Post Hoc Analysis of a Randomized Double-Blind Trial of the Correlation of Functional and Binding Antibody Responses Elicited by 13-Valent and 7-Valent Pneumococcal Conjugate Vaccines and Association with Nasopharyngeal Colonization

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In a randomized double-blind trial in healthy Israeli infants in Israel who received the 13-valent or 7-valent pneumococcal conjugate vaccine (PCV13 or PCV7, respectively) at 2, 4, 6, and 12 months, PCV13 significantly reduced nasopharyngeal (NP) colonization of serotypes 1, 6A, 7F, 19A, cross-reacting 6C, and the common PCV7 serotype 19F, from ages 7 to 24 months. No differences were observed between the vaccine groups for serotype 3 or for the remaining common PCV7 serotypes. For serotype 5, too few events were observed to draw an inference. Generally consistent with these findings, PCV13 elicited significantly higher enzyme-linked immunosorbent assay (ELISA) IgG-binding antibody responses than did PCV7 for the additional PCV13 serotypes 1, 3, 5, 6A, 7F, 19A, and for the common serotype 19F, with similar or lower responses for the remaining common serotypes. To further assess immunogenicity and colonization, we conducted a post hoc analysis of PCV13 functional antibody responses measured by opsonophagocytic activity (OPA) assays in a randomly selected subset of subjects. The pattern of functional antibody OPA responses elicited by PCV13 relative to PCV7 was similar to that of the ELISA anticapsular IgG-binding antibody responses described above. In addition, the OPA responses generally correlated positively with IgG responses for all 13 serotypes among the PCV13 recipients and for all 7 common serotypes and the additional serotype 6A but not for 19A or the other serotypes unique to PCV13 among the PCV7 recipients. This post hoc analysis supports an association between serum OPA functional and IgG-binding antibody levels, allowing for a transfer of inferred associations between IgG responses and NP colonization to OPA responses.

We previously reported a randomized double-blind trial involving 1,866 healthy infants in Israel, which assessed the effectiveness of the 13-valent and 7-valent pneumococcal conjugate vaccines (PCV13 and PCV7, respectively), administered at 2, 4, 6, and 12 months of age, in reducing nasopharyngeal (NP) colonization from ages 7 to 24 months, and we compared the immunogenicity of the two vaccines measured by enzyme-linked immunosorbent assay (ELISA) 1 month after the infant series and 1 month after the toddler dose (1). The impact on NP colonization was generally consistent with the higher immune responses elicited by PCV13 (PCV7 plus serotypes 1, 3, 5, 6A, 7F, and 19A) compared with those of PCV7 (serotypes 4, 6B, 9V, 14, 18C, 19F, and 23F), as measured by ELISA, in particular for serotypes 1, 6A, 7F, and 19A, for which immune responses were significantly higher and NP colonization was significantly lower in the PCV13 group. For serotype 5, there were too few acquisitions to draw inferences, and for serotype 3, which elicited the lowest IgG immune response, no impact on colonization was observed (1).

To further assess immune responses and colonization, functional antibodies were measured by opsonophagocytic activity (OPA) assays in the remaining serum. A post hoc analysis was then performed to assess whether there is an association between anticapsular IgG-binding antibody responses measured by ELISA and the functional antibodies measured by OPA assays, in order to ascertain whether the transfer of inferred associations between IgG responses and NP colonization to OPA responses is appropriate.

MATERIALS AND METHODS

Trial design. This randomized double-blind trial was conducted in Israel by a single coordinating center overseeing activities at 11 clinical sites. The details of the study, including vaccine formulations, are described elsewhere (1). In brief, eligible subjects were randomly assigned at a 1:1 ratio to receive PCV13 or PCV7. PCV13 or PCV7 was administered at approximately 2, 4, and 6 months of age (infant series), as well as at 12 months of age (toddler dose), by intramuscular injection into the anterolateral left thigh. Other pediatric vaccines were administered according to national recommendations into the anterolateral right thigh. Blood samples for serology were obtained at 1 month after the infant series and at 1 month after the toddler dose. NP swabs for culture were taken at 2, 4, and 6 months to establish a baseline, and also at 7, 12, 13, 18, and 24 months to assess NP acquisition over time and the prevalence of serotypes at each age point. The trial was approved by the Institutional Ethics Committee of the...
TABLE 1 Demographic characteristics of subjects with OPA assays

