Predicting Antidisease Immunity Using Proteome Arrays and Sera from Children Naturally Exposed to Malaria*§

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Malaria remains one of the most prevalent and lethal human infectious diseases worldwide. A comprehensive characterization of antibody responses to blood stage malaria is essential to support the development of future vaccines, sero-diagnostic tests, and sero-surveillance methods. We constructed a proteome array containing 4441 recombinant proteins expressed by the blood stages of the two most common human malaria parasites, *P. falciparum* (*Pf*) and *P. vivax* (*Pv*), and used this array to screen sera of Papua New Guinea children infected with *Pf*, *Pv*, or both (*Pf*/*Pv*) that were either symptomatic (febrile), or asymptomatic but had parasitemia detectable via microscopy or PCR. We hypothesized that asymptomatic children would develop antigen-specific antibody profiles associated with antidisease immunity, as compared with symptomatic children. The sera from these children recognized hundreds of the arrayed recombinant *Pf* and *Pv* proteins. In general, responses in asymptomatic children were highest in those with high parasitemia, suggesting that antibody levels are associated with parasite burden. In contrast, symptomatic children carried fewer antibodies than asymptomatic children with infections detectable by microscopy, particularly in *Pv* and *Pf*/*Pv* groups, suggesting that antibody production may be impaired during asymptomatic infections. We used machine-learning algorithms to investigate the relationship between antibody responses and symptoms, and we identified antibody responses to sets of *Plasmodium* proteins that could predict clinical status of the donors. Several of these antibody responses were identified by multiple comparisons, including those against members of the serine enriched repeat antigen family and merozoite protein 4. Interestingly, both *P. falciparum* serine enriched repeat antigen-5 and merozoite protein 4 have been previously investigated for use in vaccines. This machine learning approach, never previously applied to proteome arrays, can be used to generate a list of potential seroprotective and/or diagnostic antigens candidates that can be further evaluated in longitudinal studies. *Molecular & Cellular Proteomics* 13: 10.1074/mcp.M113.036632, 2646–2660, 2014.

Of the five species of malaria parasites that infect humans, *Plasmodium falciparum* (*Pf*)¹ and *P. vivax* (*Pv*) are the most common. Interventions aimed at reducing transmission and improving diagnosis and treatment have led to a dramatic reduction in morbidity and mortality (1, 2). For example, *Pf* fatalities have declined from an estimated one million to 655,000 annually (2). Although *Pv* is now recognized as the most widespread species worldwide and a significant cause of severe disease, this parasite, which can relapse months to years after the initial blood stage infection, is still largely ignored (3, 4). Furthermore, mixed-species infections, most commonly of *Pf* and *Pv*, are more frequent than previously thought. Although blood smears suggest that <2% of cases are mixed-species infections, PCR-based diagnoses suggest that 55–65% of infections in Thailand, Papua New Guinea

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¹ The abbreviations used are: *Pf*, *P. falciparum*; ADCI, antibody-dependent cellular inhibition; EBNA1, Epstein-Barr nuclear antigen 1; EST, expressed sequence tag; HA, hemagglutinin; HIS, polyhistidine; IFN-γ, interferon gamma; LM, asymptomatic donors with parasites detected by light microscopy; PCR, asymptomatic donors with parasites detectable only by PCR; *Pf*/*Pv*, donors infected with both *P. falciparum* and *P. vivax*; PNG, Papua New Guinea; *Pv*, *P. vivax*; S, symptomatic donors.
(PNG), and other countries in south-east Asia (5–7) are mixed-species infections.

Natural immunity can be subdivided into antidisease immunity and antiparasitic immunity. Antidisease immunity (defined as the absence of symptoms) develops quickly, sometimes requiring only one or two infections in high transmission areas (8–11). However, individuals living in high transmission areas develop non-sterile antiparasitic immunity, resulting in low-level parasitemia and asymptomatic infections. This immunity is acquired much more slowly than antidisease immunity, may require repeated infections depending on the transmission rate, and is rarely sterilizing (12). Parasite densities in individuals that have acquired antiparasite immunity are average $10^{14}$- to $10^{16}$-fold lower than those in non-immune individuals (13).

Blood stage parasites activate innate responses, which in turn lead to significant levels of humoral and cellular adaptive immunity (reviewed in (14)). Antiparasitic immunity appears to be mediated primarily by antibody responses against blood stage antigens (15, 16). Specific antimalarial antibodies can block invasion of host erythrocytes in vitro by both Pf (17) and Pv merozoites (18–21). Additionally, certain antibody iso-types, in particular IgG3, can induce antibody-dependent cellular inhibition (ADCI) of parasite invasion and development in erythrocytes, which is strongly associated with protection against malaria parasites (13, 22). Moreover, passive transfer of Pf antimalarial antibodies to infected patients can result in parasite clearance (15, 16). Evidence from field studies suggests that the slow acquisition of antibodies to genetically variant circulating strains over several years is associated with antiparasite immunity to Pf (23), but to a lesser extent to Pv (24). Cell-mediated immune responses also play a role in protection, particularly early in the immune response. A strong pro-inflammatory response mediated primarily by interferon-gamma (IFN-γ) and tumor necrosis factor-α (TNF-α) contributes to the initial killing and clearance of parasite-infected red blood cells (25).

