New antigens for multi-component blood-stage vaccines against *Plasmodium falciparum* malaria

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Abstract

An effective blood stage vaccine against *Plasmodium falciparum* remains a research priority but the number of antigens that have been translated to candidates for testing in clinical trials remains limited. Investigations of the large number of potential targets found in the parasite proteome have been constrained by an inability to produce natively folded recombinant antigens for immunological studies. We overcame these constraints by generating a large library of demonstrably biochemically active merozoite surface and secreted full-length ectodomain proteins. We then systematically examined the antibody reactivity against these proteins in a cohort of Kenyan children (n=286) who were sampled at the start of a malaria transmission season and prospectively monitored for clinical episodes of malaria over the ensuing six months. We found that antibodies to previously untested or little-studied proteins had superior or equivalent potential protective efficacy to the handful of current leading malaria vaccine candidates. Moreover, cumulative responses to combinations comprising five of the ten top ranked antigens, including PF3D7_1136200, MSP2, RhopH3, P41, MSP11, MSP3, PF3D7_0606800, AMA1, Pf113 and MSRP1 were associated with 100% protection against clinical episodes of malaria. These data suggest that not only are there many more potential vaccine candidates for the vaccine development pipeline, but also that highly effective vaccination may be achieved through combining a selection of these antigens as observed in nature.

Introduction

Immunity to *Plasmodium falciparum* malaria can be acquired, although the underlying mechanisms remain poorly understood (1). Passive transfer of purified gamma immunoglobulins from immune adults to infected children was shown to significantly reduce parasitaemia and clinical symptoms of malaria more than fifty years ago (2) but the number, identity, and relative importance of the protective antibody targets remains unknown (3, 4). Proteins displayed at the surface of the merozoite, the blood-stage form of the parasite, constitute prime targets because of their repeated exposure to the host immune system. Prospective population-based immuno-epidemiological studies that establish temporal associations between the presence of antibodies and subsequent clinical outcome are powerful tools often employed to identify targets of protective immunity against malaria. Such studies have either focused on a small number of antigens (4), or used proteins or more commonly protein subfragments expressed using *in vitro* bacterial (5–7) or wheat-germ cell-free (8) translation systems. Whilst a clear advantage of *in vitro* translation systems is the ability to test a large number of polypeptides simultaneously, they leave open the possibility that some proteins or protein subfragments may be incorrectly folded and as such biologically relevant antigenic epitopes may be overlooked. The native conformation of proteins appears to be important for the induction of protective antibodies in some cases (9–11).

Here, we systematically analyze responses to a protein library comprising 39 correctly folded merozoite surface and secreted proteins expressed using a mammalian expression system, many of which are either being studied here for the first time or have not been widely studied in the context of acquired immunity. We assess their potential protective efficacy against clinical episodes of malaria in a prospectively monitored longitudinal cohort of Kenyan children and perform comprehensive combinatorial analyses to identify cumulative responses predictive of high levels of protection. Our data suggests that the pool from which novel antigens can be selected for the generation of new malaria vaccines is considerably larger than previously anticipated.

Results

Serological validation of the protein library

We first checked the validity of serological responses observed against proteins in the merozoite library. We analyzed seroprevalence with regards to antigen localization, established patterns of age- and exposure-dependent antibody acquisition, as well as correlations with previously published responses to well-studied merozoite antigens in the same Kenyan cohort (n=286). Of the 39 antigens, antibodies to 36 were recognized by sera from children in the cohort, and not by malaria-naive sera and these are reported here. Sero-prevalence varied from 5% (ETRAMP10.2) to 96% (P12), being generally highest for GPI-anchored antigens and AMA1, and then sequentially lower for peripheral surface, and micronemal antigens (Fig. 1). This distribution fits broadly with current understanding of antigen location, and/or timing of secretion (12). Similarly, sero-prevalence and ELISA OD levels increased significantly with age for most antigens as expected, and were higher in children with concurrent asymptomatic parasitemia at sampling (parasite positive) than in

those without (parasite negative) (Table S1). Finally, antibody responses against AMA1, MSP2 and MSP3 from the protein library were highly correlated with those against the same full-length proteins produced in *Escherichia coli* (pairwise correlation coefficients 0.88, 0.53, and 0.72, respectively, P <0.001), and validated using classical immuno-fluorescence assays (13–15)(Fig. S1).

