



Antibody Levels to *Plasmodium falciparum* Erythrocyte Membrane Protein 1-DBL γ 11 and DBL δ -1 Predict Reduction in Parasite Density

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ABSTRACT *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1) is a variant surface antigen family expressed on infected red blood cells that plays a role in immune evasion and mediates adhesion to vascular endothelium. PfEMP1s are potential targets of protective antibodies as suggested by previous seroepidemiology studies. Here, we used previously reported proteomic analyses of PfEMP1s of clinical parasite isolates collected from Malian children to identify targets of immunity. We designed a peptide library representing 11 PfEMP1 domains commonly identified on clinical isolates by membrane proteomics and then examined peptide-specific antibody responses in Malian children. The number of previous malaria infections was associated with development of PfEMP1 antibodies to peptides from domains CIDR α 1.4, DBL γ 11, DBL β 3, and DBL δ 1. A zero-inflated negative binomial model with random effects (ZINBRE) was used to identify peptide reactivities that were associated with malaria risk. This peptide selection and serosurvey strategy revealed that high antibody levels to peptides from DBL γ 11 and DBL δ 1 domains correlated with decreased parasite burden in future infections, supporting the notion that specific PfEMP1 domains play a role in protective immunity.

IMPORTANCE *Plasmodium* infection causes devastating disease and high mortality in young children. Immunity develops progressively as children acquire protection against severe disease, although reinfections and recrudescences still occur throughout life in areas of endemicity, partly due to parasite immunoevasion via switching of variant proteins such as *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1) expressed on the infected erythrocyte surface. Understanding the mechanisms behind antibody protection can advance development of new therapeutic interventions that address this challenge. PfEMP1 domain-specific antibodies have been linked to reduction in severe malaria; however, the large diversity of PfEMP1 domains in circulating parasites has not been fully investigated. We designed representative peptides based on B cell epitopes of PfEMP1 domains identified in membranes of clinical parasite isolates and surveyed peptide-specific antibody responses among young Malian children in a longitudinal birth cohort. We examined previous infections and age as factors contributing to antibody acquisition and identified antibody specificities that predict malaria risk.

KEYWORDS *Plasmodium falciparum*, *Plasmodium falciparum* erythrocyte membrane protein 1, proteomics

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With over half the world's population at risk, malaria remains one of the deadliest infectious diseases. *Plasmodium falciparum* is responsible for a large proportion of malaria infections and resulted in 409,000 deaths worldwide in 2019, primarily in young children with limited prior exposure (1). Individuals living in areas of high *P. falciparum* transmission develop immunity to malaria, resulting in fewer infections with less severe symptoms than in early in life; however, sterile protection is thought to be rarely achieved (2, 3).

The *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) family has ~60 allelic variants encoded in each parasite genome, is expressed on the surface of infected red blood cells (iRBCs), and plays an important role in malaria pathogenesis by mediating cytoadhesion (4, 5). PfEMP1 proteins share a relatively conserved structure consisting of an extracellular binding region with an N-terminal segment (NTS), multiple Duffy binding-like (DBL) and cysteine-rich interdomain region (CIDR) domains, occasional interdomains, a transmembrane (TM) segment, and a conserved acidic terminal segment (ATS) (6). The DBL and CIDR domains are classified into six (α , β , δ , γ , ϵ , and ζ) and five (α , β , δ , γ , and pam) subtypes, respectively, based on sequence similarities (6, 7). PfEMP1s are classified into four groups (A, B, C, and E) and two intermediate groups (B/A and B/C) (8). Further sequence analysis performed by Rask et al. on *var* genes from seven genomes resulted in the classification of tandem domains into conserved structural units called domain cassettes (DCs) (6). Multiple extracellular DBL and CIDR domains, as well as specific DCs, have been implicated in adhesion of iRBCs to receptors on the host endothelium, which allows parasites to sequester in the vasculature of specific organs, obstruct blood flow, and prevent splenic clearance (4, 5).

PfEMP1s are displayed on the iRBC surface and are thus a prime target of the host immune response. PfEMP1s are highly polymorphic and undergo clonal antigenic variation. As the host mounts an immune response to one PfEMP1, iRBCs expressing a different PfEMP1 expand, enabling recrudescence and repeated infections to occur (9). The host immune response yields antigen-specific antibodies to each new infection, leading to a growing repertoire of PfEMP1 antibodies over time. However, some parasites survive within the host due to sequential expression of PfEMP1 variants not yet targeted by the existing repertoire of host antibodies (10, 11).