Identifying antibody targets that are associated with infection, disease, or immunity will support the development of vaccines, diagnostics, and tools for sero-surveillance. By comparing the humoral response profiles of defined populations possessing varying degrees of antidisease and/or antiparasite immunity, it may be possible to identify combinations of responses that are associated with protection against clinical disease and/or parasitemia. These responses could guide selection of antigen(s) for blood stage vaccines. Here, we applied Plasmodium genome sequence, proteomics, bioinformatics, and proteome array fabrication technologies to construct a Pf/Pv blood stage proteome array. The Plasmodium genome encodes over 5000 proteins (5538 and 5435 in Pf and Pv, respectively (26)), nearly half of which have been identified via proteomics at the different stages of malaria parasite life cycle (27, 28). A total of 4441 recombinant proteins, representing 1922 Pf and 1936 Pv native proteins previously reported or predicted to be expressed by the blood stages of these parasites were included on the Pf/Pv proteome arrays, which were then used to analyze antibody responses to both Plasmodium species in naturally-exposed individuals with clinically characterized infections.

The resulting data were interrogated using machine-learning algorithms to identify antibody responses that associated with disease status. We identified sets of antigen-specific antibody responses that can be used to distinguish between asymptomatic donors with parasitemias detectable by light microscopy or PCR and asymptomatic donors, some of which were identified by multiple comparisons. This study is a proof-of-concept of the power of applying machine learning algorithms to biomarker discovery, and paves the way for future, more robust studies to identify novel malaria vaccine targets.

**EXPERIMENTAL PROCEDURES**

*Serological Samples—* Serum samples from children aged 6 months to 10 years in the Madang area on the north coast of Papua New Guinea (PNG) were used for this study. The study sites have a tropical, humid climate year-round, with a rainy season from Nov. to May; malaria is hyperendemic with limited seasonality (29). Both P. falciparum (Pf) and P. vivax (Pv) are common, and the disease burden is mainly in children under 10 years of age (29, 30). To establish parasitemia, two thick and two thin smears per participant were evaluated under 1000× oil immersion and parasitemia was calculated from the number of asexual parasites per 200 leukocytes, assuming mean leukocyte counts of 8000/µL. Two independent reads were performed, and a third of the first two reads disagreed. This was later confirmed by PCR: whole blood genomic DNA containing parasite DNA was prepared for species-specific PCR/LDR-based diagnosis of Plasmodium infection (31) to confirm parasitemia and species identification. Among samples that were positive for light microscopy, there were very few discrepancies between microscopy and PCR regarding the identified species; in these cases the species identification was changed to reflect the species-specific PCR result. Plasma was collected and stored for future studies.

Samples from symptomatic (clinical) cases ($n = 108$) were collected as part of a morbidity surveillance study of children aged 0–5 at three health centers near Madang (Yagaum, Mugil, and Alexishafen) in 2005–2006 (32, 33), and were defined as those individuals presenting at local health clinics with parasitemia over 1000 Pf asexual stage parasites per µL of whole blood, or 250 Pv asexual stage parasites per µL of whole blood, and fever within the last 24 h but without any severe malaria symptoms (32). Samples from asymptomatic children used in this study ($n = 116$) were collected as part of a larger ($n = 1275$) cross-sectional community survey in 2005 that included individuals of all ages (34, 35) presenting with no fever or history of fever in the last 48 h, but with parasitemias detectable by light microscopy (LM) or PCR. For this study, we selected a subset of sera from children aged 0–10 with PCR-confirmed, asymptomatic infection that were either positive (LM, $n = 55$) or negative (PCR, $n = 61$) by LM. Because of the rapid acquisition of antiparasite immunity, in particular to P. vivax (36), a full age-matching to the symptomatic cases was not possible in the study population. However, in order to minimize the difference in age between the groups, samples from the youngest children with asymptomatic infection were preferentially selected. These groups were further subdivided into nine final groups according to the parasite species detected (Pf, Pv, or Pf/Pv infections) (Table I). 18 US malaria-naïve control donors were used to determine the malaria-specificity of the responses.
Recombinant proteins were expressed using H11032 tagged with 5 cloned into the PXT7 plasmid with a T7 transcription terminator, and Pf coding sequences were PCR-amplified from surfins, the active cytoplasmic proteins, lacking both signal peptides and trans-cision by microarray (39, 40). These included both unique Pv proteins and Pv orthologs of Pf proteins. Proteins predicted to be on the surface of the parasite were defined as genes with signal peptides and/or transmembrane domain(s) according to PlasmoDB (26). Putative cytoplasmic proteins, lacking both signal peptides and transmembrane domains, were also selected (pl < 9.0 for Pf genes and pl < 7.6 for Pv genes, respectively). Genes encoding antigenically variant proteins, such as the Pf gene families PfEMP1s, rifins, stevors, surfins, the Pv vir genes, as well as pseudogenes were excluded. Both single and multi-exon genes were included, but exons of multi-exon genes were cloned into separate plasmids. Furthermore, large genes or exons were further divided into overlapping segments in order to limit amplicon length to between 300 and 3000 nt. Amplicons were labeled with the 5′ end of a 2-exon gene. Genes that were further divided into segments that overlapped by 1000 nt. Genes had evidence for blood stage expres-sion by microarray (39, 40). Genes that were further divided into overlapping segments in order to limit amplicon length to between 300 and 3000 nt. For each recombinant protein spotted once in each array (41). Each array contained 256 negative control spots made with an negative control DNA and incubated at room temperature for 30 min with constant mixing to remove E. coli specific antibodies. Anti-EBNA1 or anti-human IgG were used as controls for the primary antibodies, and serially diluted anti-human IgG was used as a control for the secondary antibody. After rehydration for 30 min, the arrays were probed in triplicate with the pre-treated sera overnight at 4°C with constant agitation, then washed and incubated with Cy3-labeled antihuman immunoglobulin. The slides were washed seven times, air-dried, and analyzed using a Perkin Elmer ScanArray Express HT microarray scanner. Intensities were quantified using QuantArray software and corrected for spot-specific background. Statistical analyses were performed as described previously (42). Briefly, the triplicate data points were averaged, and the data were calibrated and transformed using the variance stabilizing normalization (vsn) package (43) in the R statistical environment (www.r-project.org).