| Patient characteristic | PCV13 (n = 174) | PCV7 (n = 177) |
|------------------------|----------------|----------------|
| Gender (n [%])          |                |                |
| Male                   | 87 (50.0)      | 93 (52.5)      |
| Female                 | 87 (50.0)      | 84 (47.5)      |
| Race (n [%])           |                |                |
| White                  | 169 (97.1)     | 170 (96.0)     |
| Black or African American | 5 (2.9)       | 7 (4.0)        |
| Ethnicity (n [%])      |                |                |
| Bedouin                | 91 (52.3)      | 92 (52.0)      |
| Jewish                 | 83 (47.7)      | 85 (48.0)      |
| Age (mean ± SD) (mo) at time of dose: | | |
| 1                      | 2.2 ± 0.3      | 2.2 ± 0.3      |
| 2                      | 3.9 ± 0.4      | 4.0 ± 0.5      |
| 3                      | 5.7 ± 0.5      | 5.8 ± 0.6      |
| 4                      | 12.5 ± 0.5     | 12.5 ± 0.7     |

*PCV13, 13-valent pneumococcal conjugate vaccine.

Soroka University Medical Center and the National Ethics Committee. PCV13 was not available in Israel during the period when subjects were to be vaccinated, which allowed for a comparison with licensed PCV7. Assessment of capsular IgG binding antibodies was a predefined endpoint of the study, but assessment of functional antibody responses was not. The study protocol and informed consent permitted additional assays of the remaining serum samples to further assess the immune response to the administered vaccines.

**Participants.** Healthy 2-month-old infants (age range, 42 to 98 days) were enrolled after their parent(s)/legal guardian(s) provided informed consent. The details of the eligibility criteria are included in Dagan et al. (1). Subjects eligible for the OPA analyses were those included in the pneumococcal IgG analysis who had sufficient serum for the OPA assays. The evaluable immunogenicity population included participants who adhered to the protocol requirements, had valid and determinate assay results, and had no other major protocol violations. The all-available immunogenicity population included all randomized participants who had ≥1 valid and determinate assay result. Sufficient subjects (n = 354) were selected randomly to allow approximately 100 evaluable subjects (50 Jewish and 50 Bedouin) from the PCV13 and PCV7 vaccine groups to be assessed by OPA at each sampling time. The results of the all-available population were similar to that of the evaluable immunogenicity population and are not presented here.

**Opsonophagocytic activity assays.** Validated micro colony OPA assays were used to measure the 13 pneumococcal serotypes contained in the PCV13. These assays were based on previously described methods (2, 3). OPA assay titers were defined as the interpolated reciprocal serum dilution that results in complement-mediated killing of 50% of the bacteria in the assay. The assay sensitivity is the limit of detection (LOD), which is normally a titer of 8 and is the same for each serotype-specific OPA assay. However, because of the limited volumes of the available samples for the post hoc analyses in this study, the samples were diluted 1:2 before testing for the OPA. Therefore, the LOD for a 1:2 prediluted sample was 16 (instead of 8). The lower limit of quantitation (LLOQ) was determined for each serotype-specific assay during assay validation; OPA titers higher than the LLOQ were considered accurate, and their quantified values were reported. Titers lower than the LLOQ were set to 0.5 × LOD for analysis, which here is a titer of 8. In addition, a sensitivity analysis was performed in which OPA titers lower than the LLOQ were set to 0.5 × LLOQ for each serotype. The LLOQ in the titers for each serotype was doubled, given the 1:2 prediluted sample. The LLOQ for the various serotypes were: serotype 1, 36; serotype 3, 24; serotype 4, 42; serotype 5, 58; serotype 6A, 74; serotype 6B, 86; serotype 7F, 420; serotype 9V, 690; serotype 14, 70; serotype 18C, 62; serotype 19A, 36; serotype 19F, 96; and

**FIG 1 Immune responses as measured by ELISA and OPA assays.** The immune responses were significantly higher when the lower limit of the 95% CI for the ratio was >1 and were significantly lower when the upper limit of 95% CI for the ratio was <1.
serotype 23F, 26. Serotype-specific assay titers as determined from the assay plates were adjusted for the dilution factor (i.e., doubled) in reporting the final results.