Previous studies have demonstrated that parasites infecting young children and children with severe malaria have more conserved PfEMP1 variants (12–15). Hence, the identification of conserved PfEMP1 variants may be crucial to determine targets of protective antibodies and putative vaccine candidates. Multiple studies have utilized real-time PCR to identify transcript levels of PfEMP1 domains of interest (14, 16–21). This approach associated upregulation of DC8 and DC13 transcripts with severe malaria, using parasite samples from children with severe malaria, as well as parasite lines selected for adhesion to brain endothelial cells (15, 17, 22, 23). Previous studies that evaluated humoral immune responses to PfEMP1 domains relied on genomic data from either clinical parasite isolates or laboratory parasite lines to identify and select PfEMP1s to investigate sera reactivity toward specific PfEMP1 domains (24–28). The majority of previous studies examined antibody levels during and after infection (14, 16–21, 24, 25), while fewer related PfEMP1 antibody levels to protection from severe or clinical malaria (27–29).

Here, we describe a new approach to identify PfEMP1 targets of protective antibodies, utilizing proteomic analysis of clinical parasite isolates. Our proteomic analysis previously identified the PfEMP1 repertoire in 31 clinical isolates collected from young Malian children that were followed from birth up to 5 years of age (30). We utilized this data set to (i) identify PfEMP1 domains expressed by clinical isolates and (ii) design a peptide library representing 11 PfEMP1 domains selected due to their identification in multiple child isolates, high peptide coverage, or domain cassette classification. Antibody levels to peptides were measured in plasma samples from young Malian children (6 to 43 months) enrolled in a longitudinal birth cohort, which allowed us to relate antibody responses to past infection history as well as future malaria risk.

