Detection of opsonizing antibodies directed against a recently circulating *Bordetella pertussis* strain in paired plasma samples from symptomatic and recovered pertussis patients

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Correlates of protection (CoPs) against the highly contagious respiratory disease whooping cough, caused by *Bordetella pertussis*, remain elusive. Characterizing the antibody response to this pathogen is essential towards identifying potential CoPs. Here, we evaluate levels, avidity and functionality of *B. pertussis*-specific-antibodies from paired plasma samples derived from symptomatic and recovered pertussis patients, as well as controls. Natural infection is expected to induce protective immunity. IgG levels and avidity to nine *B. pertussis* antigens were determined using a novel multiplex panel. Furthermore, opsonophagocytosis of a *B. pertussis* clinical isolate by neutrophils was measured. Findings indicate that following infection, *B. pertussis*-specific antibody levels of (ex-) pertussis patients waned, while the avidity of antibodies directed against the majority of studied antigens increased. Opsonophagocytosis indices decreased upon recovery, but remained higher than controls. Random forest analysis of all the data revealed that 28% of the opsonophagocytosis index variances could be explained by filamentous hemagglutinin- followed by pertussis toxin-specific antibodies. We propose to further explore which other *B. pertussis*-specific antibodies can better predict opsonophagocytosis. Moreover, other *B. pertussis*-specific antibody functions as well as the possible integration of these functions in combination with other immune cell properties should be evaluated towards the identification of CoPs against pertussis.

Whooping cough, a highly contagious respiratory disease caused by the Gram-negative bacterium *Bordetella pertussis*, has resurged despite high vaccine coverage\(^1,2\). In the 1990s, the whole cell pertussis vaccine was replaced by the current acellular pertussis vaccine (ACV) in many industrialized countries. The ACV effectively prevents disease. However, protective immunity is lost 4–7 years post-vaccination and this vaccine does not protect against transmission\(^3,5\) urging the development of an improved vaccine. Evaluating pertussis vaccine-induced protection poses a problem as correlates of protection (CoPs)\(^6\) against pertussis have not been defined. A CoP can be defined as an (immune) marker that statistically correlates with vaccine efficacy but is not necessarily mechanistically

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CoP will not be unveiled merely by monitoring the antibody levels induced by the ACV. In addition to the
studied in pertussis, as typically IgG levels and isotypes are measured. The antibody response to
vaccine-induced protection, it provides the longest protection from disease with estimates of up to 20 years. In an
opsonophagocytosis wanes within years after infection but remains higher compared to controls.

Avidity maturation of BrkA-specific antibodies depends on antigen specificity. In addition to IgG levels in plasma, we determined the avidity of antigen-specific IgG antibodies in plasma of symptomatic and recovered pertussis patients and controls (Fig. 1).

The levels of antibodies against Ptx, FHA, Prn, OMVs, LOS, BrkA and Vag8 were significantly higher in plasma of symptomatic patients compared to the controls (Fig. 2A–G). The levels of antibodies against Fim2 and Fim3 did not significantly differ from controls (Fig. 2H and I). For the recovered patients, the levels of the specific antibodies for all tested antigens were significantly lower compared to that of the symptomatic patients. The Ptx-, FHA- and Prn-specific antibody levels for the recovered patients remained significantly higher than the controls (Fig. 2A–C) whereas those of OMV-, LOS-, BrkA-, Vag8-, Fim2- and Fim3-specific antibodies did not (Fig. 2D–I).

Opsonophagocytosis wanes within years after infection but remains higher compared to controls. Since antibody-mediated opsonophagocytosis is important for the clearance of B. pertussis during infection, we analysed the functionality of the B. pertussis- specific antibodies using the OPA. Opsonophagocytosis by primary neutrophils was assessed using the clinical B. pertussis isolate B191717, which was modified to express...
green fluorescent protein (GFP). We observed significantly lower opsonophagocytosis indices in plasma samples from recovered compared to symptomatic patients. Notably, the opsonophagocytosis indices from the recovered patients remained significantly higher than those of the controls (Fig. 4).

