-adjusted p-value $\ll$0.001), resulting in post-booster GMCs between 7.33 ng/ml (95% CI 5.75–9.33) for serotype 23F and 27.30 ng/ml (95% CI, 22.14–33.67) for serotype 19F.

Because the PCV10 immunization schedule changed in 2013, 51 (42.5%) infants who were immunized according to the 'old' 3 + 1 schedule with priming doses at 2, 3 and 4 months, were compared with 68 (56.7%) infants who received the 'new' 2 + 1 schedule, dropping the 3-month dose. Regarding post-primary IgG concentrations, there were no differences between the schedules, although we observed a trend towards higher IgG concentrations against serotype 4 in children who received the 2 + 1 schedule compared with the 3 + 1 schedule (p = 0.064) (Fig. 2B). By contrast, post-booster IgG concentrations were significantly lower following the 2 + 1 in comparison with the 3 + 1 schedule for serotypes 4 (p = 0.035), 7F (p = 0.048) and 23F (p = 0.0056), with a borderline significant difference for serotype 9 V (p = 0.058) (Fig. 2C).

To investigate independent effects of immunization schedule and other factors known to influence PCV immunogenicity, i.e. age at first immunization, baseline anti-pneumococcal IgG concentrations, and male compared to female gender, on the IgG response to PCV10, we performed a multivariable analysis (Fig. 3). These host factors were comparable between subjects receiving different schedules (Table 1). We found no significant associations between immunization schedule and post-primary or post-booster salivary anti-pneumococcal IgG concentrations in multivariable analysis, though for serotype 23F a trend towards lower post-booster IgG concentrations was observed for the 2 + 1 group (p = 0.079). Older age at time of first immunization was associated with higher post-primary IgG concentrations against serotypes 1, 4, 18C and 19F (GMR 1.05–1.08, p < 0.030), but not with post-booster salivary IgG concentrations. Finally, we observed negative correlations between baseline and post-primary IgG concentrations for serotypes 5, 6B, 7F, 14, 18C and 23F (GMR 0.70–0.86, all p < 0.005), and between baseline and post-booster IgG concentrations for serotype 14 (GMR 0.84, 95% CI 0.73–0.96, p = 0.014). Male compared to female gender was not associated with IgG responses to PCV10.

4. Discussion

To our knowledge, this is the first study that compared serotype-specific anti-pneumococcal IgG in saliva following pneumococcal conjugate vaccination with PCV10 between different immunization schedules. Comparable IgG concentrations were observed 2 months after completion of a 2-dose (2–4 months) and a 3-dose (2–3-4 months) primary series of PCV10. However, IgG concentrations against serotypes 4, 7F and 23F were modestly but significantly lower following the booster dose in children who received PCV10 in a 2 + 1 schedule. Studies comparing serum IgG responses between the 3 + 1 and 2 + 1 PCV10 schedule have not been published, and it remains unclear whether our findings in saliva align with results in serum. However, a large trial comparing schedules with PCV13 showed that post-booster serum responses following the 2 + 1 schedule were non-inferior to the 3 + 1 schedule [10]. Previous studies have demonstrated strong correlations between saliva and serum anti-pneumococcal IgG [7,16], suggesting that salivary antibodies can be a useful indicator of serum antibody status. Furthermore, IgG at mucosal surfaces may contribute to protection against pneumococcal infection by inhibiting nasopharyngeal colonization. Findings from a human challenge model showed that mucosal IgG effectively prevented acquisition of *S. pneumoniae* through bacterial agglutination [9]. Future studies should strive to establish correlates of protection against pneumococcal colonization for mucosal antibodies.