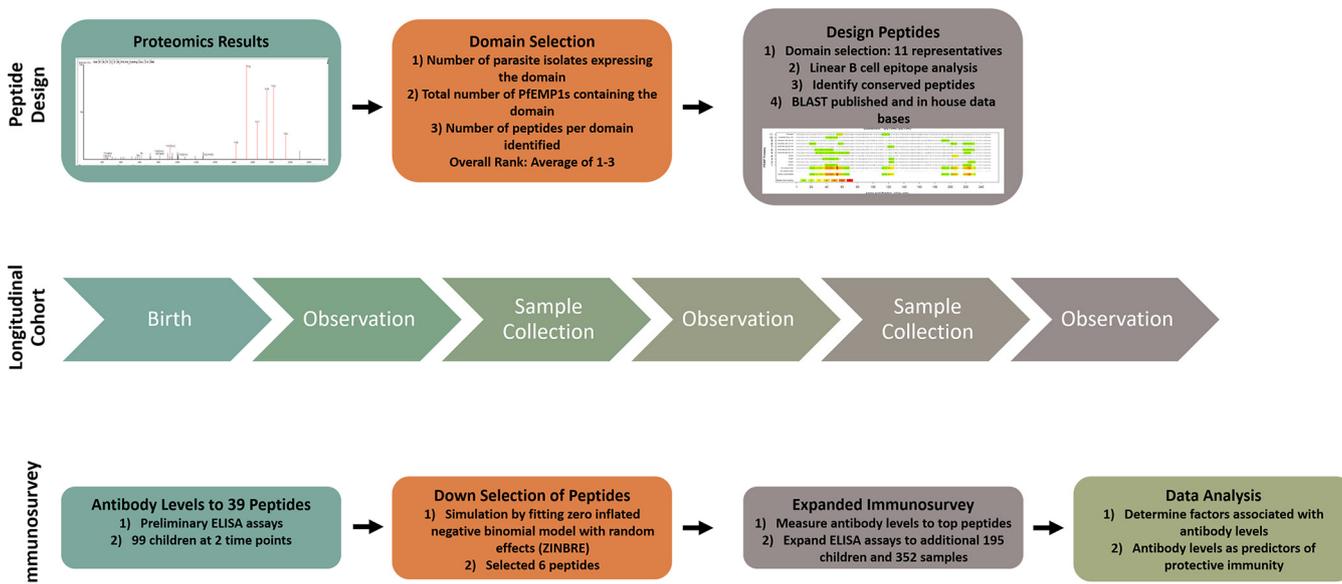


FIG 1 Experimental approach in the current study. Peptide selection began with the identification of PfEMP1 domains from clinical isolates in a previous proteomic analysis, followed here by down-selection of domains of interest, linear B cell epitope predictions, and peptide selection. The longitudinal cohort consisted of children from birth for up to 5 years of age who provided monthly blood smears during the malaria transmission season and during sick visits. Participants were monitored for illnesses between sample collections. The immunosurvey consisted of a preliminary analysis examining antibody levels toward 39 PfEMP1 peptides in 99 children at 2 time points. After down-selection of peptides through ZINBRE analysis of the initial cohort, antibody levels toward the selected 6 peptides were measured in the expanded cohort for a total of 294 children and 550 samples.

RESULTS

Previous work in our lab developed a new proteogenomic pipeline that combined high-resolution mass spectrometry, a custom protein sequence database, and *de novo* sequencing of peptides (30). This work improved the identification of PfEMP1 proteins in clinical parasite samples by expanding the publicly available *P. falciparum* databases and developing a novel algorithm for peptide alignment, referred to as the LAX algorithm, that matches peptides identified by *de novo* sequencing. The implementation of this approach enabled the identification of the PfEMP1 repertoire expressed by parasites infecting young children (3 to 62 months) and increased the number of PfEMP1 proteins identified in clinical samples compared to the standard method of database search of spectra identified by tandem mass spectrometry (30). PfEMP1 information collected in our previous proteomics study was applied to the current study as described in the experimental workflow depicted in Fig. 1.

PfEMP1 domains identified in this previous work were ranked by three criteria: (i) number of clinical samples from children that had proteomic evidence for a specific domain, (ii) number of PfEMP1 proteins containing the specific domain, and (iii) number of peptides in the domain identified by tandem mass spectrometry. The three rankings were averaged to determine the overall rank for all identified PfEMP1 domains (Table S1). The top four domains, DBL δ 1, CIDR β 1, CIDR α 3.1, and DBL β 3, were selected based on this ranking system. DBL δ 1 is the most common domain, present in 51.5% of PfEMP1s included in the database. Three other domains, DBL γ 11, DBL ζ 5, and DBL α 2, were chosen as relatively common domains identified in approximately 45%, 39%, and 23%, respectively, of Malian children in the proteomic study. This allowed comparisons of PfEMP1 domains with various frequency in clinical isolates as targets of acquired immunity. Two additional domains, DBL β 7 and DBL β 12, were selected because of their identification in 50% of isolates collected from children with severe malaria. The final two domains, CIDR α 1.1 and CIDR α 1.4, were included as representative domains found solely in PfEMP1s with DC8 and DC13 domain cassettes that were previously shown to be upregulated during severe malaria episodes (15, 17). The characteristics of the 11 domains are depicted in Table 1.

To identify peptides for the immunosurvey study, sequences of the 11 PfEMP1 domains identified in our previous proteomic analysis were subjected to linear B cell epitope prediction.

TABLE 1 Selection of PfEMP1 domains previously identified by proteomics analysis of clinical isolates from young Malian children

Domain	Domain cassette(s) ^a	Isolates (%) ^b	No. of PfEMP1s ^c	Mean (range) peptides per domain ^d
DBL δ 1	None	90.3	285	2.21 (2–7)
CIDR β 1	None	54.8	49	2.14 (2–4)
CIDR α 3.1	DC14	77.4	35	2.11 (2–4)
DBL β 3	DC4 (29%), DC5 (5%), or none (66%)	64.5	40	2.27 (2–5)
DBL β 7	DC5 (44%) or none (56%)	45.2	20	2.48 (2–3)
DBL α 2	DC8 (93%) or none (7%)	22.6	12	2.44 (2–7)
DBL β 12	DC8 (83%) or none (17%)	48.4	17	2.31 (2–8)
CIDR α 1.1	DC8	12.9	5	2.43 (2–4)
CIDR α 1.4	DC13	9.7	3	2.00 (2–2)
DBL ζ 5	None	38.7	12	2.68 (2–9)
DBL γ 11	None	45.2	16	2.12 (2–3)

^aDomain cassette classification according to Rask, et al. (6).