**Lower IgG levels, opsonophagocytosis indices and higher avidities of B. pertussis-antibodies are generally observed for recovered compared to symptomatic patients.** In order to analyse all the obtained data, a PCA was performed based on the IgG levels and avidity as well as the opsonophagocytosis indices. As shown in Fig. 5, the three groups of the cohort form clusters, with the symptomatic patients positioned furthest away from the controls and the recovered patients being positioned in between. The lines depict the inter-correlations of the various parameters measured in all samples. Firstly, the lines indicate that the axes of differentiation of the majority of the antibody levels had similar alignments and were mainly directed towards the
higher scores of the two principal components, mostly populated by the symptomatic group. Secondly, the axes of differentiation of the avidities pointed mainly in the direction of the recovered group. This analysis reflects the findings represented in Figs 2 and 3 which show lower antibody levels and higher AI in the recovered versus symptomatic patients for most of the studied antigens. Likewise, the PCA reflects the findings presented in Fig. 4 where the axes of differentiation of the opsonophagocytosis indices pointed in the direction of the symptomatic group.

Random forest analysis reveals predictive value of FHA and Ptx antibodies for opsonophagocytosis indices. A random forest (RF) analysis was performed to determine which of our measured variables, namely effective-IgG levels as well as age and sex, was most predictive for the observed opsonophagocytosis and may serve as potential CoPs. The analysis showed that 28% of the variance of the opsonophagocytosis indices is explained by our model. Results indicate that FHA- followed by Ptx-specific antibodies are most predictive for the accounted variances in the opsonophagocytosis indices (Fig. 6). Multiple linear regression analysis with the same variables considered in the RF analysis revealed significant $p$-values for FHA and Ptx effective-IgGs supporting their potential predictive role (Table 1).

**Figure 3.** Avidity of IgG antibodies directed against nine different pertussis antigens. Avidity Index (AI) of antibodies directed against (A) OMVs, (B) LOS, (C) BrkA, (D) Vag8, (E) Fim2, (F) Fim3, (G) FHA, (H) Ptx and (I) Prn were determined in collected from symptomatic (circles) and recovered (squares) pertussis patients. Statistical testing: paired $t$-test. The false discovery rate was controlled at the level of 10% by applying the Benjamini-Hochberg method. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$ ****$p \leq 0.0001$ ns = non-significant. Data shown in A–I represent the mean ± SD.
Discussion

In the attempt to identify serological CoPs against pertussis, our study pioneers by integrating the dynamics of different antibody properties. These include their specificity to nine different *B. pertussis* antigens, their avidity and functional capacity to opsonise live *B. pertussis*. These assays were performed using paired plasma samples from symptomatic and recovered pertussis patients, who are expected to have protective immunity conferred by natural infection. As previously shown, the levels of antibodies to Ptx, FHA, Prn, Fim2, Fim3 and OMVs waned years after infection\(^1\). Antibodies against the other studied antigens, BrkA, Vag8 and LOS, also decreased when patients had recovered from clinical disease. BrkA, Vag8 and LOS were included in this study because they are outer membrane antigens and could be anchors for antibody-mediated phagocytosis.

Besides the waning of *B. pertussis*-specific antibody levels, we also show avidity maturation for the majority of specific antibodies years after *B. pertussis* infection with the exception of Prn, FHA and Ptx. High avidity

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**Figure 4.** Opsonophagocytosis indices wane years after pertussis infection. Antibody-mediated opsonophagocytosis indices were determined using paired plasma samples collected from symptomatic (circles) and recovered (squares) pertussis patients. Plasma samples from controls (triangles) were also included. Statistical testing: one-way analysis of variance (ANOVA) followed by post-hoc tests (Welch Two Sample t-test or paired t-test). The false discovery rate was controlled at the level of 10% by applying the Benjamini-Hochberg method. ***p ≤ 0.0001. Data shown represent the mean ± SD.