Statistical analysis. The serotype-specific OPA titers were logarithmically transformed for analysis. Within each vaccine group, serotype-specific OPA geometric mean titers (GMTs) were calculated. Two-sided 95% confidence intervals (CIs) for the GMTs and the ratio of GMTs (GMR) for each group on the logarithmic scale. Comparisons of the GMTs between the vaccine groups using the same statistical methods were available from the main study (1). In addition, analyses were performed to assess whether there was a correlation between the IgG concentration and the OPA titer by vaccine group for all serotypes.

RESULTS
The evaluable immunogenicity population with OPA results consisted of 351 subjects. The two vaccine groups were similar with respect to their demographic characteristics (Table 1).

After the infant series, PCV13 elicited significantly higher OPA responses than PCV7 for the six PCV13 additional serotypes and for common serotype 19F (lower limit of GMR 95% CI, >1); the responses for the remaining six common serotypes were similar (serotypes 4, 6B, 9V, and 18C) or lower (serotypes 14 and 23F, with upper limit of GMR 95% CI, <1) in the PCV13 group (Fig. 1; see also see Table S1 in the supplemental material). A generally similar response pattern was observed after the toddler dose. The pattern of functional antibody responses measured by OPA assays after the infant series and after the toddler dose was generally similar to that observed by IgG-binding antibody responses measured by ELISA (Fig. 1; see also Table S1); the previously reported NP acquisition data (see Table S1) show that the general similarity between the OPA and ELISA results extends to the relationship between the ELISA results and NP colonization.

In the PCV13 group, the OPA and IgG responses correlated significantly (lower limit of the 95% CI, >0) for all 13 serotypes.

In the PCV7 group after the infant series, the OPA and IgG responses correlated positively for all 7 common serotypes and for additional serotypes unique to PCV13, including serotype 19A (which showed no cross-reaction with 19F). Generally, similar results were seen after the toddler dose, but in addition, serotypes 3 and 7F correlated positively; however, the immune responses for these serotypes were minimal (Table 3).

The above analyses were repeated, this time with OPA titers.