Potential protective efficacy of responses to individual antigens

We assessed and ranked the individual potential protective efficacy of each of the 36 proteins in children monitored for clinical episodes of malaria over six-months. To minimize the misclassification bias that arises due to difficulties in ascertaining exposure to infectious mosquitoes, we restricted this analysis to 121 children with documented parasitaemia at sampling (parasite positive). We classified antibody reactivity as either high versus low (16), or positive versus negative, depending on the antigen (see Statistical Analyses). Antibody reactivity was then fitted as an independent variable in modified Poisson regression models, separately for each antigen, with the binary outcome of whether a child did or did not develop a clinical episode of malaria during follow-up. We adjusted for age as a confounder in each model since it is correlated with exposure (antibodies) and outcome (clinical malaria). We also adjusted for total parasite exposure, which is correlated with age by including reactivity to parasite schizont extract in each model. Antibody levels against parasite schizont extract were significantly and positively associated with age amongst parasite negative individuals in this cohort (but not in parasite positive individuals), indicating that they generally reflect exposure over a longer period. We found that antibody responses to many newly or little-studied proteins had point estimates of the risk ratio (RR) that were better than or equivalent to those of leading malaria vaccine candidates (Table 1). Although none of these estimates remained significant at the P < 0.05 level after adjusting for multiple comparisons, these results nevertheless suggested that responses to many more antigens potentially contributed to protective immunity. Interestingly, the most potentially protective antibodies were not the most prevalent in the cohort. For example, antibodies to PF3D7_1136200 were associated with an 83% reduction in the risk of malaria (P = 0.06), but were present in only 10% of children (Table 1).

We considered the possibility that the potential protective effects of responses to one antigen merely mirrored the effect of other responses, because antibodies to parasite antigens are co-acquired and thus may be highly correlated. We therefore analyzed all pairwise correlation coefficients, ranking these as low, moderate or high (coefficients <0.3, 0.3-0.5, 0.5-0.99, respectively) based on our experience with the analysis of antibodies to allelic versions of the same antigen (16–19). We found high correlations for only a small proportion of all pairs of antibodies, 29 of 648 (4.5%), suggesting that the observed individual associations with protection could not be simply explained by their co-acquisition (Fig. S2).

A lower proportion of children developed malaria in the subgroup that were aparasitaemic at screening, 30/165(18%) versus 40/121 (33%), P = 0.004. We repeated the analyses above in this parasite-negative group and found as we had observed previously (17, 20–22) that for most responses, antibodies were associated with a non-significant increase in the risk of malaria (Table S2).

Heterogeneity in the breadth of responses to merozoite antigens

We, and others, have previously shown that the breadth of the antibody response is an important predictor of clinical outcome (16, 23, 24). To examine this, we converted the risk ratios from Table 1 into potential protective efficacy (PPE= (1-RR)*100). Next we classified antibody reactivity as either high (or seropositive) or low (or seronegative), and potentially protective (PPE >= 20%) or non-protective (<20%), and asked whether it differed between protected versus non-protected children. This threshold for potential protective efficacy was arbitrary, but guided by the lower bound efficacy of the most successful malaria vaccine to date, RTS,S (34.8%, 95% CI 16.2-49.2). Using this scheme, the breadth of antibody reactivity for individual children was compared between those who developed a clinical episode of malaria and those who did not (Fig. 2A). We observed a marked inter-individual variation in the breadth of responses, with each child having a unique reactivity profile. Notably, there were four children who did not develop malaria and did not have any of the responses we had defined as potentially protective. If immunity is mediated primarily through antibodies, this suggests that there are still other important antigens yet to be identified. As we had previously observed with a limited panel of antigens (16), breadth increased significantly with age (Cuzick non-parametric rank sum test for trend across ordered groups Z = 6.66, P < 0.001), Fig. S3. We compared the difference in the breadth of the response between the two groups of children separately for the potentially protective responses and the non-protective ones. Children who did not develop malaria had a median count of 9 potentially protective responses compared to 3.5 in those who developed malaria, P < 0.001, two-sample Wilcoxon rank sum test (Fig. 2B). In contrast, for the non-protective responses, the breadth of the response was similar in both groups of children, 5 versus 4 respectively, P =0.09, two-sample Wilcoxon rank sum test (Fig. 2C). These results suggested to us that antibodies to some antigens potentially contributed to protective immunity while others did not. We therefore explored this further by analyzing the PPE of combinations of specific responses in a comprehensive combinatorial analysis.

Potential protective efficacy of combinations of responses

Combinations were restricted to five proteins to ensure sufficient statistical power. The 36 proteins were first grouped into all of the 376,992 possible five-way combinations. For each combination, the 121 parasite-positive children were each assigned a breadth score, which ranged from zero (no response) to five (all five responses present). For each combination, we fitted the breadth score as a continuous variable in the regression model and estimated the PPE of a breadth score of one, through to five, relative to a breadth score of zero, termed PPE_{comb} hereon.

Children with a breadth score of five versus zero had, on average, 50% PPE_{comb} compared to 14% in those with a breadth score of one versus zero (Fig. 3A). This illustrates that for the majority (though not all) combinations of antigens the PPE of multiple antigens operates in a cumulative rather than an "either/or" fashion. Thus antibody against only one antigen does not appear to be sufficient. When the ten most protective antibodies were analyzed in five-way combinations (n = 252), the average PPE_{comb} for a breadth score of five versus zero was 88% (Fig. 3A). Indeed, the PPE_{comb} for these 252 combinations constituted the top 5% of the distribution of PPE_{comb} for all possible random combinations thus showing that the

breadth effect is best observed when it involves the antibodies that were individually the most strongly associated with protection (Fig. 3A). For example, the PPE$_{comb}$ of combinations containing the five top-ranking proteins, PF3D7_1136200, MSP2, RhopH3, P41, MSP11, rose to 100% when responses to any three of the five were present (Fig. 3B). By contrast, responses to multiple antigens of low to medium individual rank did not show the same effect when in combination. To further illustrate these findings, we asked which antibody responses were overrepresented in five-way combinations for which the rise in PPE$_{comb}$ with breadth score above 1 was highly significant ($P < 0.01$, n = 7,731). Antibodies associated with the highest individual PPE tended to be the same as those that improved PPE upon combination with other antigens ($P < 0.01$, n = 3,515, Fig. 3C). Again, the proportion of children who recognized all five antigens was low: 3.8% averaged across all 7,731 possible combinations.