Furthermore, anti-pneumococcal IgG patterns in saliva were comparable to those observed in studies of systemic IgG responses in serum following vaccination with PCV-10, which suggests that saliva may be a good, non-invasive specimen next to serum to monitor IgG responses to vaccination. In line with earlier observations, we confirmed that older age at time of the first PCV10 dose was associated with higher post-primary IgG concentrations [10,17]. Likewise, we confirmed that pre-vaccination IgG concen-

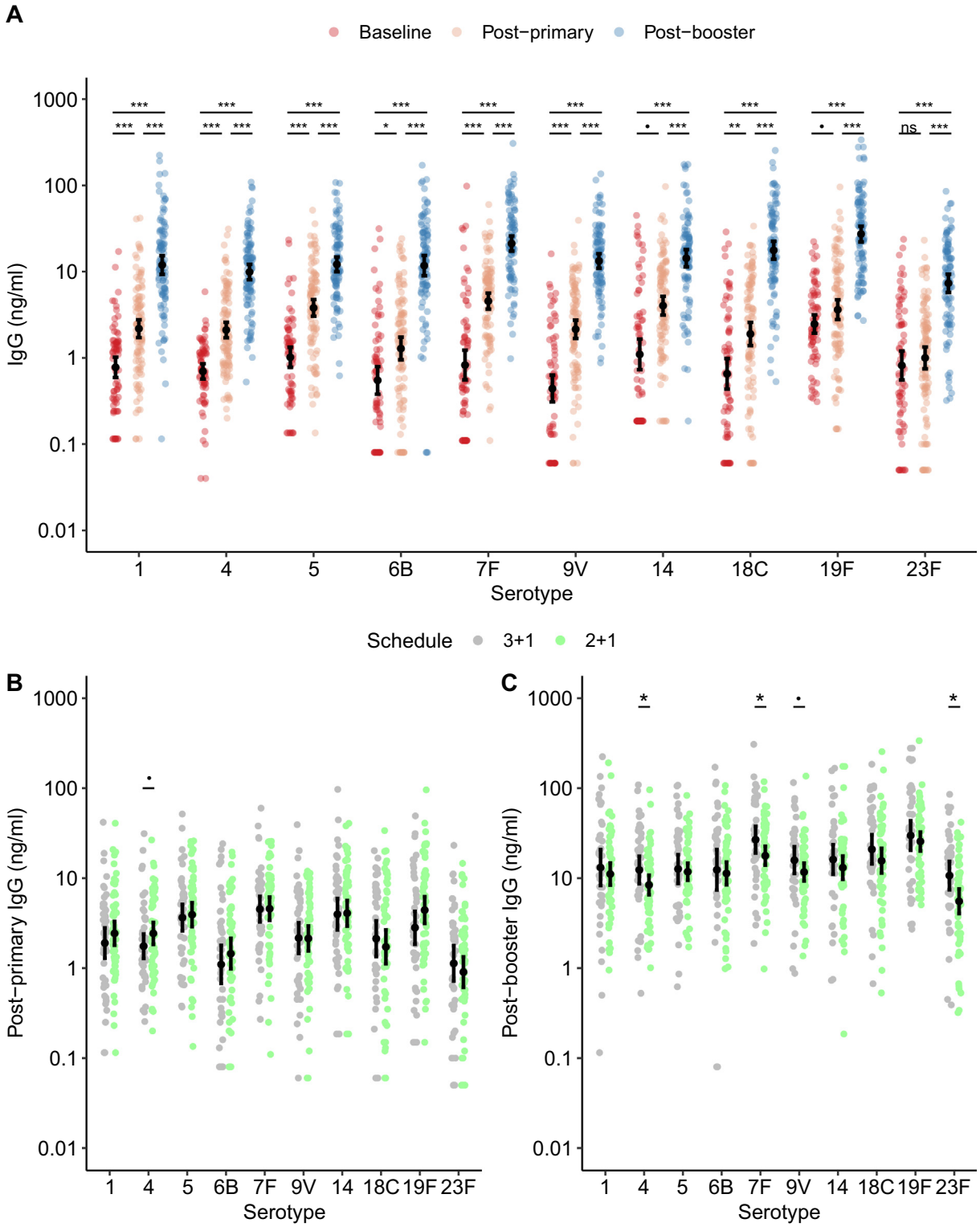

Fig. 2. Serotype-specific anti-pneumococcal IgG responses following the priming doses and the booster dose of PCV10. Serotype-specific anti-pneumococcal IgG concentrations were compared (A.) before PCV10 vaccination, after the primary series and after the booster dose, and between the 3 + 1 (at 2–3-4–11 months) and the 2 + 1 (at 2–4–11 months) PCV10 immunization schedule (B.) after the primary series, or (C.) after the booster dose. Black dots and error bars represent geometric mean concentrations with 95% confidence intervals. Significance was assessed by pairwise Wilcoxon rank-sum tests, and was indicated by ***: $p < 0.001$; **: $p < 0.005$; *: $p < 0.05$; •: $p < 0.10$; ns: not significant.

trations at 1 month of age were negatively related to post-primary IgG concentrations. Similar associations were previously reported for serum antibody concentrations, and likely reflect that maternally derived IgG, which predominates in early life, inhibits the IgG response to pneumococcal vaccination [17].