Computational Methods—To identify antibody responses that discriminated between the different groups, we first filtered the dataset. First, we removed antibody responses with signal intensities that were not significantly greater in malaria-exposed children than in the 256 empty wells. To do this, we fit the signals arising from the 256 empty wells to a normal distribution. Next, we assigned the response to each recombinant protein to a p value by testing it against the right tail of this distribution. These p values were subjected to a standard FDR correction and those signal intensities with a resulting q-value > 0.01 (44) were set to zero. Any recombinant protein that did not elicit significant signal intensity by this analysis was removed. Next, we filtered out responses that were not significantly different in exposed children and the 18 malaria-naive US donors. For each recombinant protein, we used a Welch’s two-tailed t test to test the hypothesis that the signal intensities in 224 infected children were significantly different than those of the 18 US controls. The resulting p values were converted to q-values using Benjamini and Hochberg false discovery rate (FDR) correction (n = 4441)(44). Those recombinant proteins with a q-value > 0.01 were removed. Next, we performed clustering analysis and replaced clusters of proteins that elicited similar re-sponses in all exposed donors with a representative reactivity profile for each cluster. In brief, we clustered proteins that had positive signals in more than 75% of children into groups, and we replaced the reactivities to individual recombinant proteins with a logical “or”ing representative signal of the reactivity ratios.

We further reduced the remaining antibody responses using an age filter to select for antibody responses that were associated with asymptomatic infections regardless of age group. Briefly, as asymptom-atic children tended to be older than symptomatic children, and as the breadth and intensity of reactivities are associated with in-creasing pathogen exposure, age was significantly correlated with mean reactivity before filtering and clustering (for the entire data set, Pearson’s r = 0.15, p value = 0.026, n = 224) and this was exac-erbated by the filtering (r = 0.18, p value = 0.006, n = 224). We calculated the mutual information (45) between the reactivity to each recombinant protein and the individual’s age and the disease status of the individual. Those recombinant proteins that shared more infor-mation with the disease status than with age (i.e. those that showed a stronger association with the individual’s phenotype than with the individual’s age (resulting r = 0.10, p value = 0.120, n = 224) were preserved, whereas the others were filtered out. In other words, if a recombinant protein was more predictive of age than of disease status, it was removed. Because this filtering required the algorithm to use the individual disease status, it was performed inside the cross-validation loop as necessary. Next, we used machine-learning ap-proaches to identify biomarkers that associated with disease status by comparing each of the six different asymptomatic groups sepa-rately to the corresponding symptomatic control. We first used cross-validation to estimate whether a classifier was effective at predicting...
Proteome Array Analyses of Humans Exposed to Malaria

**TABLE I**

Description of clinical groups of P. falciparum and P. vivax infections

The characteristics of the 9 clinical groups and the non-exposed donors are indicated, including the *Plasmodium* species detected, the abbreviation used to designate the group throughout the manuscript and a short description of the clinical group (PCR: asymptomatic donors with parasites detected by PCR, LM: asymptomatic donors with parasites detected by light microscopy, S: symptomatic donors, Pf: donors with detectable *P. falciparum* infection; Pv: donors with detectable *P. vivax* infection; Pf/Pv: donors with detectable *P. falciparum* and *P. vivax* infection); as well the mean and range of parasitemia values observed by LM, the result of the PCR test for parasitemia, the presence or absence of fever, the mean and range of the age of donors, the percentage of donors older than 5 years, and the number of donors in each group (N). * - all symptomatic samples are from a study that includes only children younger than 5 years; ´ - all malaria naïve controls are older than 18 years.