^bPercent of child isolates in which a specific domain was identified by mass spectrometry with a minimum of 2 peptides.

^cNumber of unique PfEMP1 proteins identified containing the domain of interest with a minimum of 2 peptides.

^dMean and range of peptides used for the identification of a specific domain within individual PfEMP1 proteins.

The B cell epitope-predicted peptides present in the highest proportion of the domains identified by proteomics were selected for each PfEMP1 domain. The resulting peptides are listed in Table S2. A BLAST search of published PfEMP1 sequences, as well as those identified in our lab previously, was conducted to verify that these peptides were unique for a given domain.

Immunosurvey analysis included a total of 550 plasma samples from 294 children. Three different time points were examined for one child, two time points were examined for 254 children, and one time point was examined for 39 children. The average age at the first sample collection was 15.1 months (range, 5.2 to 41.8 months), and the average age at the second sample collection was 26.9 months (range, 11.7 to 48.1 months). Malaria transmission in the study area is highly seasonal, with most infections occurring between July and December. To reduce the likelihood of concurrent parasitemia that can boost PfEMP1 antibodies, 70.2% and 16.4% of the samples were collected during the dry season or the beginning of malaria transmission season, respectively; only 5.1% of samples were collected at the time of infection detected by blood smear microscopy.

To relate specific PfEMP1 domains to acquired immunity, a two-step analysis was conducted. First, we measured antibody levels against 39 peptides corresponding to 11 unique PfEMP1 domains, using plasma samples from 99 children randomly selected from the group of the 294 children. Peptides selected in the first stage were used in the second stage with plasma samples from the remaining 195 children. The average age of the 99 children included in the initial analysis was similar to that of the whole group of 294 children. Age at the first sample collection was 16.3 months (range, 5.7 to 41.8 months), and the average age at the second sample collection was 29.4 months (range, 12.4 to 48.1 months).

In samples from 99 children, the frequency of a positive response to an antigen ranged from 13% to 74%. The lowest seropositivity rate was obtained with peptide 23 from the DBL γ 11 domain, and the highest positivity rates were obtained with peptides 10 and 14, corresponding to the DBL α 2 and CIDR β 1 domains, respectively (Table S3).

Zero-inflated negative binomial model with random effects (ZINBRE) simulations with discovery phase were performed to select peptides that were assessed in a larger analysis for their ability to predict a reduction in parasite density during subsequent infections (Fig. 2). The peptides with the largest coefficients and smallest standard error combinations were selected, including the following six peptides: peptide 37 (DBL δ 1), peptide 35 (DBL δ 1), peptide 24 (DBL γ 11), peptide 22 (CIDR α 1.4), peptide 33 (DBL β 3), and peptide 34 (DBL β 3). This is consistent with the domain expression ranking described above; DBL δ 1 and DBL β 3 were the most common domains, and DBL γ 11

