International Journal of Infectious Diseases 153 (2025) 107804

Contents lists available at ScienceDirect

International Journal of Infectious Diseases

journal homepage: www.elsevier.com/locate/ijid

Wider antibody breadth against multiple *Plasmodium falciparum* antigens is associated with reduced risk of malaria in a transmission hotspot in southern Ghana

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ARTICLE INFO

Article history:
Received 23 August 2024
Revised 23 December 2024
Accepted 25 January 2025

Keywords:
Malaria
Antibodies
Antibody breadth
Merozoite protein
Elisa

ABSTRACT

Objectives: Naturally acquired immunity to malaria results from repeated infection with *Plasmodium* parasites. However, identifying immune correlates of immunity against febrile malaria is quite challenging. Here we investigated antigenic targets of malaria protective antibodies in populations residing a malaria transmission hotspot in southern Ghana.
Method: We enrolled 973 children, aged 6 months to 12 years, in southern Ghana out of which 211 were infected at least once with *Plasmodium falciparum* in a 50-week longitudinal cohort study. Total IgG levels in baseline plasma samples were determined using indirect ELISA.
Results: We found a significant association between higher IgG levels to MSP3 (adjusted *P*-value [aP] = 0.0002), GLURP-R2 (aP = 0.0026), MSP DBL2 (aP = 0.004) and N-MSP3 (aP = 0.002), and protection from febrile malaria. A negative association between higher antibody levels to MSP3, GMZ2, GLURP-R2 and MSPDBL2 and parasite density was also observed. Wider antibody breadth was associated with protection against febrile malaria and single, compared to multiple malaria episodes.
Conclusions: Specific antibody levels and breadth of responses against multiple *P. falciparum* surface antigens protect against febrile malaria, parasitaemia and multiple malaria episodes. This data supports the development of multivalent vaccines targeting *P. falciparum* surface antigens in high malaria endemic settings.

Background

Plasmodium falciparum malaria results in significant morbidity and mortality, especially in children below 5 years of age and pregnant women in endemic areas. In Ghana, malaria is endemic across the country with seasonal variations across the country [1]. The Greater Accra region in southern Ghana is generally considered a low transmission area with about 2% of children below 5 years being malaria-positive by microscopy compared to other regions of the country [2]. Notwithstanding, hotspots of high malaria transmission areas exists within the Greater Accra region [3].

Individuals in malaria-endemic areas are thought to acquire protective immunity due to repeated infections [4]. Naturally, acquired anti-malarial immunity may be mediated by a broad repertoire of parasite-specific antibodies working in concert with immune cells [5]. The exact antigenic targets of malaria protective

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https://doi.org/10.1016/j.ijid.2025.107804

antibodies have not been clearly defined and remain a high priority in malaria vaccine studies. Indeed, antibodies against different stages of the parasite, and proteins at different locations of the merozoite have been associated with protection against clinical malaria [6]. However, the purported protective anti-malarial antibodies in some studies [6,7] are often associated with contrary findings [6] in others, thus underscoring the need for conducting studies that will help identify the most important targets of naturally acquired malaria immunity. Several malaria antigens are therefore increasingly being identified as targets of protective antibodies and are being tested as potential markers for vaccine development [8].

Different antibody-mediated mechanisms are involved in protection from malaria [9–12]. Protection from antibodies is a result of the acquisition of antibodies from a repertoire of different strains of parasites over time. While most studies that have associated protection from malaria with higher antibody levels relied on single or few antigens, individuals in malaria-endemic areas are repeatedly challenged with different strains of parasites, probably introducing new variants of parasite antigens with each bite. It is also known that wider antibody breadth (the number of malaria antigens to which an individual responds) is likely to be more protective against febrile malaria [7,13]. It has also been suggested that breadth is associated with multiclonal infections [13], and transmission intensity [14] indicating that persistent parasitaemia is necessary to maintain wider antibody breadth and hence protection from malaria.

With multiple malaria antigens identified as targets of protective immunity, the rate and pattern of acquisition of antibodies to these targets are expected to differ in an antigen-specific manner [15]. While this may be true, antibodies may also be co-acquired or elicited at similar levels due to the antigen's similarity of function, proximity and location on the merozoite.

It is not clear whether a vaccine design strategy that incorporates multiple antigens to boost a broader antibody breadth would be more effective. Here, we assessed anti-malarial antibody levels and antibody breadth against a panel of ten (10) *P. falciparum* antigens in children aged 12 years and below and determined how this impact the risk of febrile malaria in a transmission hotspot in southern Ghana.

Methods

Study site and participants

This longitudinal cohort study was undertaken at Danfa and the surrounding communities (Otinibi, Kweiman and Adoteiman) in the La-Nkwantanang Madina Municipality and two communities in the adjoining Ga-East Municipality (Ayi Mensah and Ghana Flag) of the Greater Accra Region in southern Ghana. While Greater Accra may be considered a low malaria transmission zone, the study communities were identified to be hotspots during the period of the study [3]. A total of 973 children were recruited into the study after informed consent was obtained from parents and guardians. Those included in the study were asymptomatic children aged between 0.5 and 12 years, and permanently resident in the communities. Those excluded at baseline were children with symptoms of clinical malaria (axillary temperature, >37.5°C with malaria parasites) and any other acute or chronic illness. Participants received free antimalarial care throughout the follow-up period. The children were categorized into infected and uninfected based on whether they had parasites at any time during the 50-week follow-up period. The categorization was done using microscopy. A detailed description of the study area, design and participants has been described elsewhere [16].