| Abbreviation | Clinical group | Mean parasitemia (range) | Parasitemia (by PCR) | Fever | Mean age, yrs (range) | % of donors > 5 Yrs | N |
|--------------|----------------|--------------------------|----------------------|-------|----------------------|-------------------|---|
| Pf infection | Pf/PCR Asymptomatic Pf infection, PCR+ | 0 (0–0) | Yes | No | 5.83 (2–9) | 65.5 | 29 |
|              | Pf/LM Asymptomatic Pf infection, LM+ | 798.8 (40–2,015) | Yes | No | 6.21 (0–10) | 73.7 | 19 |
|              | Pf/S Symptomatic Pf infection | 85,480.0 (3,000–378,000) | Yes | Yes | 2.83 (1–5) | 0* | 40 |
| Pv infection | Pv/PCR Asymptomatic Pv infection, PCR+ | 0 (0–0) | Yes | No | 5.32 (0.5–10) | 50 | 20 |
|              | Pv/LM Asymptomatic Pv infection, LM+ | 544.5 (80–1,482) | Yes | Yes | 4.90 (2–6) | 65 | 20 |
|              | Pv/S Symptomatic Pv infection | 21,622.40 (280–223,040) | Yes | Yes | 1.32 (0–4) | 0* | 50 |
| Pf&Pv co-infection | Pf/Pv/PCR Asymptomatic mixed infection, PCR+ | 0 (0–0) | Yes | No | 7.25 (2–10) | 83.3 | 12 |
|              | Pf/Pv/LM Asymptomatic mixed infection, LM+ | Pf: 604.5 (40–2,180) | Yes | No | 5.87 (2–9) | 75 | 16 |
|              | Pf/Pv/S Symptomatic mixed infection | Pf: 112,073.3 (5,160–326,720) | Yes | Yes | 2.56 (1–4) | 0* | 18 |
|              | Pv: 1966.7 (360–10,160) | Pv: 381.9 (65–1,612) | Yes | No | Adults 100* | 18 |

| Non-exposed controls | Malaria naïve | Mean parasitemia (range) | Parasitemia (by PCR) | Fever | Mean age, yrs (range) | % of donors > 5 Yrs | N |
|----------------------|---------------|--------------------------|----------------------|-------|----------------------|-------------------|---|
| 0 (0–0) | No | No | Adults 100* | 18 |

a given clinical category. We constructed evolutionary trees using the evTree algorithm (46), and performed eightfold cross-validation to measure the agreement and the Pearson’s correlation of the predicted and actual categories. These metrics were reported alongside the final decision tree as a general measure of classifier accuracy. Finally, to select which recombinant proteins would be most informative as biomarkers associated with disease status when working in concert with other biomarkers, we used the mProbes (47) feature ranking algorithm with Random Forest (48) feature selection. mProbes estimates a false discovery rate by repeatedly running feature selection after adding noise features, generated by assigning the signal intensities for a real protein to random patients, to the data set. The reported false discovery rate is the fraction of times that a noise feature was ranked as being more informative that the signal intensity to the recombinant protein in question. It should be noted that this latter analysis using the mProbes algorithm was not performed for the Pf/PvLM versus Pf/PvS comparison because the cross-validation analysis revealed it did not possess sufficient information to predict donor status.

RESULTS

**Children Rapidly Acquire Immunity Against Development of Fever**—In order to define the serological responses to *P. falciparum* (*Pf*) and *P. vivax* (*Pv*) infection in relation to host immunity, we obtained samples from 224 children in PNG, where both parasite species are endemic (29, 30). Symptomatic cases (*n* = 108) correspond to donors <5 years of age presenting at local health clinics with positive parasitemia, defined as having over 1000 *Pf* and/or 250 *Pv* asexual blood stage parasites per μl of whole blood, and fever within the last 24 h, but without any severe malaria symptoms (32). Asymptomatic cases (*n* = 116) were selected from a wider cross-sectional field study that included all age groups, and were defined as children <10 years of age with no fever or history of fever in the last 48 h, but with *Pf* and/or *Pv* parasitemia detectable by PCR or light microscopy (LM) (33). Although symptomatic and asymptomatic samples were collected in different studies, and were not matched for age or parasitemia, this data set allowed us to query the natural immune response to both *Pf* and *Pv* simultaneously.

Nine groups were defined based on the combination of clinical status (*i.e.* symptomatic (S), asymptomatic but with parasitemia detectable by light microscopy (LM), or asymptomatic but with parasitemia detectable only by PCR (PCR)), and the parasite species detected (*i.e.* *Pf*, *Pv*, or both, indicated as *Pf/Pv*) (Table I). The parasitemias of all samples from LM and S children are shown in Fig. 1A; parasitemia levels were similar in all LM asymptomatic children, regardless of the parasite species detected (Kruskal-Wallis, *p* value = 0.24), and were lower than in symptomatic cases, although this difference was less pronounced in *Pv* infections. Mean parasitemias in symptomatic *Pf* infections were sevenfold higher than in symptomatic *Pv* infections (Kruskal-Wallis, *p* value <0.0001). These high *Pf* parasitemia levels are due in part to *Pf*’s ability to invade both mature erythrocytes and reticulocytes, whereas *Pv* preferentially invades reticulocytes (49) and generally produces lower parasitemias than *Pf*. Moreover, symptomatic children who were infected only with *Pv* carried higher *Pv* parasite loads than symptomatic children with *Pf/Pv* infections (Fig. 1A).

Among the symptomatic children (all under the age of 5 years as per the original study design), those infected only with *Pv* were significantly younger than all other infected children (one-way ANOVA, *p* value <0.0001), with an average
age of 1.3 years, compared with 2.8 and 2.6 years for those with Pf and Pf/Pv infections, respectively (Table I). This suggests that children acquire anti-Pv disease immunity (i.e., fever) more rapidly than anti-Pf disease immunity, in agreement with previous findings in PNG (36). However, it should be noted that the age distribution of the asymptomatic samples does not correspond to a random sample of asymptomatic infections in the general population because we selected samples from the youngest children in this group for our study. Despite this, the mean age of the children in the asymptomatic groups was higher than that of children in the symptomatic groups (5.6 and 5.9 years for the LM and the PCR children, respectively, one-way ANOVA, p value <0.0001). Among the asymptomatic children, there was no significant difference in the age range of those infected with different species of Plasmodium (one-way ANOVA, p value = 0.15).