Discussion

Optimism that completion of the *P. falciparum* genome would bring an effective malaria vaccine closer to the clinic has been tempered by the sheer complexity and quantity of the proteins contained therein (25), and the limited, though growing understanding of their biological roles in nature (12). The targets and mechanisms that define a protective human immune response against malaria remain elusive despite the explosion of technology in the "omic" era. To home in on potentially important antigens, we employed a hypothesis driven approach, tethered on the principle that proteins associated with the merozoite surface are exposed repeatedly to the immune system during natural infections, making them prime targets for protective antibodies. We maximized the opportunity to detect antibodies against important epitopes by analyzing entire surface and secreted full-length ectodomains of unique proteins, as opposed to subfragments of the same or different proteins (5, 26). In addition, since proteins in our library have been shown to be demonstrably biochemically active (27, 28) and therefore highly likely to be correctly folded, we anticipate that all potentially protective epitopes were accessible to antibodies.

We observed high correlations between antibody reactivities detected against three mammalian cell versus *E. coli* expressed full-length proteins. In keeping with this, responses against these three antigens, AMA1, MSP2 and MSP3 which had previously been shown to be associated with protection against malaria in this population (16) were ranked among the top ten potentially protective antibodies in the current analysis. These results are reassuring, and validated not only the previous results, but also the quality of the proteins that despite being produced independently in different expression systems, nevertheless led to the same conclusion. Direct functional activity of antibodies targeting proteins in our library has only been demonstrated for PfRh5 (29, 30) and overall, the approaches to antigen expression are complementary (5, 31, 32).

Our new data strongly reinforces previous observations that the breadth of the antibody response is a better correlate of protection than responses to individual antigens (16). Whereas this finding was previously limited to a small number of proteins, the quality and quantity of proteins in our library allowed us for the first time to make a clear distinction between combinations of responses that were strongly associated with protection from those

that were not, and more likely reflected cumulative exposure to malaria parasites. The distribution of protective efficacy for combinations of responses to the top ten ranked antigens was consistently at the top end of that of all possible random five-way combinations, an observation that is unlikely to be due to chance if all antigens were equally protective when in combinations. Thus, despite the extensive inter-individual heterogeneity of antibody responses, there existed certain combinations that conferred very high levels of protection.

The most highly ranked antibody responses tended to be those against which it was possible to define a threshold level that was associated with protection. This fits with our observations from multiple studies (16, 33, 34) and those of others (23, 24, 32, 35–37), that high levels of antibodies are strong correlates of immunity, and suggests that this is an important biological phenomenon. It is also generally in keeping with a larger body of evidence that shows that for vaccine-induced protection against many infectious diseases, a certain threshold concentration of antibodies is required (38). However, antibodies against our most highly ranked antigen PF3D7_1136200, and indeed 3 of our top 10 antibodies were classified as seropositive/seronegative, suggesting that for some antigens, only a low concentration of antibodies may be sufficient for protection. Such targets may be more attractive for vaccine development as protective levels could be achieved more easily. Whilst the data presented in this report need to reproduced in other settings, ultimately, only head to head comparisons of antigens in clinical trials will reveal which types of responses are superior with regards to protection.

Immunity against *P. falciparum* malaria is acquired slowly and is never complete, a situation largely attributed to the antigenic diversity of immune targets (39). Our current study did not address this as we analyzed responses to just the 3D7-variant. However, our previous studies using several variants of AMA1 (18) and MSP2 (19) found that antibodies to different variants of the same protein were highly correlated and although allele-specific antibodies were readily detectable, the evidence that they played a major role in naturally acquired immunity was relatively weak. In contrast, vaccine-induced allele-specific antibodies against MSP2 and AMA1 appear to be important for the limited protection that was observed in clinical trials (40, 41). Whilst the advantages of conserved versus polymorphic antigens continue to generate debate, clear evidence from studies in humans is lacking and studies to address this are urgently needed to inform vaccine development.

We observed mainly low to modest correlations between antibodies to different antigens in this cohort. This may be a surprising finding given that the responses are co-acquired, as in theory all antigens are encountered upon infection. The reasons why some make responses to one antigen and not another in the face of challenge are not well understood. Potential explanations include: i) antigenic diversity, ii) the short-lived nature of antibodies in children (42, 43), iii) the relatively young age of the cohort studied, iv) the differential immunogenicity of different antigens against a background of an outbred population, and v) measurement error.