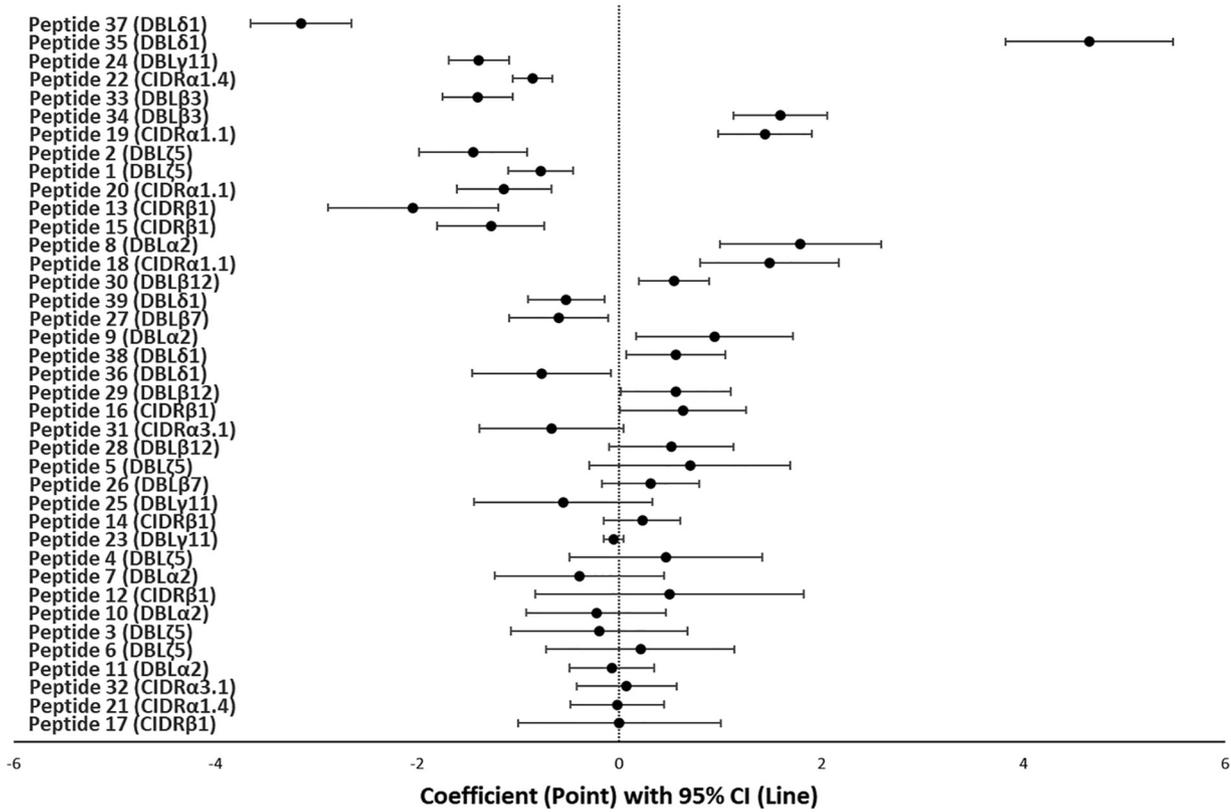


FIG 2 Down-selection of PfEMP1 domain peptides for large scale immunosurveillance. ZINBRE simulation depicting the coefficient size and the certainty of the estimate used for the down-selection of PfEMP1 peptides.

was a relatively common domain expressed in clinical isolates. We investigated further to determine whether antibodies toward these peptides predicted a reduction in parasite density by analyzing samples from the remaining 195 children in addition to the original 99 children.

Among the 294 children tested, the positivity rate ranged from 24% against peptide 33 of domain DBLβ3 to 67% against peptide 35 of DBLδ1. These rates were similar to those seen in the initial survey of 99 children, with 24% and 58% positive, respectively, in that subset (Table 2).

We fitted linear regression models to evaluate factors contributing to various antibody levels among the children. First, we investigated the relationship between age and antibody levels to PfEMP1s; no significant association was seen between a child’s age and their antibody levels specific to any of the six peptides in the 294 children and 550 plasma samples examined (data not shown). We then examined whether the number of previous malaria infections from the time of birth impacted domain-specific antibody levels in the 294 children. Antibody levels to all 6 peptides were positively associated with the number of previous infections, with peptide 24 from DBLγ11 showing the greatest increase in OD value of 0.008 for each infection (Table 3, $P < 0.0001$). These results demonstrate that a higher number of previous infections predicted higher antibody levels (Table 3).

We analyzed antibody responses in the 294 children to the six selected peptides for their ability to predict malaria infection severity. An ordinal logistic random-intercept mixed model was fitted to evaluate the effect of antibody response to the six peptides on parasite density during subsequent infections. This analysis exploits the longitudinal study design in which blood smears were routinely collected once a month during the malaria transmission season and during sick visits. Antibody levels were stratified by tertile, where the highest tertile was analyzed for the likelihood of predicting decreased parasite

TABLE 2 Seropositivity and antibody levels measured to down-selected peptides

Peptide	Seropositivity (%) ^a	Mean level (range) ^a
Pep 22 (CIDR α 1.4)	29	0.16 (0.03–1.19)
Pep 24 (DBL γ 11)	37	0.13 (0.03–0.92)
Pep 33 (DBL β 3)	24	0.11 (0.02–1.07)
Pep 34 (DBL β 3)	51	0.15 (0.02–1.32)
Pep 35 (DBL δ 1)	67	0.14 (0.02–1.80)
Pep 37 (DBL δ 1)	64	0.14 (0.01–1.31)