In general, younger children were more likely to carry high parasitemias, whereas older children had lower parasitemias (Fig. 1B). Although we selected the youngest children from the asymptomatic study, between 65 and 83.3% of the asymptomatic LM cases, depending on the infecting Plasmodium
species, occurred in children that were between 5 and 10 years of age (Table I and Fig. 1B), suggesting that children in this endemic site rapidly acquired antidisease immunity to the development of fever. Finally, we compared the cumulative incidence of symptomatic *Pv*, *Pf*, and *Pf*/*Pv* cases over time (Fig. 1C). We saw that in this cohort, the majority of symptomatic *Pv* infections are detected earlier in life than symptomatic *Pf* or *Pf*/*Pv* infections. For example, 85% of the symptomatic *Pv* cases were detected by age 2, whereas only 40% of the symptomatic *Pf* and *Pf*/*Pv* cases had occurred by that age. Although our dataset does not allow us to evaluate the effect of previous exposure to malaria parasites on the development of immunity, this result suggests that children acquire antidisease immunity to *Pv* more rapidly than they do to *Pf* (30, 36, 50).

**Antibody Responses Against Parasite Proteins Depend on Symptomatic Status and the Infecting Parasite Species**—Next, we assessed the reactivity of the children’s sera to *Pf* and *Pv* proteins using a proteome array that contains 4441 recombinant polypeptides (2208 from *Pf* and 2233 from *Pv*) representing 1922 *Pf* and 1936 *Pv* native proteins, respectively. Proteins with evidence or prediction of expression during blood stages and secretion or surface localization were selected for inclusion in the array. We probed the arrays with sera from the 224 PNG children, as well as sera from 18 US malaria naïve controls (Table I), and quantified the resulting signals as previously described (41). The strategy used to analyze antibody responses to the *Plasmodium* recombinant proteins contained in the array is illustrated in Fig. 2. We first selected antibody responses against arrayed proteins (or indicators) that were significantly higher in sera from malaria-exposed children than in US adult controls or in empty wells, using both z and t-tests, with a cut-off of *q* = 0.01 (Fig. 2A). This step excluded 635 (14.3%) antibody responses that did not differ between exposed children and empty wells, as well as 1586 (35.7%) antibody responses that were similar in malaria-exposed children and malaria-naïve US controls. The 2220 responses that remained were directed against 1026 *Pf* recombinant proteins (out of 2208 *Pf* proteins in the array, or 46.5%) and 1194 *Pv* recombinant proteins (out of 2233 in the array, or 53.5%) derived from 2021 native proteins (928 *Pf* and 1093 *Pv*, respectively). Next, we performed clustering analysis, and identified sets of antibody responses that were highly similar among all exposed donors. These 373 antibody responses (8.4%) grouped into 16 clusters, each of which was replaced in the final dataset with a signature profile, resulting in the net removal of 357 antibody responses. The remaining 1863 antibody responses were then passed through an age filter that removed those that associated more strongly with the age of the donor than with their symptomatic status (*n* = 1206, Fig. 2A). After applying these filters, 657 antibody responses (directed against 344 *Pf* and 313 *Pv* recombinant proteins, respectively, Fig. 2B) were retained for further analysis.

As shown in Fig. 2B and 2C, the nine groups defined on the basis of the infecting parasite species and symptomatic status exhibited distinct serological profiles. For example, among symptomatic children, those infected only with *Pf* (*Pf*.S) recognized significantly more of the arrayed *Pf* and *Pv* polypeptides than *Pv*.S or *Pf*/*Pv*.S children (Fig. 2B and 2C and supplemental Table S1). On the other hand, there was no significant difference in the number of polypeptides recognized by sera from *Pv*.S or *Pf*/*Pv*.S children. Moreover, the mean numbers of polypeptides recognized by *Pv*.S or *Pf*/*Pv*.S children were not significantly different from those recognized by *Pv*.PCR or *Pf*/*Pv*.PCR children, but was significantly lower than those recognized by *Pv*.LM or *Pf*/*Pv*.LM children. However, this was not the case for children infected only with *Pf*, because *Pf*.S children recognized significantly more polypeptides than *Pf*.PCR children, but not *Pf*.LM children.

The number of polypeptides recognized by *Pf*.PCR, *Pv*.PCR, and *Pf*/*Pv*.PCR children did not differ significantly. Among LM children, those with *Pf*/*Pv* recognized significantly more *Pf* and *Pv* proteins than those infected only with *Pv*, but curiously, those infected with *Pf* recognized significantly more *Pv* proteins than those infected with *Pv*. The mean fraction of *Pf* polypeptides recognized was 13.40% for *Pf*.LM, 7.28% for *Pv*.LM, and 24.06% for *Pf*/*Pv*.LM children (Fig. 2C). Overall, fewer *Pv* polypeptides were recognized by sera from LM children (9.22% for *Pf*.LM, 4.59% for *Pv*.LM, and 18.17% for *Pf*/*Pv*.LM children; Fig. 2C and supplemental Table S1; two-tailed Wilcoxon p value = .0313, t test p value = .0017, n = 6). Finally, for both *Pf* and *Pf*/*Pv* asymptomatic infections, the mean fraction of positive responses was significantly higher in LM cases than in PCR cases.