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**Figure 5.** PCA of all obtained data. PCA was performed based on the antibody levels, AI and opsonophagocytosis indices for all plasma samples used in this study. The axes of differentiation of the antibody levels and AI are shown by the lines. Plasma samples collected from symptomatic patients are depicted as red dots, from recovered patients as blue squares and from controls as green triangles. The red, blue and green ellipses indicate clustering of the symptomatic, recovered and control samples, respectively. Lines indicate the axes of differentiation of the different parameters measured in this study.
Antibodies are classically secreted by specific B cells that are positively selected during repeated exposures in germinal centre reactions. In contrast to our findings, avidity maturation of Ptx antibodies was previously shown in paired samples of pertussis patients. This difference may be due to the time between collections of the paired samples, which in our case was on average three years instead of four weeks after diagnosis as performed in the before mentioned study. Avidity of antibodies has also been measured following ACV booster vaccination. The authors showed that Ptx and Prn antibody avidity increased one year post-vaccination. This emphasizes that in the search for CoPs against pertussis it is important to consider the antibody kinetics, not only of the levels but also of the avidity, which may differ following infection or vaccination.

During an infection with *B. pertussis*, neutrophils infiltrate the lung where they are important for the clearance of this respiratory pathogen. In contrast to our findings, avidity maturation of Ptx antibodies was previously shown in paired samples of pertussis patients. This difference may be due to the time between collections of the paired samples, which in our case was on average three years instead of four weeks after diagnosis as performed in the before mentioned study. Avidity of antibodies has also been measured following ACV booster vaccination. The authors showed that Ptx and Prn antibody avidity increased one year post-vaccination. This emphasizes that in the search for CoPs against pertussis it is important to consider the antibody kinetics, not only of the levels but also of the avidity, which may differ following infection or vaccination.

Table 1. Multi-linear regression analysis with the same parameters considered in the random forest analysis using the effective-IgG levels (IgG levels and avidity).

| (log10 (IgG x Avidity)) | Estimate | Std. Error | p-value |
|--------------------------|----------|------------|---------|
| Prn                      | 0.002    | 0.064      | 0.977   |
| FHA                      | 0.350    | 0.110      | 0.002   |
| Fim2                     | −0.183   | 0.079      | 0.023   |
| Fim3                     | 0.172    | 0.085      | 0.045   |
| Ptx                      | 0.225    | 0.092      | 0.017   |
| LOS                      | −0.055   | 0.168      | 0.743   |
| Vag8                     | 0.073    | 0.125      | 0.562   |
| OMV                      | 0.062    | 0.196      | 0.753   |
| BrkA                     | 0.117    | 0.088      | 0.187   |
| Age                      | 0.003    | 0.002      | 0.247   |
| Sex                      | 0.009    | 0.011      | 0.433   |

Figure 6. Random Forest analysis integrating all available data. Random Forest analysis was performed using a model that includes the log transformed values of the effective-IgG levels directed against the nine different antigens, as well as the age and sex of the participants. $\text{R}^2$ represents the fraction of the total opsonophagocytosis indices variance which can be explained by variations in the effective-IgG levels, where a value of 1 would mean that the variance in the measured opsonophagocytosis indices is solely explained by the considered predictive variables. The percentage of increase in the mean square error (%IncMSE) after randomly permuting the values of the considered predictive variables gives an indication of how influential each considered variable is on predicting OPA values to the extent of the OPA variance which can be explained by the model ($\text{R}^2$).
that were circulating at the time of our clinical study. The OPA here described can discriminate between controls, symptomatic and recovered (ex) pertussis patients. Based on these findings, we propose antibody-mediated opsonophagocytosis of B. pertussis as a possible CoP against pertussis. Assays which measure the capacity of antibodies to opsonize pathogens for phagocytosis are currently used in the field of pneumococcal vaccination. Opsonophagocytosis has also been shown to correlate with protection against malaria.