As previously reported, antibodies were associated with an increased risk of clinical malaria amongst children who were parasite-negative at screening (17, 20–22). This subgroup is

likely to have lower exposure to infectious mosquitoes, and they experienced fewer clinical episodes and had lower antibody levels. We hypothesize that in this sub-group, antibodies identify individuals getting some exposure to parasites, hence the apparent increased risk of malaria, but that their antibodies have not reached protective levels (33).

Literature on the novel and little-studied antigens is scanty at present (12), impeding efforts to marry parasite biology to protective immunity in humans. An increasing body of evidence points to the potential role of antibodies against the EBA and Rh families of proteins (24, 29, 30, 32, 36, 44, 45), known for their involvement in parasite-host ligand interactions during red cell invasion (28, 46, 47). However, biological function remains incompletely understood even for the most extensively studied vaccine candidates (12). Antibodies to the *P. falciparum* erythrocyte membrane protein 1(PfEMP1) were also associated with protection in this cohort (48), but the extensive variability encoded in this protein may limit its potential utility as viable vaccine candidate (49). Nevertheless, the current study suggests that a core subset of proteins drive protective immunity, with responses against these working together additively. We hypothesize that the ability to make high titer responses against multiple members of this subset interrupts the parasite life cycle by inhibiting erythrocyte invasion at multiple points (50, 51), enhancing antibody-dependent mechanisms of parasite clearance (52, 53) and that targeting different antigens contributes to overcoming parasite diversity and consequent immune escape.

Translation of these findings to the clinic will require further preclinical validation of the candidates in other countries across sub-Saharan Africa that experience differing levels of malaria transmission intensity. Efforts in this regard are already underway. Our analysis supports the view that high levels of protection against *P. falciparum* malaria *can* be achieved with multi-component vaccines incorporating a limited number of correctly-folded blood-stage antigens, and formulated to induce high titer antibodies.

Materials and Methods

Study site and population

The Chonyi cohort in Kilifi, a rural district along the Kenyan coast has been studied previously to identify correlates of naturally acquired immunity to malaria (16, 22). Details of the study area are published (54). Briefly, it typically experiences two seasonal peaks in malaria transmission (June to August, November to December), with an average annual entomological inoculation rate of 20 – 100 infective bites/person/year, when the study was conducted (55). Venous blood was collected from study participants (N=536) at the start of the malaria transmission season in October 2000. Participants were actively followed up for clinical malaria episodes in the ensuing six months. Detection of clinical malaria was achieved through weekly visits to participants' homes, and included a morbidity questionnaire, and documentation of axillary temperatures. Participants had open access to a dedicated outpatient clinic at the local district hospital (passive case detection). Age-specific criteria for defining clinical malaria in this area are: children < 1 year, axillary temperature >37.5°C plus any parasitaemia; children > 1 year, axillary temperature >37.5°C plus a parasitaemia > 2500/µl (54). Participants were excluded if they were absent at three follow-up visits. Although the original cohort comprised children and adults, the present analysis

was restricted to children up to ten years of age (N = 286) because they accounted for 90% of all clinical episodes observed in the cohort. Previous studies (20–22) indicated that the inclusion of antibody data from adults made little difference to the estimates of risk. The Kenya National Research Ethics Committee gave permission for the study (REF CTMDR/SCC/1340) and all participants (or guardians) gave written informed consent.

Recombinant merozoite proteins

A description of the proteins analyzed in this report has been recently published (56). Briefly, sequences encoding the extracellular domains of these proteins, excluding their signal peptide, were made by gene synthesis (GeneartAG, Germany). All sequences were derived from the *P. falciparum* 3D7 strain and were codon-optimized for expression in human cells. All antigens were expressed as biotinylated proteins in HEK293E cells. The soluble biotinylated recombinant proteins were collected from the cell culture supernatant six days post transfection, and dialyzed into HEPES-buffered saline (HBS).

Cohort antibody assays

ELISAs against each recombinant antigen were performed in duplicate according to a standard protocol. Individual wells of Streptavidin Coated 96 Microwell ELISA plates (NUNC, Denmark) were washed with PBST (Phosphate Buffered Saline/0.1% Tween 20) prior to coating with 100 μl of normalized concentrations of biotinylated protein (~300 ng/ml). Plates were incubated for approximately 45 minutes at room temperature and subsequently washed four times in HBST (0.14M NaCl, 5mM KCl, 2mM $CaCl_2$, 1mM $MgCl_2$, 10mM HEPES/0.1% Tween 20). Individual wells were then incubated with 100 μl of test sera (1:1000 dilution in HBST) for one hour before washing four times in HBST, with a final wash in HBS. Plates were then incubated at room temperature for one hour with 100 μl of HRP-conjugated rabbit anti-human IgG (Dako) at 1:5000 dilution in HBST before final washing and detection with H_2O_2 and O-phenylenediamine (Sigma). The reaction was stopped with 30 μl of 2M H_2SO_4 per well and absorbance read at 492nm. Standard positive controls ("immune" sera) were run in duplicate on each day of the experiment, on each plate, to allow for standardization of day-to-day and plate-to-plate variation.