Our findings suggest that older, asymptomatic children with infections detectable by LM have broader serological responses than younger, symptomatic children, and that the breadth of these responses may be linked to protection against fever. Furthermore, although the interpretation of these data is limited by the fact that we cannot distinguish between infections detectable only by PCR that may reflect existing antiparasite immunity resulting from previous episodes of parasitemia or resolving infections in which antibodies have waned, our results suggest that asymptomatic infections detectable by LM may associate with an active and effective immunological response against fever.

**A Machine-learning Approach Can Classify Children into Different Disease Groups Based on Antibody Responses Determined via Proteome Arrays**—Having used the proteome arrays to profile antibody responses against *Pf* and *Pv* proteins in PNG children infected with *Pf*, *Pv*, or both species, next we asked whether these reactivity profiles could be used to accurately classify children into the different disease groups described in Table I. First, we used the evTree decision tree construction algorithm (46), to assess the dataset and determine whether the observed antibody responses to the arrayed proteins could be used to accurately predict...
whether any individual donor belonged to a symptomatic or asymptomatic group. To do this, we compared the antibody responses of donors in each of the six asymptomatic groups to those in the corresponding symptomatic groups (i.e. Pf\textsubscript{LM} versus Pf\textsubscript{S}; Pf\textsubscript{PCR} versus Pf\textsubscript{S}; Pv\textsubscript{LM} versus Pv\textsubscript{S}; Pv\textsubscript{PCR} versus Pv\textsubscript{S}; Pf/Pf\textsubscript{v}\textsubscript{LM} versus Pf/Pf\textsubscript{v}\textsubscript{S}; and Pf/Pf\textsubscript{v}\textsubscript{PCR} versus Pf/Pf\textsubscript{v}\textsubscript{S}). We applied the filters described previously to eliminate responses that were similar to those observed in empty wells, as well as those that were not significantly different between PNG children and naïve U.S. donors or were nearly identical for all PNG donors. Then, to estimate whether or not the donors in each dataset could be accurately assigned to the symptomatic and asymptomatic groups, we used the evTree machine-learning algorithm with 8-fold cross validation (46), as illustrated in Fig. 3A. In brief, we divided the dataset into an 88% training set and a 12% validation set, and applied the age filter described above to remove responses that associated more strongly with the age of the donor than with their clinical status. Next, a decision tree was built from the training dataset and used to classify the validation dataset into the symptomatic and asymptomatic categories. This process was repeated eight times until the status of each donor was predicted exactly once in the validation dataset. Each cycle of cross-validation produced a different tree that
predicted the symptomatic and asymptomatic status of each donor. These assignments were then used to calculate the cross-validated accuracy and correlation of each classifier, as shown in Fig. 3B. With the exception of the Pf/Pv.LM versus Pf/Pv.S comparison, all the pairwise comparisons classified donors into the symptomatic and asymptomatic groups with
We developed a *P. falciparum* (*Pf*)-*P. vivax* (*Pv*) proteome array containing 4441 proteins to characterize antibody responses to both parasite species in children under 10 years old living in endemic areas in PNG. Sera were obtained from symptomatic children who had attended local health clinics, and from asymptomatic but infected children identified in cross-sectional surveys. We found that asymptomatic children with high parasite loads detectable by light microscopy had antibody responses to more antigens than asymptomatic children with very low parasite loads detectable only by PCR, suggesting that antibody responses in the asymptomatic children were associated with parasite load. Conversely, children with symptoms exhibited very few humoral responses, suggesting a possible disregulation of the antibody response during acute infection. Using a machine-learning approach, we identified humoral responses against subsets of parasite proteins that can be used to predict whether a child is symptomatic or asymptomatic. The approaches we describe may be applied to a well-characterized study population to identify potential antigens for vaccine research or serological surveillance.

The proteome array technology has been used to identify novel antigens in several infectious agents, including *Francisella tularensis* (51), *Burkholderia pseudomallei* (52), *Pf* (53–56), and *Pv* (41). The array described here detects antibody responses to both *Pf* and *Pv* proteins simultaneously, enabling the analysis of sera from malaria endemic areas where both parasites are transmitted. Combining whole proteome arrays with modern analytical tools is a very effective strategy to discover novel antigens for vaccines or diagnostics. Despite the advantages offered by proteome array technology, it is important to note that folding, multimerization, and post-translational modifications such as phosphorylation or glycosylation of arrayed proteins synthesized *in vitro* transcription–translation will differ from the native proteins. However, the ability to screen thousands of potential antigens simultaneously is a substantial advantage over conventional approaches.