Analysis of our data with the RF model using effective-IgG levels of B. pertussis-specific antibodies uncovered FHA- followed by Ptx-antibodies as being the two variables that best predict the opsonophagocytosis indices. These findings corroborate earlier studies in which the authors also found a correlation between FHA- and to a lesser extend Ptx-specific antibodies and opsonophagocytosis. In contrast to our findings, others have shown either that high anti-FHA levels inhibit opsonophagocytosis or that anti-Ptn is important for opsonophagocytosis. These differences may be explained by the various read-outs (bacterial uptake by flow cytometry, respiratory burst or microscopy) used to determine opsonophagocytosis, as well as the timing of serum or plasma sample collection. Effective-IgG levels to FHA, a vaccine antigen and major adhesion of B. pertussis, best predicted the variances of the opsonophagocytosis indices. FHA-antibodies are expected to bind to the bacterial membrane and mediate bacterial uptake by engaging Fcγ-receptors on neutrophils. It remains unknown whether FHA-specific antibodies generated upon infection and vaccination are similar in functionality. Antibodies against the additionally included outer membrane-localised antigens LOS, Vag8 and BrkA, important immune modulators mediating resistance against antimicrobial peptides (LOS) and complement (LOS, Vag8 and BrkA), were not predictive for the opsonophagocytosis indices according to the RF analysis. It is possible that these antibodies are of an IgG subclass that does not mediate opsonophagocytosis or that the antigen is masked on the bacterial membrane. However, this does not exclude other functional roles for human antibodies against these additional surface antigens. Monoclonal antibodies directed against LOS were found to be important in preventing B. pertussis infection in a mouse model and BrkA-specific antibodies have been implicated in bactericidal killing of B. pertussis. Further studies should address these matters. The second best predictor for opsonophagocytosis was the presence of antibodies directed to Ptx. Because this toxin is generally secreted by B. pertussis and induces high levels of antibodies following infection, we speculate that the association found in the RF model is not mechanistically related. Interestingly, FHA- and Ptx-specific antibodies did not show an increased avidity in the recovered pertussis patients suggesting that perhaps high antibody avidity for these antigens is not necessarily important for opsonophagocytosis of B. pertussis. Whether the FHA, and possibly Ptx, antibodies are mechanistically related to neutrophil opsonophagocytosis will have to be verified by either depleting or and/or isolating these antibodies from plasma, as well as by performing the OPA with B. pertussis that do not express FHA or Ptx. To determine whether these antibodies indeed also protect from colonisation with B. pertussis, an in vitro model using differentiated human airway epithelial cells may be used for validation. Moreover, eventually the novel controlled human infection pertussis model could be valuable to determine protection from colonization by specific antibodies.

Although FHA- and Ptx-specific antibodies were the best predictors for opsonophagocytosis in our model, their presence only explained 28% of the opsonophagocytosis indices variances. Adenylate cyclase toxin (ACT) produced by B. pertussis reduces phagocytosis and subsequent bacterial killing by neutrophils. Previously, antibodies directed against this toxin have been shown to promote phagocytosis of B. pertussis via toxin-neutralization. ACT is not only secreted but also remains associated to the bacterial membrane by interacting with FHA. ACT-specific antibodies could hence also opsonize B. pertussis. Although ACT was not included in the antigen panel of this study, it is likely that part of the unexplained variances can be attributed to ACT-specific antibodies. Also, other specificities cannot be excluded, as the naturally acquired or vaccine-induced B. pertussis serum reactomes are very diverse and broad. Future studies should explore whether ACT-specific or other B. pertussis-specific antibodies further predict opsonophagocytosis.

This work represents a first step towards identifying CoPs by the combined analysis of different parameters of B. pertussis-specific antibodies from (ex) pertussis patients. These findings should be interpreted with caution as the longitudinal pertussis cohort used is small and includes individuals of different ages with different vaccination status and most likely previous infection backgrounds. Due to the low sample size we were unable to analyse our data by grouping the individuals according to age. Furthermore, our cohort does not include young infants whom are the main victims of severe pertussis. The induction of B. pertussis-specific antibodies and their functional properties may differ between infants and the older individuals in our cohort, possibly due to the presence of maternal antibodies, which should be taken into account when developing novel childhood pertussis vaccines.