Statistical analyses

Analyses were performed in STATA version 11 (StataCorp, College Station, TX), Excel 2011 (Microsoft Corp, Redmond, WA) and R (57). All analyses on the protective efficacy of individual or combinations of antigens are presented for the subgroup of children who were parasite positive at sampling (n = 121). This ensured that the analysis was focused on children with unequivocal exposure to malaria parasites and thus the opportunity to develop protective immunity. High levels of antibodies were defined using probability plots (16) and represent the OD levels for individual antigens above which the risk of disease was lower than the population's average risk. For 16/36 antigens analyzed in the study, it was not possible to define high levels of antibodies as described above because the ELISA OD values were low (ELISA OD < 0.3 for > 99% of all samples). For these, the conventional cutoffs for positive versus negative were used (positive = mean plus 3 standard deviations of negative control sera).

Individual antibody analyses

The modified Poisson regression model (58) was used for all regression analyses and its suitably for this type of analysis was verified in our previous study (16). For antibodies to single antigens, protective efficacy was estimated by fitting a model with antibody response (binary outcome; either high versus low levels or positive versus negative), age (fixed factor with five levels; two-year age categories) and schizont extract (continuous covariate) as covariates. The inclusion of age as a covariate increased the estimate of risk by an average of 0.08 (range -0.39 to 0.37), while the inclusion of parasite schizont extract increased the estimate by a further 0.07 on average (range -0.05 to 0.26). The Benjamini-Hochberg method was used to adjust for the false discovery rate.

Combinatorial analyses

All possible five-way combinations of antibodies were generated from the data (n = 372,996). A breadth score was then created for each combination, for each child, and ranged from 0 (no response) to 5 (all five antibody responses present). For the purpose of estimating the significance of the combination ('breadth') effect, the breadth score was fitted as a continuous covariate, along with terms for 'baseline', age, the antibody response to schizont extract and an intercept, in the modified Poisson regression model (58) using the glm package in R (57). The baseline term modelled the effect of having responses to at least one vs. zero antigens (the intercept) while the breadth effect modelled the effect of a response to each additional antigen among those with at least one response (i.e., the slope of the line relating risk to breadth score). The P-values for the breadth effect were calculated from the t-values of the coefficient for breadth. For ease of presentation of the results, a similar model was fitted, replacing the continuous variable for breadth with a 5-level factor, from which the PPE was calculated for children with a breadth score of five, four, three, two, and one relative to children with a breadth score of zero. In this analysis, 100% protective efficacy refers to the situation in which none of the children with a particular combination of antibodies develop malaria, in contrast to the proportion of children without those specific responses, some of whom develop malaria.

Alternative analytical approaches

Consideration was given to alternative methods of analyses. Fitting antibodies as continuous variables to minimize loss of information yielded low estimates of risk but with extremely wide confidence intervals, and complicated the combinatorial analyses. Time-dependent measures of analysis yielded estimates of risk that were very similar to those presented (Table 1), and did not change the interpretation (Supplementary Table S3 and Fig. S4). An analysis based on multiple episodes was precluded by their low incidence; 7.6% overall, 14 children with 2, 6 with 3, 1 with 4 and 1 with 5 episodes each.