^aSeropositivity measured in 294 children and 550 serum samples.

burden in future infections. Parasite density was categorized into five levels of outcomes based on the distribution of parasite density in the cohort: (i) all 0s (meaning the child did not have a malaria infection during the follow up period), (ii) 1 to 7,475 parasites/ μ l, (iii) >7,475 to 37,475 parasites/ μ l, (iv) >37,475 to 112,475 parasites/ μ l, and (v) >112,475 to 800,000 parasites/ μ l. We observed that high antibody levels (top tertile) to peptide 24 (DBL γ 11) and peptide 37 (DBL δ 1) significantly reduced the likelihood of a high-parasite-density infection (>112,475 parasites/ μ l) (Table 4). The odds of having an infection with a parasite density of >112,475 parasites/ μ l were 0.58 (95% confidence interval [CI], 0.46 to 0.73) and 0.57 (95% CI, 0.45 to 0.73) for children with antibody levels in the top tertile, corresponding to peptides 24 and 37 (Table 4). On the other hand, the peptide representing CIDR α 1.4, which is part of DC13 previously associated with severe malaria, was predictive of a higher-density parasite infection. Specifically, the odds of having an infection with parasite density at the highest category was 1.99 (95% CI, 1.55 to 2.56) times more likely for a child with antibody levels in the top tertile versus lower tertiles, suggesting that antibodies toward this peptide may act as a biomarker of infection severity.

Lastly, we explored the association between peptide-specific antibody levels and the risk of severe malaria in future infections. Of the 294 children, 8.2% were diagnosed with severe malaria (following World Health Organization criteria) within 12 months of sample collection. In a logistic regression model adjusted for age and hemoglobin type, antibody levels to any of the six selected peptides did not predict a reduced likelihood of a severe malaria episode (data not shown).

DISCUSSION

The immense sequence diversity of PfEMP1 domains complicates the study of naturally acquired immunity toward this target as well as the use of this antigen for vaccine development. In the present study, we used proteomics data from young children in Mali to exploit the PfEMP1 repertoire expressed by clinical parasite isolates and prospectively relate the antibody response to these PfEMP1s with subsequent clinical presentations. We selected peptides representing 11 PfEMP1 domains (DBL δ 1, CIDR β 1, CIDR α 3.1, DBL β 3, DBL β 7, DBL α 2, DBL β 12, CIDR α 1.1, CIDR α 1.4, DBL ζ 5, and DBL γ 11) as targets for longitudinal serological studies in Malian children.

Our results show a wide range of positivity rates across PfEMP1s, confirming that some PfEMP1 peptides are more frequently recognized by naturally acquired antibodies than others. Thirteen peptides from four domains reacted with plasma samples of

TABLE 3 Associations between prior *Plasmodium falciparum* infections and PfEMP1 peptide antibody levels^a

Peptide	Coefficient	95% CI	P
Pep 22 (CIDR α 1.4)	0.006	0.002–0.009	7.03e-04
Pep 24 (DBL γ 11)	0.008	0.005–0.010	3.27e-08
Pep 33 (DBL β 3)	0.004	0.002–0.007	6.97e-04
Pep 34 (DBL β 3)	0.005	0.002–0.008	2.93e-03
Pep 35 (DBL δ 1)	0.005	0.001–0.008	7.51e-03
Pep 37 (DBL δ 1)	0.005	0.002–0.008	2.23e-03

^aAbbreviations: CI, confidence interval; P, Holms-adjusted P value. Model was adjusted for hemoglobin type.

TABLE 4 High antibody levels to DBL γ 11 and DBL δ 1 predict reduced parasite density in future infections^a

Antigen	OR	95% CI	P
Pep 22 (CIDR α 1.4)	1.99	1.55–2.56	3.18e-07
Pep 24 (DBL γ 11)	0.58	0.46–0.73	1.80e-05
Pep 33 (DBL β 3)	0.90	0.71–1.15	1.00e+00
Pep 34 (DBL β 3)	1.02	0.81–1.29	1.00e+00
Pep 35 (DBL δ 1)	1.08	0.84–1.39	1.00e+00
Pep 37 (DBL δ 1)	0.57	0.45–0.73	2.46e-05