### Table 2 Correlation of immune responses measured by ELISA and OPA in the PCV13 group

| Serotype | Assay | After the infant series (n = 90–118) | After the toddler dose (n = 97–117) |
|----------|-------|------------------------------------|------------------------------------|
|          | GM (95% CI) | r (95% CI) | GM (95% CI) | r (95% CI) |
| Common   |       |         |             |             |
| 4        | IgG   | 1.95 (1.67, 2.27) | 0.46 (0.29, 0.60) | 4.19 (3.52, 4.99) |
|          | OPA   | 441.2 (307.0, 634.1) | 2,119.1 (1,651.7, 2,718.8) | 0.38 (0.21, 0.53) |
| 6B       | IgG   | 2.10 (1.66, 2.66) | 0.65 (0.52, 0.75) | 8.97 (7.47, 10.78) |
|          | OPA   | 546.3 (366.2, 814.9) | 1,887.2 (1,318.4, 2,701.5) | 0.63 (0.49, 0.74) |
| 9V       | IgG   | 1.42 (1.25, 1.61) | 0.36 (0.18, 0.52) | 4.00 (3.41, 4.70) |
|          | OPA   | 55.5 (33.4, 92.4) | 706.1 (435.3, 1,145.6) | 0.43 (0.26, 0.57) |
| 14       | IgG   | 4.68 (3.83, 5.72) | 0.46 (0.30, 0.59) | 10.66 (8.78, 12.95) |
|          | OPA   | 666.1 (505.7, 877.3) | 1,777.1 (1,485.4, 2,126.1) | 0.38 (0.21, 0.52) |
| 18C      | IgG   | 1.45 (1.26, 1.66) | 0.48 (0.32, 0.61) | 3.75 (3.18, 4.42) |
|          | OPA   | 968.4 (764.7, 1,226.4) | 3,596.9 (2,879.2, 4,493.5) | 0.61 (0.48, 0.72) |
| 19F      | IgG   | 2.52 (2.10, 3.01) | 0.48 (0.31, 0.62) | 9.27 (7.43, 11.55) |
|          | OPA   | 77.1 (51.7, 114.9) | 579.7 (395.8, 849.0) | 0.57 (0.42, 0.69) |
| 23F      | IgG   | 1.12 (0.93, 1.35) | 0.65 (0.52, 0.74) | 5.18 (4.40, 6.08) |
|          | OPA   | 349.1 (257.7, 512.6) | 2,195.4 (1,716.1, 2,808.5) | 0.65 (0.53, 0.75) |
| Additional |       |         |             |             |
| 1        | IgG   | 1.88 (1.62, 2.19) | 0.54 (0.38, 0.67) | 4.77 (4.05, 5.61) |
|          | OPA   | 28.8 (21.3, 38.9) | 117.6 (87.7, 157.6) | 0.69 (0.58, 0.78) |
| 3        | IgG   | 0.90 (0.77, 1.04) | 0.54 (0.39, 0.67) | 1.40 (1.20, 1.64) |
|          | OPA   | 80.0 (64.7, 99.0) | 210.8 (178.2, 249.4) | 0.60 (0.47, 0.70) |
| 5        | IgG   | 1.40 (1.19, 1.65) | 0.62 (0.47, 0.73) | 3.95 (3.35, 4.65) |
|          | OPA   | 37.6 (26.8, 52.8) | 201.8 (154.4, 263.9) | 0.83 (0.77, 0.88) |
| 6A       | IgG   | 2.39 (2.02, 2.83) | 0.42 (0.24, 0.57) | 9.44 (8.01, 11.12) |
|          | OPA   | 1,000.2 (778.5, 1,284.9) | 3,128.5 (2,413.5, 4,055.2) | 0.43 (0.26, 0.57) |
| 7F       | IgG   | 2.89 (2.50, 3.35) | 0.47 (0.31, 0.60) | 7.39 (6.86, 8.27) |
|          | OPA   | 1,040.0 (754.7, 1,433.2) | 3,611.5 (3,124.9, 4,174.0) | 0.57 (0.43, 0.69) |
| 19A      | IgG   | 1.88 (1.57, 2.25) | 0.65 (0.51, 0.76) | 8.08 (7.03, 9.27) |
|          | OPA   | 40.2 (27.9, 57.9) | 453.9 (357.0, 577.2) | 0.58 (0.44, 0.69) |

a IgG, immunoglobulin G measured by ELISA; OPA, antibody titer measured by opsonophagocytic activity assay.
b Number of subjects with valid and determinate IgG and OPA assay results for the specified serotype at the given visit.
c Cls (confidence intervals) are back transformations of a CI based on the Student t distribution for the mean logarithm of the titers/concentrations. GMT, geometric mean titer/concentration.
d Correlation (correlation coefficient [r]) between OPA and IgG for the specified serotype. A correlation was statistically significant when the lower limit of the 95% CI was >0.
lower than the LLOQ, which were set to $0.5 \times$ LLOQ. Higher OPA GMTs were achieved using $0.5 \times$ LLOQ for titers lower than the LLOQ compared with OPA GMTs using $0.5 \times$ LOD for titers lower than the LLOQ. In some cases, this resulted in lower GMT ratios, but the statistical inferences of the comparisons of the two vaccines did not change (comparisons were based on the assessment of the 95% CI of the GMT ratios; see Table S1 in the supplemental material). For the correlation of IgG geometric mean concentrations (GMs) and OPA GMTs using $0.5 \times$ LLOQ for titers lower than the LLOQ, changes in the correlation values were minimal, and there were no changes in the statistical inferences, where correlations were statistically significant when the lower limit of the 95% CI was $>0$ (data not shown).