Supplementary Materials

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Funding

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One Sentence Summary

Uncharacterized proteins from the merozoite stage of *Plasmodium falciparum* offer new hope for effective multi-component blood stage vaccines against malaria
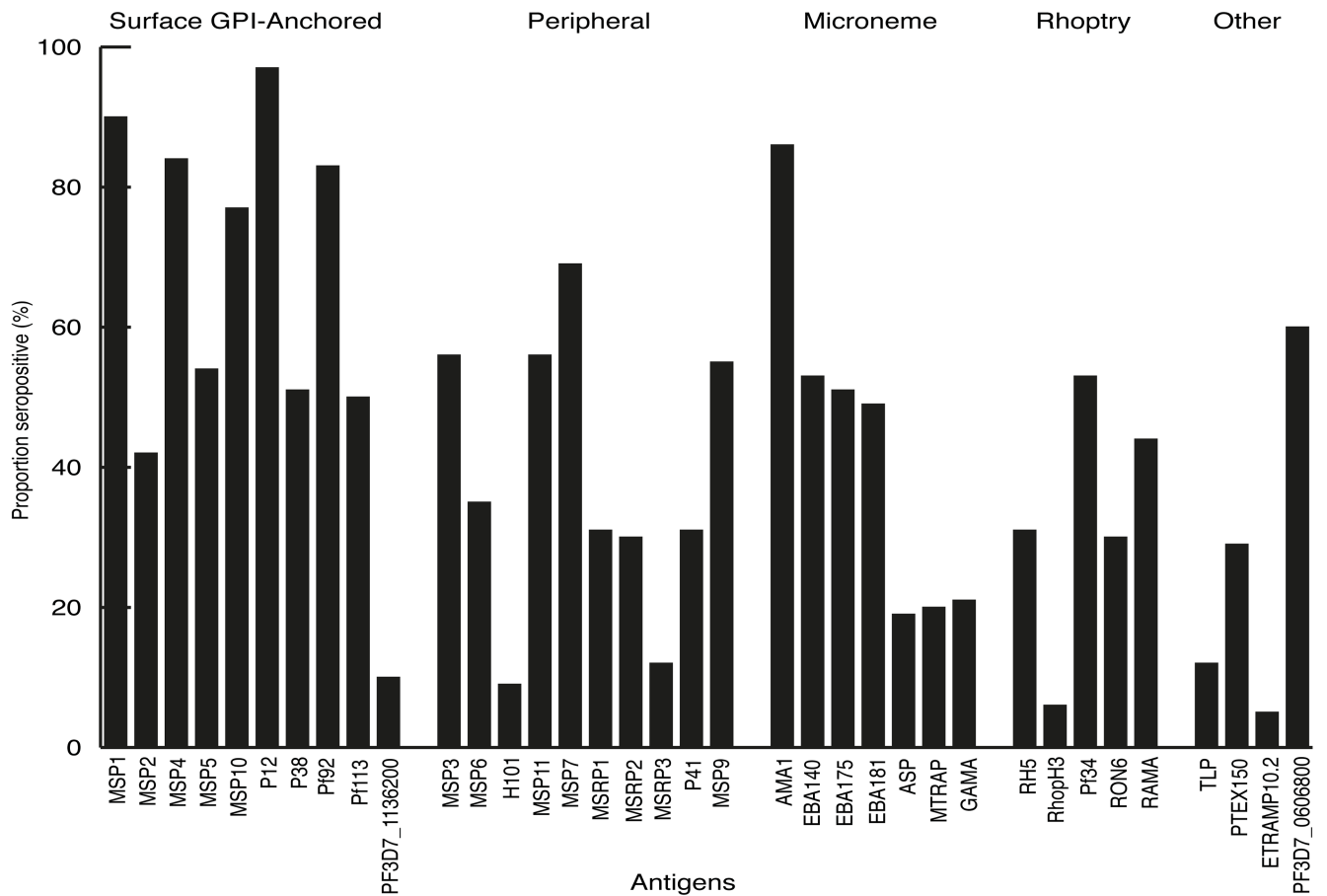
Fig. 1.
Sero-prevalence of each of the 36 immunoreactive antigens within the recombinant merozoite protein library, grouped by antigen localization within the parasite. The threshold for positivity was defined as an absorbance reading greater than the mean + 3 SD of 20 non-malaria-exposed control sera. Antibodies to AARP, SPATR and P12p were not included.
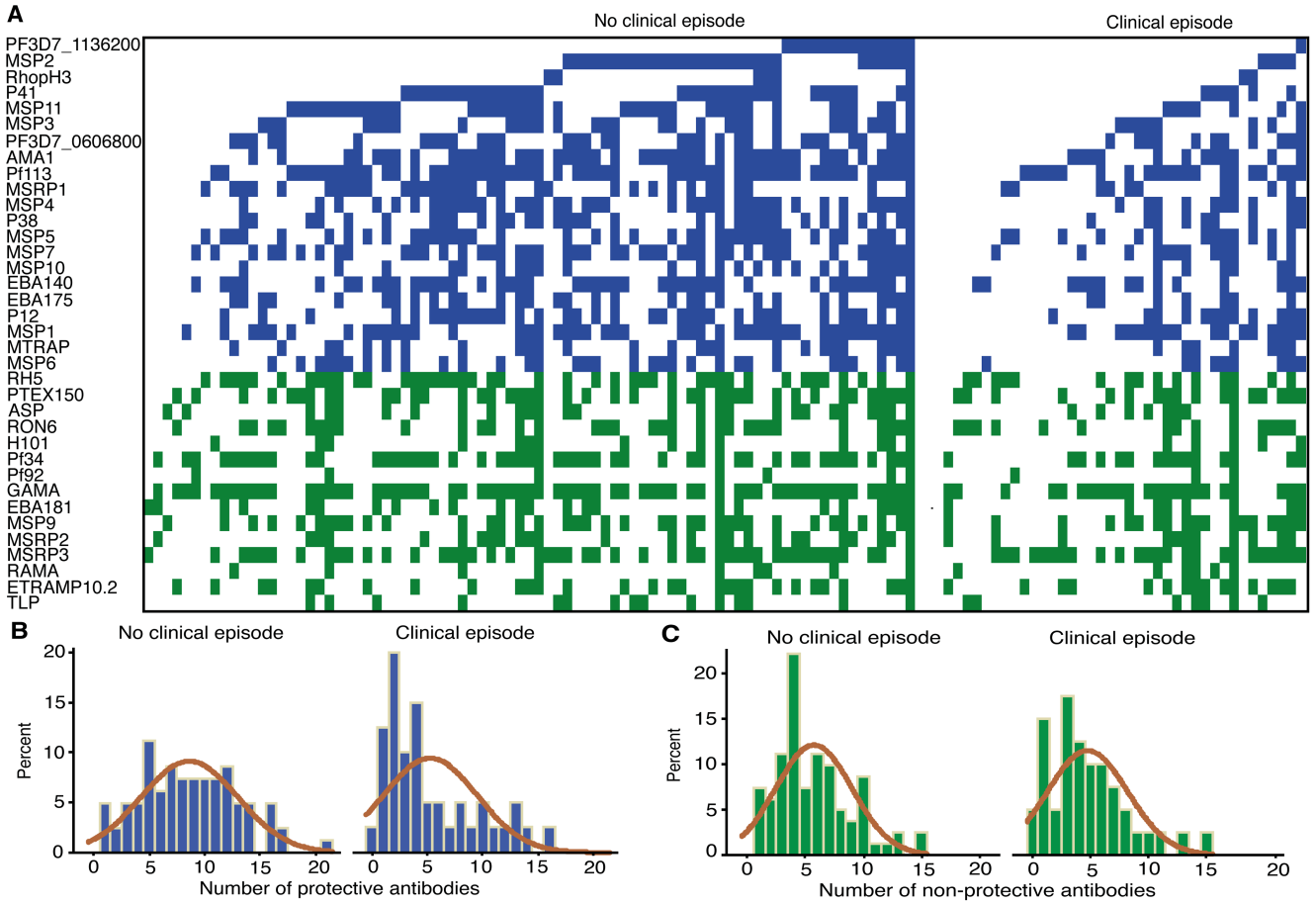
Fig. 2.