We furthermore suggest exploring antibody characteristics such as subclass and fucosylation status which can influence opsonizing capacity or other antibody effector functions. Additionally, protective mechanisms including neutrophil respiratory burst and subsequent killing, as well as complement-mediated killing of B. pertussis could be studied. Recently, levels of specific memory B cells, that can give rise to a fast increase in antibody levels upon an infection with B. pertussis, were implied in protection. Moreover, it is clear that not only antibodies or B cells are involved in protection against pertussis, as T helper (Th1) and Th17 cells have also been implicated as very important players in the protective immune response against B. pertussis. Taken together, the pertussis field should broaden the way identification of CoPs is approached and be open to multifactorial CoPs against pertussis, which could be a combination of antibody levels, antibody functionality as well as B. pertussis specific T and B cell responses. With respect to antibodies, integration of different specific antibody parameters may bring the field closer to the identification of the required CoPs against pertussis.

Materials and Methods

Ethics. Participants donating blood for plasma isolation were Dutch symptomatic (ex-) pertussis patients selected from a cross-sectional observational study previously described (Specifieke Kinkhoest Immunititeit; SKI). The study was approved by the accredited Medical Research Ethics Committee (MREC) STEG followed by management of the METC UMC Utrecht (Dutch Central Committee on Research Involving Human Subjects)
in the opsonophagocytosis assay shortly after isolation.

RPMI-1640 (Gibco), supplemented with 0.05% human serum albumin (HSA, Sanquin). Neutrophils were used (MIA) described earlier13. The International WHO pertussis standard (NIBSC 06/140) served as reference for Fim3, Vag8, BrkA, LOS and OMV were measured in HI-plasma samples by an in-house multiplex immunoassay found to interfere with one another.

version 5.5 (Bio-Rad Laboratories, West Grove, PA, USA). The various antigen coupled bead regions were not fluorescence intensity where KIOVIG was set at 500 AU/ml. For detection, goat-anti-human IgG-PE (Jackson for infusion (KIOVIG, Baxalta, Belgium) was used as an in-house reference to measure levels of IgG against the IgG levels against Ptx, FHA and Prn (International units, IU/ml). Human Normal Immunoglobulin solution (HSA, Sanquin) for 20 minutes at 37 °C. Subsequently, freshly isolated neutrophils (7.5 in infection is 70) were opsonized with 5% HI-plasma in RPMI supplemented with 0.3% human serum albumin (BD Biosciences) supplemented with 30 μg/ml gentamycin (Merck, Darmstadt, Germany) at 35 °C, 5% CO2, for four days. Subsequently, bacteria were recultured in Thalen-Tjssel medium56 overnight (start OD 0.05, 35 °C, 130 rpm) until mid-log phase (OD 0.7) was reached before use.

Pertussis antigens. Ptx and FHA were obtained from Kaketsuken (Obuko, Japan). Fim2 was a kind gift from Dr. A Gorringe, Fim3 and Prn were purified in-house. LOS was extracted from strain B1917 by means of hot phenol-water as previously described43. OMVs of B1917, containing various outer membrane antigens, were generated as previously described55 with some modifications and recombinant passenger domains of Vag8 and BrkA were expressed in Escherichia. coli as described elsewhere47.