Strengths of our study include the extensive participant data including vaccination dates. The sensitive MIA technology also allowed us to accurately measure IgG concentrations, even in very low volumes of saliva. A limitation of our study is the lack of serum; therefore, salivary IgG could not be correlated with serum

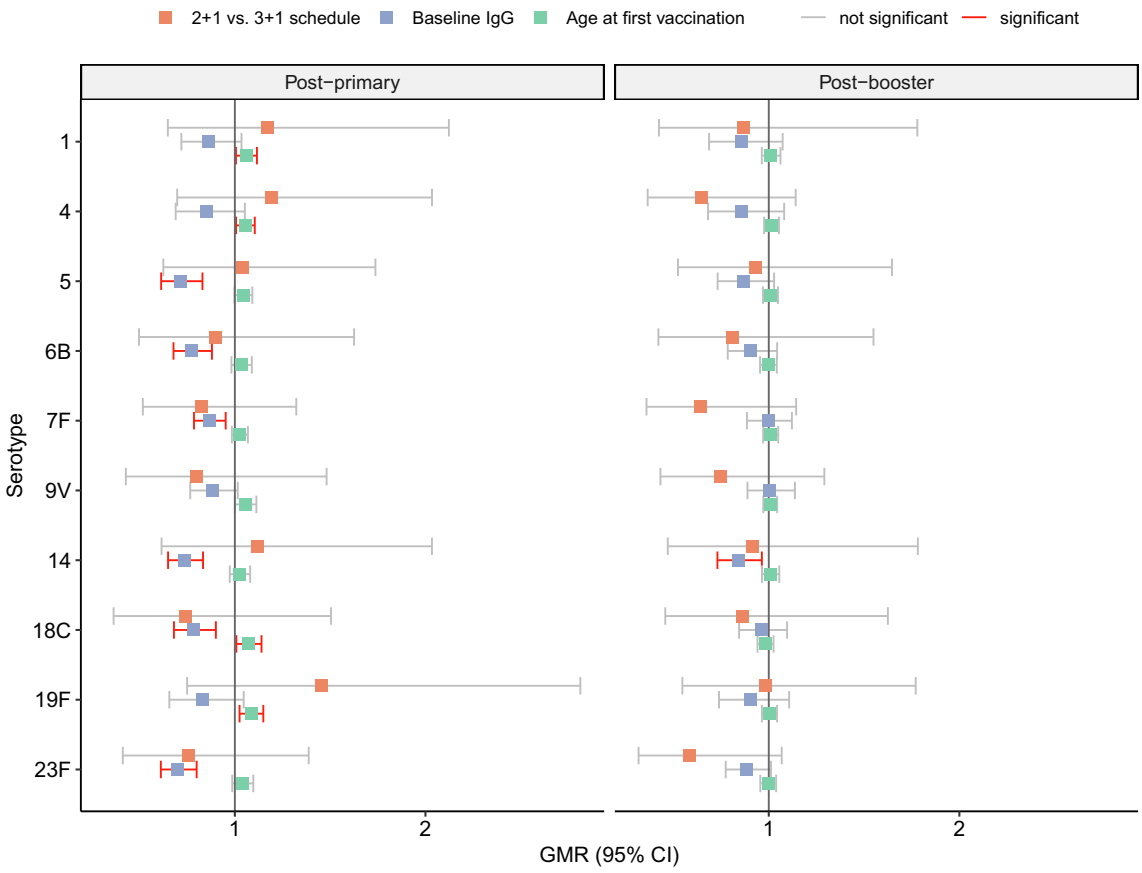

Fig. 3. Independent associations between PCV10 immunization schedule, baseline antibody concentrations, age at first vaccination, and sex and vaccine responses. Significant findings ($p < 0.050$) are shown in red. For PCV10 schedule, geometric mean ratios (GMRs) greater than 1.0 indicate that children who received the new 2 + 1 (2–4–11 months) schedule, have higher IgG concentrations than children who received the old 3 + 1 (2–3-4–11 months) schedule after the primary series or the booster dose. For baseline IgG, the GMR indicates the relative increase in IgG concentrations associated with a doubling of baseline IgG concentrations. For age at first PCV10 vaccination, the GMR indicates the relative increase in IgG concentrations associated with a 1 day increase. Male vs. female gender was not significantly associated with serotype-specific IgG concentrations and was therefore not included in the figure. The analyses also corrected for time between vaccination and saliva collection using a second degree polynomial.

Table 1
Gender, age at first immunization, and baseline anti-pneumococcal IgG in infants receiving the 3 + 1 and the 2 + 1 PCV10 schedule.

	3 + 1 schedule	2 + 1 schedule	p-value
	51	68	
Basic characteristics			
Male, *n (%)*	23 (45.1)	33 (48.5)	0.85
Age at first immunization (months), *median [IQR]*	1.9 [1.8, 2.1]	1.9 [1.8, 2.0]	0.74
Baseline anti-pneumococcal IgG per vaccine serotype (ng/ml), *GMC (95% CI)*			
1	0.75 (0.54–1.06)	0.80 (0.51–1.26)	0.80
4	0.79 (0.60–1.04)	0.61 (0.45–0.83)	0.28
5	0.94 (0.63–1.39)	1.11 (0.76–1.62)	0.52
6B	0.45 (0.26–0.76)	0.69 (0.40–1.17)	0.19
7F	1.08 (0.58–2.01)	0.61 (0.37–1.01)	0.25
9V	0.52 (0.29–0.92)	0.37 (0.24–0.57)	0.62
14	1.05 (0.60–1.84)	1.15 (0.62–2.16)	0.84
18C	0.69 (0.40–1.18)	0.62 (0.32–1.18)	0.80
19F	2.16 (1.50–3.10)	2.86 (2.04–4.00)	0.32
23F	0.82 (0.48–1.40)	0.81 (0.44–1.47)	0.93