Unique antibody response profiles in protected children. **(A)** Each row represents an antigen ranked by individual protective efficacy (n = 36, Table 1); each column a parasite-positive child (n = 121). Children with no clinical episode of malaria (n = 80) and those with (n = 41) are sorted in increasing age from left to right within each category except for the first four non-protected children who did not have antibodies to the protective antigens studied. Protective responses are indicated in blue and non-protective in green. The breadth score was compared for **(B)** protective (blue) and **(C)** non-protective (green) antibodies in children with and without malaria. Normal density curves are shown in red.
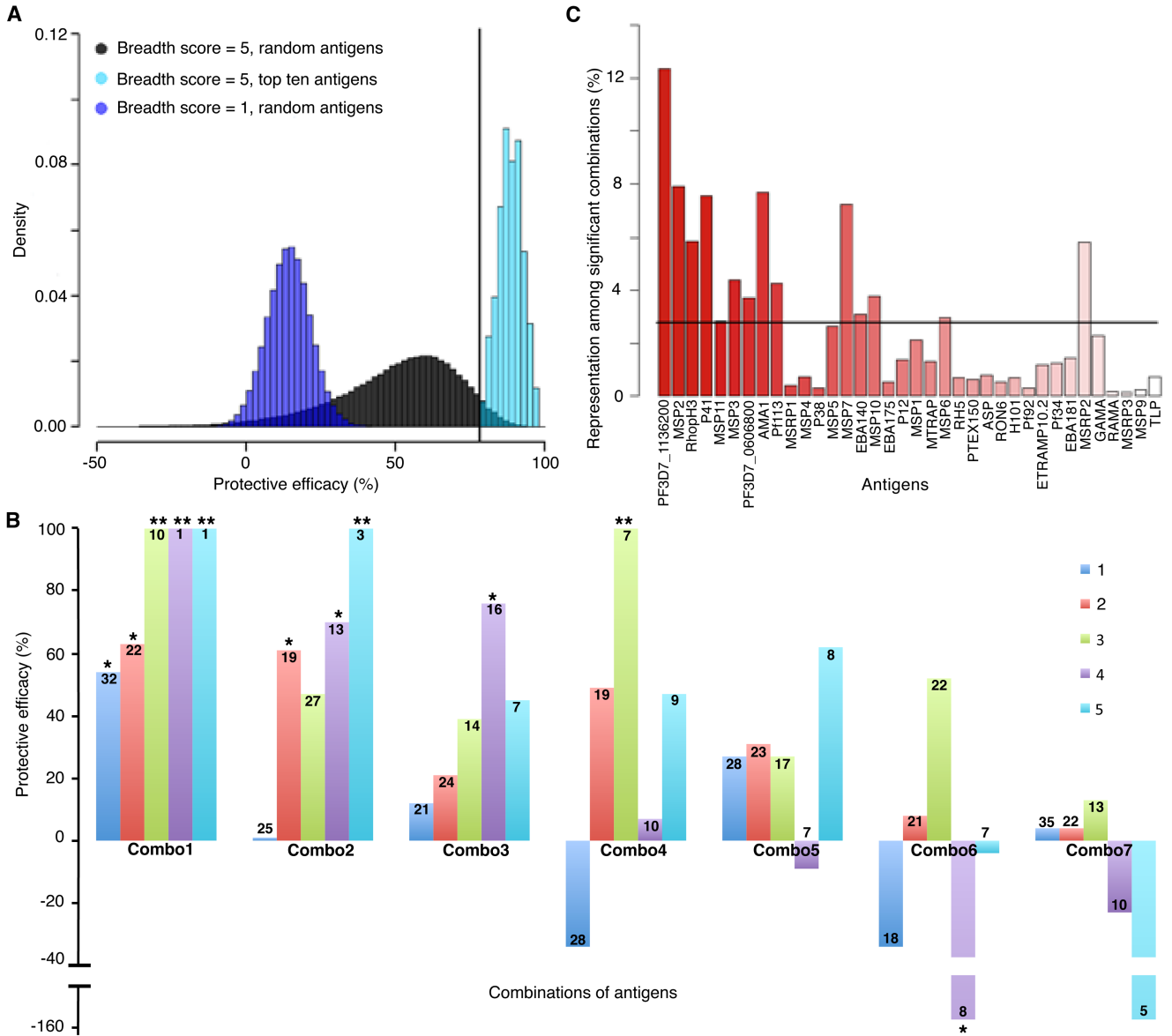
Fig. 3.