**DISCUSSION**

This post hoc analysis showed that the immune responses elicited by PCV13 compared to those elicited by PCV7 measured by serotype-specific OPA assays (functional antibodies) showed a pattern of responses similar to those measured by ELISA (binding antibodies). For the 6 serotypes unique to PCV13 and for common serotype 19F, the IgG and OPA levels were significantly higher after PCV13 than after PCV7; for the other 6 common serotypes, the immune responses elicited by PCV13 were similar to or lower than those elicited by PCV7. In addition, in the PCV13 group, the IgG levels correlated significantly with the OPA levels. In the PCV7 group, a correlation between the IgG and OPA levels was observed for the PCV7 serotypes and cross-reacting serotype 6A but not for 19A or the other PCV13 additional serotypes (with the exception of serotypes 3 and 7F after the toddler dose, but the immune responses for these serotypes were very low). The correlation between the OPA and ELISA results has also been demonstrated in other studies (4, 5). The degree of OPA-to-IgG correlation can be affected by the OPA assay type used, e.g., killing OPA assay versus uptake assay. As uptake assays are of limited value for the assessment of functional pneumococcal responses, a functional immune response to *Streptococcus pneumoniae* should be measured by true OPA killing assays, as were performed in our studies. Moreover, others have shown that the OPA killing assay correlates better with IgG concentrations than do radiolabeled or flow cytometric-based uptake assays (6).

It has been shown that when a correlation was found between OPA and ELISA, protection against invasive pneumococcal disease (IPD) was demonstrated (6). The cross-reacting serotype 6B in PCV7, for example, elicited both IgG and OPA antibody responses to serotype 6A, which were consistent with protection against serotype 6A IPD. In contrast, the cross-reacting serotype 19F in PCV7, which elicited IgG responses for serotype 19A but...
only minimal OPA responses for 19A, was not effective against this serotype (7). These data further support functional antibodies, measured by OPA assays, as the basis for protection against pneumococcal disease (8). Therefore, OPA titers may serve not only as a surrogate for protection against pneumococcal disease but, by extrapolation, as a surrogate for protection against NP colonization. No thresholds for protection have been defined for OPA titers for each serotype. For ELISA, a cumulative threshold of 0.35 μg/ml has been defined for all serotypes using the WHO assay against IPD, but this may not accurately predict effectiveness against mucosal diseases, such as pneumonia and otitis media (9).

One study showed that a much higher serum IgG level of protection than that defined by the WHO against IPD may be required to reduce NP colonization, and that absolute levels may differ between the serotypes (10). Based on the need for correlation of the ELISA and OPA titers to offer protection, we may conclude that higher functional antibody titers may be required for protection against NP colonization and mucosal disease than that required for IPD. In addition, although vaccination with conjugate vaccines is considered the main mechanism for the reduction of NP carriage and for direct prevention and transmission prevention (herd effect) of pneumococcal disease (11), other factors may play a role in pneumococcal disease reduction. These include geographical location, socioeconomic status, age, maturation of the immune system, smoking, antibiotic use, health, and innate immunological mechanisms for mucosal clearance, including the role of interleukin-17A (IL-17A)-producing Th17 cells, which are considered important effectors against S. pneumoniae colonization and subsequent disease (12, 13).

In conclusion, this study supports an association between serotype-specific IgG concentrations and OPA titers in children <24 months of age, which allows a transfer of associations between higher IgG responses and reduced NP colonization (1) to OPA responses after vaccination with PCV13. Higher IgG antibody binding levels may achieve higher protection against NP colonization (1, 14). Similarly, higher OPA functional antibody levels may be associated with increased reduction in the carriage of vaccine type serotypes, with subsequent protection against pneumonia, otitis media, and IPD in the individual, as well as in the community via herd immunity.

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