The samples used in this work were collected in two different studies: one hospital-based and the other field-based. As a result of this, all children in the symptomatic group were younger than 5 years of age, whereas those in the asymptomatic group were older. In fact, although we selected the youngest children among donors in the asymptomatic group, we were unable to age-match the symptomatic and asymptomatic cases because of the fact that there were few asymptomatic children younger than 6 years of age in the asymptomatic cohort. Unsurprisingly, symptomatic cases also carried higher parasitemia than asymptomatic cases. Furthermore, although we utilized a powerful algorithm to analyze antibody responses in the sera from 224 children to >4000 parasite proteins, this study relied on a limited number of banked samples collected in previous studies and
we did not know the donors' sex, malaria history, or other factors that impact infection. Future studies with larger sample sizes will be required to increase statistical power and confirm that the responses we identified here with banked serum samples are associated with asymptomatic infections. These caveats, along with the cross-sectional nature of this study, do not allow for the detection of immune correlates of protection. However, this dataset allowed us to describe humoral responses to both Pf and Pv simultaneously, and to develop an analysis pipeline for use in future studies.

It has been previously shown in PNG (57–59) and other locations (23, 60), that antibody titers to Pf proteins are higher in children with LM-detectable parasitemia than in children with LM-undetectable or no parasitemia. We confirmed this finding in Pf and Pf/Pv infections, for which the percentage of positive responses was significantly higher in the LM children than the PCR children. The high antibody titers in asymptomatic children with high parasitemia (LM children) compared with asymptomatic children with very low parasitemia (PCR children) suggests that in asymptomatic donors, the breadth of the antibody response correlates with antigen load, potentially because of the critical mass of antigen required to elicit a response. This may also indicate poor boosting of the memory response, as the very few parasites detectable by PCR in these children may not have been sufficient to boost antibody responses. Alternatively, these children may have developed cellular immunity through past infections that is able to control parasitemia independently of antibodies (61).

Long-term IgG production is maintained by short-lived plasma cells derived from a memory B cell population, or by long-lived plasma cells, which secrete antibodies for as long as several months after immunization (62, 63). Studies in mice

| Pf and Pv Orthology Groups               | Arrayed Proteins | Pairwise Comparisons | # of times identified |
|-----------------------------------------|------------------|----------------------|----------------------|
| Serine-repeat antigen (SERA)            | PFB0330c_204     |                      | 7                    |
|                                         | PVX_003830_204   |                      |                      |
|                                         | PFB0345c_404     |                      |                      |
|                                         | PVX_003835_204   |                      |                      |
|                                         | PVX_003800_204   |                      |                      |
|                                         | PFI0135c_204     |                      |                      |
| Merozoite surface protein 4 (MSP4)     | PFB0310c_101     |                      | 6                    |
|                                         | PVX_003775_102   |                      |                      |
|                                         | PVX_003776_202   |                      |                      |
|                                         | PFB0310c_202     |                      |                      |
| Hypothetical protein, conserved         | MAL7P1.203_308   |                      | 4                    |
|                                         | MAL7P1.203_108   |                      |                      |
| DNA repair protein RAD23                | PVX_094806_101   |                      | 3                    |
|                                         | PF10_0114_101    |                      |                      |
| Hypothetical protein, conserved         | PFE1240w_101     |                      | 3                    |
| Hypothetical protein, conserved         | PFC0690c_101     |                      | 3                    |
| Hypothetical protein                    | PF14_0250_101    |                      | 3                    |
| Hypothetical protein                    | PFB0775w_101     |                      | 3                    |
| Hypothetical protein, conserved         | PVX_089030_101   |                      | 3                    |
| Hypothetical protein, conserved         | PFD0235c_102     |                      | 3                    |
| Hypothetical protein, conserved         | PVX_115450_102   |                      | 3                    |
have shown that younger individuals develop fewer and shorter-lived plasma cells than adults (64, 65). If this result is applicable to humans, it is possible that young children with malaria may require constant antigen stimulation in order to produce antibodies until they are able to develop long-lived plasma cells. This view is supported by several studies showing that antibody responses against Pf antigens are more persistent in adults than in children (66–68). Furthermore, a more recent study showed that antibody titers declined more slowly with time in both older children and asymptomatic children than they did in younger children, suggesting roles for both antigen persistence and immunological memory in determining the longevity of the antibody response (69). Young symptomatic children may therefore have low antibodies because they have not been sufficiently exposed to the parasite and cannot sustain an antibody response. Alternatively, acute malaria has also been associated with a decreased response to tetanus toxoid, meningococcal polysaccharide, Hib conjugate, and whole cell vaccines for typhoid fever (70–72). Acute malaria itself may therefore disregulate the immune response and prevent generation of antibody responses of sufficient magnitude for protection. However, it may also be the case that younger children rapidly acquire antidiase immnity independently of antibodies, such as a strong cellular response and prevent generation of antibody responses of sufficient magnitude for protection. However, it may also be the case that younger children rapidly acquire antidiase immnity independently of antibodies, such as a strong cellular response and then slowly acquire antiparasite immunity as they grow older.

In this study we attempted to identify antibody responses that could be used to predict whether a child was asymptomatic, as these antibody responses could protect against development of clinical disease. For this, we turned to machine learning algorithms to identify sets of antibody responses that could discriminate between symptomatic (febrile) and asymptomatic (afebrile) cases detectable by LM or PCR for subsets of children infected with P. falciparum, P. vivax, or both. The machine learning community has long grappled with how to best determine which features, in this case antibody responses, are most informative. Typically these methods focus on selecting a subset of features (e.g., biomarkers) that maximize predictive accuracy, such as support vector machine (SVM) with backward feature selection (73) or partial area under the curve (AUC) (74), algorithms. However, recent research using simulated data implies that for biomarker discovery the best feature-ranking methods also estimate false discovery rates, which are not calculated by SVM and AUC algorithms. For example, the mProbes algorithm with Random Forest (47, 48) seemed especially well suited for problems such as the one discussed here where the accumulated evidence suggests that immunity to malaria only develops after exposure to numerous different antigens, as mProbes with Random Forest can detect groups of antibody responses that work well in concert. However, to our knowledge this is the first time that mProbes has been used in the context of biomarker discovery.