Increasing breadth of responses to combinations of the most protective antigens is strongly associated with protection from malaria. **(A)** Normalized distributions of protective efficacies for all five-way combinations of 36 antibodies (n = 376,992) when all five (black) or only one (dark blue) responses were present. All combinations restricted to the ten top-ranked antibodies (n = 252) in which all five responses were present were significantly (P < 0.05) more protective than random combinations (light blue, top 5% marked by vertical line). **(B)** In this example, "Combo1" contains the five individually most protective antibodies, "Combo2" the subsequent five etc. Combo7 contains the five least protective antigens, TLP was excluded. Numbers on bars indicate the percentage of individuals with corresponding antibody responses. **P <0.001; *P <0.05. **(C)** Representation of antibodies to individual antigens amongst combinations that showed significant breadth effects on

protection. Antibodies were ranked (x-axis) by their individual protective efficacy. Horizontal line indicates expected representation by chance alone.

Table 1.
Responses to individual antigens and protection from malaria

Associations between responses to individual antigens and the risk of malaria were analyzed using modified binomial regression models. Data are presented as Incidence Risk Ratios (IRR) with 95% Confidence intervals (95% CI), and are adjusted for age and reactivity to parasite schizont extract.

Rank	Antigen	Prevalence[a] (%)	IRR(95% CI)	P value	P value[b]
1	PF3D7_1136200[c]	12	0.17(0.03-1.07)	0.06	0.28
2	MSP2	27	0.33(0.14-0.76)	0.01	0.28
3	RhopH3[c]	7	0.38(0.14-1.04)	0.06	0.28
4	P41	32	0.47(0.22-1.01)	0.05	0.28
5	MSP11	38	0.51(0.25-1.04)	0.06	0.28
6	MSP3	31	0.57(0.32-1.00)	0.05	0.28
7	PF3D7_0606800	36	0.62(0.37-1.02)	0.06	0.82
8	AMA1	48	0.62(0.33-1.15)	0.13	0.48
9	Pf113[c]	63	0.64(0.41-0.99)	0.05*	0.28
10	MSRP1	36	0.64(0.41-0.99)	0.15	0.48
11	MSP4	41	0.65(0.36-1.16)	0.14	0.48
12	P38	35	0.69(0.37-1.27)	0.23	0.58
13	MSP5	43	0.70(0.41-1.19)	0.19	0.56
14	MSP7	46	0.70(0.39-1.27)	0.24	0.58
15	MSP10	47	0.71(0.34-1.48)	0.36	0.56
16	EBA140	25	0.71(0.43-1.20)	0.20	0.68
17	EBA175	31	0.74(0.34-1.59)	0.44	0.68
18	P12	40	0.76(0.45-1.27)	0.29	0.63
19	MSP1	51	0.77(0.46-1.29)	0.32	0.64
20	MTRAP[c]	27	0.78(0.40-1.52)	0.46	0.68
21	MSP6	34	0.80(0.45-1.42)	0.45	0.68
22	RH5[c]	48	0.81(0.47-1.41)	0.46	0.68
23	PTEX150[c]	48	0.91(0.58-1.41)	0.66	0.82
24	ASP[c]	29	0.95(0.48-1.88)	0.89	0.98
25	RON6[c]	39	0.96(0.58-1.57)	0.86	0.98
26	H101[c]	17	0.98(0.47-2.02)	0.95	0.98
27	Pf34[c]	70	1.01(0.57-1.78)	0.98	0.98
28	Pf92	53	1.02(0.31-3.36)	0.98	0.98
29	GAMA[c]	31	1.03(0.63-1.68)	0.90	0.72
30	EBA181	26	1.12(0.59-2.12)	0.74	0.88
31	MSP9	36	1.17(0.75-1.80)	0.49	0.63
32	MSRP2[c]	45	1.18(0.70-1.99)	0.54	0.68
33	MSRP3[c]	12	1.18(0.74-1.90)	0.49	0.80

Rank	Antigen	Prevalence[a] (%)	IRR(95% CI)	P value	P value[b]
34	RAMA[c]	57	1.19(0.60-2.37)	0.62	0.68
35	ETRAMP10.2[c]	8	1.30(0.79-2.15)	0.30	0.98
36	TLP[c]	18	1.34(0.68-2.65)	0.39	0.68

[a]Prevalence of high titer responses.

[b]Benjamini-Hochberg adjustment for false discovery rate - none of these associations remained significant.

[c]Seropositivity used for analysis (see Methods).