^aAbbreviations: OR, odds ratio; CI, confidence interval; P, Holms-adjusted P value. Model was adjusted for hemoglobin type.

more than 50% of the children tested, illustrating the high rate at which young children recognize frequently expressed PfEMP1s. Plasma positivity rates reached as high as 74% for a specific peptide, highlighting the fact that most young children can be exposed to similar PfEMP1 variants early in life. For example, peptides corresponding to DBL α 2 and CIDR β 1 were among those commonly recognized by children's plasma. DBL α 2 is typical of PfEMP1s classified as groups B/A and B, while CIDR β 1 is typical of groups B and C. Previous studies described that antibody responses to group A and B/A PfEMP1s (including DBL α 2) develop early (2, 28), while this is the first report in which CIDR β 1 was also commonly recognized by young children.

Multiple factors such as age, malaria exposure, and transmission setting have previously been shown to influence antibody levels to a variety of malaria antigens (2, 31, 32). In the present study, we examined the role that age and malaria exposure play in the generation of PfEMP1 domain-specific antibodies. The number of previous infections was associated with increasing antibody levels to the six tested peptides representing four distinct PfEMP1 domains. This is similar to our previous study describing a positive association between the number of prior infections and plasma reactivity with the surface of iRBCs (33). Age was not significantly associated with antibody levels against peptides from four PfEMP1 domains—DBL δ 1, DBL γ 11, CIDR α 1.4, and DBL β 3. One limitation of this analysis is the age range of children included in this study; our study measured PfEMP1 antibody levels in children between the ages of 6 and 48 months, while previous studies utilized a wider age range than the current study (34, 35).

Previous studies have demonstrated the ability of older children to more effectively control parasite density during malaria episodes compared to younger children, thus more effectively controlling infection (36, 37). The ability to limit parasite density is one component in the process of acquiring protective immunity; therefore, we sought to relate antibody levels against the selected PfEMP1 peptides with parasite density in future infections. Children with antibody levels in the top tertile toward DBL γ 11 and DBL δ 1 peptides had lower parasite densities in future infections. The lower parasite densities in children with DBL γ 11 and DBL δ 1 peptide-specific antibodies could be explained by PfEMP1 antibodies inhibiting parasite adhesion to vascular endothelium resulting in splenic clearance. Alternatively, antibodies targeting these conserved peptides might mediate iRBC phagocytosis. Lastly, we cannot rule out the possibility that antibodies toward DBL γ 11 and DBL δ 1 peptides may act as a marker of an elevated immune status of the children with low parasite density infections. These proposed mechanisms remain to be determined in future studies. While 30% of DBL γ 11 belongs to group B PfEMP1s, 68.3% and 22.3% of DBL δ 1 belong to groups B and C, respectively. Reduced parasite density by antibodies targeting DBL γ 11 and DBL δ 1 is consistent with previous evidence suggesting that hyperparasitemia is related to upregulation of *var* genes encoding PfEMP1s of groups B and C (38). Conversely, a previous study reported that antibody responses to DBL β 3_{PF11_0521} were associated with a 37% reduced risk of high-density clinical malaria (29), whereas we report that antibodies to 2 peptides corresponding to DBL β 3 did not predict reduction in parasite density. This

difference may be due to methodological differences, such as the use of peptides versus an entire domain to measure antibody levels. However, a recent serologic study of PF11_0521 domain cassette DC13, as well as the DBL β 3_D4 domain, utilized peptides as well as full domains and observed significant convalescent antibody increases against both (39). This finding supports the use of peptides for immunosurveillance studies such as our own.