Using this approach, we compared each asymptomatic group to the corresponding symptomatic group, and identified responses to subsets of Pf and Pv proteins that distinguished between asymptomatic and symptomatic donors within each pairwise comparison. In general, the absence of a response was indicative of a symptomatic infection, as symptomatic donors had on average very few responses. Interestingly, several proteins were identified by multiple pairwise comparisons. Moreover, when we classified these shared responses based on Pf and Pv protein orthology, we observed overlapping responses, for example, instances where sera from P. falciparum-exposed donors recognized P. vivax proteins and vice versa. This suggests that some Pf and Pv orthologs may share linear or conformational epitopes that elicit cross-reactive antibodies able to protect against symptomatic Pf, Pv, or Pf/Pv infections. Naturally-acquired cross-reactive antibody responses to MSP5 (75) and CLAG9 (76) have been observed in other endemic settings. Alternatively, it is possible that these apparent cross-reactive responses do not stem from shared epitopes between P. vivax and P. falciparum orthologous proteins, but instead could result from previous infections with the other Plasmodium species. Importantly, because this study did not track malaria infection, our data cannot distinguish between these two possibilities.

Two of the orthology groups identified by multiple analyses contain proteins that have been previously studied as vaccine candidates. MSP4 is a 40 kDa GPI-anchored membrane protein expressed on the merozoite surface that appears to be essential because it is refractory to genetic deletion (77, 78). Unlike most other known merozoite proteins, MSP4 is taken into the invaded erythrocyte without proteolytic processing and is detectable for several hours postinvasion (79). In a recent cross-sectional study in malaria-exposed individuals in the Brazilian Amazon (80), plasma from asymptomatic individuals reacted more strongly to recombinant MSP4 protein than those from symptomatic cases, but anti-MSP4 antibodies could not be independently associated with asymptomatic status. However, polyclonal antisera raised in rabbits inhibited the growth of asexual P. falciparum parasites in vitro, and in rodent models, MSP4 recombinant protein plus adjuvant (81–83) and MSP4 DNA vaccines (84, 85) provided partial protection against blood stage challenge.

The serine-repeat antigens SERAs (86) are soluble parasitophorous vacuolar proteins that are co-expressed in late trophozoite and schizont stages, released upon schizont rupture, and appear to facilitate merozoite egress from rupturing schizonts (87). P. falciparum possesses nine SERA proteins, all but one of which are encoded in an 8-gene cluster on chromosome 2; the ninth gene encoding SERA9 is on chromosome 9. All SERAs have been classified as cysteine-like proteases because in several paralogs the catalytic active site Cys residue has been substituted by Ser. Six SERA proteins, three each from P. falciparum and P. vivax, respectively, were preferentially recognized by the PNG sera (Table II). PSI5ERAS,
the most well-characterized SERAs, is abundantly expressed and is refractory to deletion in asexual stages (86). Because antibodies to SERAs have growth-inhibitory activity in vitro, including those isolated from sera of individuals naturally exposed to \textit{P. falciparum} (88), SERA5 has been studied extensively as a potential antigen for blood stage vaccines. Although this study did not find that asymptomatic status was associated with antibody responses against \textit{PI/SERA5}, it did identify two putative \textit{P. vivax} orthologs, PVX\_003830 and PVX\_003800, to which antibody responses discriminated between the Pf/Pv\_PCR and Pf/Pv\_S groups, and \textit{Pr.LM} and \textit{Pr.S} groups, respectively (Table II). Responses to other \textit{Pf} or \textit{Pv} SERAs were also associated with asymptomatic status, suggesting that antibodies to multiple \textit{S}ERA family proteins provide protection against symptomatic malaria, perhaps by interfering with release of infectious merozoites from mature schizonts.

The other proteins that were identified by more than three analyses are all annotated as “hypothetical proteins.” Our data showing that antibody responses against them associate with asymptomatic infections in naturally exposed donors suggests that they should be studied in greater detail.

Longitudinal studies in endemic areas are required to validate our findings from this cross-sectional study and to better assess the role of these antibody responses in immunity to malaria. Extending this study to distinct geographical locations might also allow protective antibody responses that are observed in multiple endemic sites to be identified. Furthermore, the use of this technology on longitudinal samples from areas of declining malaria incidence could provide a unique opportunity to identify markers of recent exposure that could be used as surveillance markers to support malaria elimination programs.

In conclusion, we have combined high-throughput laboratory methods with machine-learning analytical tools, providing a proof-of-concept for a novel approach to identify globally relevant novel antigens for vaccine research. This approach could be extended to screen the entire \textit{Pf} and \textit{Pv} proteome, and accelerate the search for new vaccine candidate antigens, diagnostic antigens, and serological surveillance markers for malaria.

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