Significance was assessed using chi-square tests for the categorical variable and Wilcoxon rank-sum tests for continuous variables. Data are summarized as n (%); median with interquartile range (IQR); or geometric mean concentrations (GMC) with 95% confidence intervals (CI).

levels. Furthermore, the relatively low number of participants limited our power to detect significant differences and may explain why vaccine schedule was not associated with the anti-pneumococcal IgG response in multivariable analysis. We did not correct IgG measurements for the dilution factor in saliva, but earlier work has shown that this has only little effect [7]. Moreover, our observational design does not allow to fully preclude the influence of potential confounding factors, including potential differences in circulation of pneumococcal vaccine serotypes in the population and thereby boosting of mucosal immunity during

the study period [7,18]. However, since 2011, temporal changes in vaccine serotype prevalence in the paediatric population in The Netherlands have been rare, so we anticipate this to be of limited effect [19].

In conclusion, modest decreases in serotype-specific anti-pneumococcal IgG concentrations in saliva following the booster PCV10 dose were observed after leaving out the middle dose of the primary series, resulting in a 2 (2–4) month interval between the first and second primary immunizations. Future research should investigate whether salivary IgG is a good correlate of protection from nasopharyngeal pneumococcal colonization after immunization. We propose that measuring IgG against vaccine serotypes in saliva next to serum may represent a valid, non-invasive method to quantify mucosal immune responses in studies investigating or monitoring changes to pneumococcal vaccines and schedules.

Author contributions

MAvH, DB and EAMS conceived and designed the study; MAvH was responsible for subject enrolment and sample collection; FdH performed the laboratory analyses; EMdK carried out the data analysis; EMdK, MAvH, GAMB, DB and EAMS interpreted the data; EMdK, DB and EAMS drafted the article; all authors critically revised the article for important intellectual content and approved the final version to be submitted.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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