Multiplex immunoassay. Levels of total IgG directed against B. pertussis antigens Ptx, FHA, Prn, Fim2, Fim3, Vag8, BrkA, LOS and OMV were measured in HI-plasma samples by an in-house multiplex immunoassay (MIA) described earlier14. The International WHO pertussis standard (NIBSC 06/140) served as reference for IgG levels against Ptx, FHA and Prn (International units, IU/ml). Human Normal Immunoglobulin solution for infusion (KIOVIG, Baxalta, Belgium) was used as an in-house reference to measure levels of IgG against the remaining six antigens, expressed as arbitrary units per ml (AU/ml) based on observed relative magnitudes of fluorescence intensity where KIOVIG was set at 500 AU/ml. For detection, goat-anti-human IgG-PE (Jackson ImmunoResearch, USA) was used. Analysis was performed with a Bio-Plex 200 using Bio-Plex Manager software version 5.5 (Bio-Rad Laboratories, West Grove, PA, USA). The various antigen coupled bead regions were not found to interfere with one another.

Avidity assay. The avidity of pertussis-specific IgG antibodies was measured in the MIA using HI-plasma as described previously44 with modifications. Briefly, plasma samples were allowed to bind for 45 minutes with the respective pertussis antigen coupled to fluorescently labelled microspheres. After washing, samples were incubated for 15 minutes in PBS either in the absence or presence of 9 M urea, to elute lower-avidity antibodies. Subsequently, samples were washed, goat-anti-human IgG-PE (Jackson ImmunoResearch, USA) was used as detection antibody and analysis was performed with a Bio-Plex 200 using Bio-Plex Manager software version 5.5 (Bio-Rad). The avidity index (AI) was expressed as a percentage of the remaining IgG levels in the presence of urea relative to the IgG levels measured in PBS only.

Opsonophagocytosis assay. Opsonophagocytosis of B. pertussis B1917 by human neutrophils was evaluated using a method described in literature with minor modifications27. Briefly, B1917-GFP (multiplicity of infection is 70) were opsonized with 5% HI-plasma in RPMI supplemented with 0.3% human serum albumin (HSA, Sanquin) for 20 minutes at 37 °C. Subsequently, freshly isolated neutrophils (7.5 × 106 cells) were added in a total volume of 50 μl and incubated for 25 minutes at 4 °C. After a wash step with cold RPMI-HSA, neutrophils were resuspended in RPMI-HSA and incubated for 30 minutes at 37 °C. The incubation times used were selected based on OPA pilot experiments. Cells were fixed with 1.5% paraformaldehyde and visualized using the FACSCanto II (BD Bioscience). The assay was performed in duplicate on three plates. To correct for plate and day differences, a standalone-control plasma sample was taken along on each plate for each day and a plate factor was calculated. The arbitrary opsonophagocytosis indices were calculated by dividing the mean fluorescent intensity (MFI) obtained in the presence of plasma by the MFI obtained upon incubating cells with bacteria without plasma, corrected for the plate factor.
Data analysis. Differences in antibody levels and opsonophagocytosis indices between the groups were tested with one-way analysis of variance (ANOVA) followed by post-hoc tests (Welch Two Sample t-test or paired t-test). For differences in the AI a paired t-test was performed. P values ≤ 0.05 were considered statistically significant. The false discovery rate was controlled at the level of 10% by applying the Benjamini-Hochberg method to the results of all tests (Supplementary Table 2).

Principal component analysis (PCA) was used to explore dis(similarities) among the antibody levels, AI and opsonophagocytosis indices of the three groups.

To determine which of the measured antibodies were most predictive for the observed opsonophagocytosis, a random forest (RF) analysis was performed. The model considered in this analysis uses for each antigen the effective-IgG level, which we define as the logarithm of the product of IgG levels and AI (IgG*AI). A multiple linear regression analysis using the same effective-IgG levels was also performed. Descriptive statistics of the data can be found in Supplementary Table 1.

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**Author Contributions**

E.S.H., B.K., H.J.H. and D.H. performed the experiments. A.A.B.M. and L.M.G. performed the statistical testing I.V.T., C.A.C.M.V.E. and E.P. were involved in the study design. E.H., A.A.B.M. and L.M.G. drafted the figures. E.H., I.J., C.A.C.M.V.E. and E.P. wrote the manuscript. All authors reviewed the manuscript.

**Additional Information**

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