In a previous longitudinal study, antibody levels toward 17 DBL α variants correlated with protection from severe malaria (28). Here, antibody levels to the six down-selected peptides did not correlate with protection from severe malaria episodes. It is possible that the selected peptides did not include epitopes targeted by immune responses associated with protection from severe malaria; this finding could also be related to the young (6 to 48 months) age group examined in this study (40). Lastly, severe malaria includes several syndromes, including cerebral malaria and severe malarial anemia. Differences in the proportion of each syndrome among severe malaria cases may account for differences between studies. A potential weakness of this study was the omission of older children (5 to 10 years old); children in this longitudinal birth cohort were not followed into these later age ranges. Another potential weakness of the current study is the use of peptides as opposed to full-length proteins; however, the lack of protein conformation was overcome by using peptides containing predicted linear B cell epitopes. The wide range of positivity rates across PfEMP1 peptides detected in this study demonstrated the notion that being a B cell epitope-predicted peptide does not guarantee that children previously exposed to malaria possessed antibodies toward these peptides. In fact, some peptides demonstrated low recognition, such as peptide 33 with only 24% of children tested showing a positive response to the peptide. The novelty and strength of this study lie in the use of proteomics data to define PfEMP1 sequence variants expressed by parasites naturally infecting children in the community, as well as the use of a longitudinal cohort, which allowed for prospective analyses that assess antibody levels as predictors of future malaria severity.

In summary, PfEMP1 antibodies toward two specific peptides from the DBL γ 11 and DBL δ 1 domains were associated with decreased parasite burden, whereas antibodies to other PfEMP1 peptides were not. Additionally, we demonstrated the utility of proteomics tools to identify potential targets of naturally acquired antibodies, which can be applied in future studies to identify new targets of protective immunity. Overall, these findings contribute to our understanding of the acquisition of PfEMP1 domain-specific antibodies and have implications for developing therapeutics to prevent severe malaria infections.

MATERIALS AND METHODS

Study setting and study participants. Mothers and their children were enrolled in the Immuno-Epidemiology Project (IMEP) in Ouélessébougou, Mali (a region of intense seasonal malaria transmission) between September 2011 and May 2015, as previously described (41). The protocol was approved by the Institutional Review Board of the National Institute of Allergy and Infectious Diseases at the National Institutes of Health (ClinicalTrials.gov ID NCT01168271) and the Ethics Committee of the Faculty of Medicine, Pharmacy, and Dentistry at the University of Bamako, Mali. All study methods were performed according to the relevant guidelines and regulations. Written informed consent was obtained from parents or guardians of all study participants.

Measuring antibodies to PfEMP1 peptides by enzyme-linked immunosorbent assay. Membrane proteomics data of clinical isolates was used to select 11 PfEMP1 domains according to criteria described below. Domain sequences corresponding to each of the domains were subjected to linear B cell epitope prediction at tools.jeddb.org/bcell. B cell epitope-predicted peptides identified in the greatest number of PfEMP1s were selected for each domain. A BLAST search of published PfEMP1 sequences, as well as those identified in our lab previously, was conducted to verify that the selected peptides were unique for a given domain. Peptides were synthesized by GenScript with a bovine serum albumin (BSA)-conjugated Cys residue added to the N terminus.

Antibodies to the synthetic PfEMP1 peptides were measured by enzyme-linked immunosorbent assay using established methods with modifications (17). Antigens were coated at a 2 μ g/ml concentration, and plasma was diluted 1:80 (41). Eight healthy U.S. donors never exposed to malaria were used as negative controls, and a plasma pool from adult Malian women was used as a positive control. All plasma samples were tested in duplicate. Antibody levels were expressed as the optical density (OD) at 405 nm after subtracting background reactivity with BSA in phosphate-buffered saline (PBS). A sample

was classified as antibody-positive if the OD was greater than the mean plus two standard deviations of the reactivity observed with malaria-naïve samples.

Statistical analysis. Antibody levels to all 39 peptides in 99 children at 2 time points were used in a zero-inflated negative binomial model with random effects (ZINBRE) simulation to down-select peptides for further examination in additional children. The 39 peptides were ranked by the absolute value of the z-statistic, which encompasses a joint measure of the coefficient size and the certainty of the estimate. The top six peptides were selected, and antibody levels toward these peptides were measured in an additional 195 children and a total of 550 serum samples.

To determine if antibody levels to the six down-selected peptides predict reduced infection severity (measured as a decrease in parasite density), ordinal logistic regression and predicted probability calculations were performed. Due to the longitudinal nature of the study, analyses were corrected for age at the time of sample collection. Infections occurring within 12 months of plasma collections were included in the analysis. The model was also adjusted for malaria infection at the time of blood sampling and hemoglobin type (AA, AS, and AC).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

TABLE S1, XLSX file, 0.03 MB.

TABLE S2, DOCX file, 0.02 MB.

TABLE S3, DOCX file, 0.01 MB.

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