Sampling

Four millilitres (4 mL) or 0.5 mL of whole blood was collected into EDTA tubes at enrolment for older children and those below 2 years respectively. The blood was centrifuged at 750 × *g* and plasma was collected and stored at −20°C for antibody analysis. Blood smears were made and stained with Giemsa to determine parasitaemia. Blood haemoglobin and sickle cell status were determined using Hemocue 200, and metabisulphite methods respectively. The enrolled children were actively followed up weekly for 50 weeks from February 2016 to January 2017 with monthly routine parasitaemia assessment by blood smear. Field assistants based in the communities paid weekly visits to the homes of the child participants to take their temperature. This passive surveillance system helped in detecting malaria early for immediate referral to the health care centre for treatment. Those with fever or a history of fever (axillary temperature, >37.5°C) within the previous 24 h and any parasitaemia were referred to the Danfa Health Centre for clinical confirmation and treatment.

Estimation of blood smears

Thick and thin blood smears were prepared and examined by an experienced microscopist. Counting was done at 100× magnification of the thick film. Parasite density was estimated by counting parasites against 500 white blood cells (WBCs). The estimated number of parasites/μL was determined using a standard assumed leucocyte count of 8000/μL. A slide was deemed negative for Plasmodium after observing about 100 high-powered fields.

Determination of total IGG levels

Total IgG titres against 10 recombinant antigens representing 8 different anti-malarial proteins were measured in plasma samples of the study children. Out of the 10 antigens studied, 8 were expressed at the blood stage (MSP3, NMSP3, CMSP3, MSPDBL2, SERA5, GLURP-R0 and GLURP-R2, MSP1-19 K), one was a blood stage fusion protein (GMZ2), and one was a pre-erythrocytic stage antigen (CSP). The proteins analysed in this study were expressed as described previously [17].

Total IgG levels were determined using an indirect ELISA protocol adopted from Adu et al. [18]. Antibody units (AU) were estimated from the OD values for the test samples using the standard curves generated with each plate with a four-parameter Excel-based logistic curve-fit, the Auditable Data Analysis and Management System for ELISA (ADAMSEL FPL b040, Ed Remarque). Plates with an $R^2 < 0.97$ or samples with a coefficient of variation from the duplicate measurements greater than 30% were retested.

Data normalization

Data were normalized for inter-plate and day-to-day variation by dividing the test sample (antibody titre) on each plate by the positive control of the assay plate. Then mean of the positive control of all the plates was calculated. This was then multiplied by the total mean positive control for all the plates to obtain the normalized AU values using the formula:

$$\frac{Each\ Sample}{Plate\ Positive\ control} \times Mean\ positive\ control\ for\ all\ plates$$

Data analysis

Individuals were grouped into infected and uninfected based on whether they had parasites by microscopy at least once during the 50-week follow-up period. Those with detectable parasites by microscopy at one time during the monthly assessment were

Table 1
Characteristics of the study population.

Characteristics	*N*	Cumulative incidence	Child month at risk	Malaria	Rate per 100 child-at-risk
Age group (years)					
Below 5 yrs	54	75.9 (62.4-86.5)	388.5	41	10.6 (7.8-14.3)
Above 5 yrs	157	51.0 (42.9-59.0)	1335.4	80	6.0 (4.8-7.5)
Gender					
Female	90	60 (49.1-70.1)	697.5	54	7.7 (5.9-10.1)
Male	121	55.4 (46.1-64.4)	1026.6	67	6.5 (5.1-8.3)

named the exposed group and all those without parasites throughout the observation period the unexposed. The unexposed were excluded from this analysis. The exposed group was further categorized based on whether they had febrile malaria (an axillary temperature of ≥37.5°C, and any other symptoms of clinical malaria such as chills and fever) or were asymptomatic. Children who had only parasites during the follow-up period but with no febrile symptoms were deemed to be protected and were called the "protected" group. Children with at least one episode of febrile malaria during the follow-up period were deemed to be in the "susceptible" group. Comparison between log-transformed antibody levels between protected and susceptible groups was performed by the Student's *t*-test. To determine antibodies that were co-acquired, a correlation matrix was drawn and a coefficient of 0.7 and above were deemed co-acquired. Logistic regression was fitted to investigate the association of antibody responses and risk factors associated with febrile malaria.

To calculate breadth scores, antibody levels were categorized based on quartiles and assigned 0 for lowest, 1 for the second, 2 for third and 3 for the highest quartile. The scores were summed up for the 10 antigens for each individual to generate breadth scores. Mann–Whitney and Kruskal–Wallis tests were used to determine differences in breadth between protection status, and malaria episodes.

Results

Study demographics

A total of 848 children successfully completed the 50-week longitudinal follow-up of which 211 were considered definitively exposed to malaria based on microscopically detected *P. falciparum* infection at any visit during the study period. Of these, 129 children had febrile malaria and were categorized as "susceptible" while 98 children did not present any symptoms of clinical malaria and were categorized as "protected." There was no difference in the distribution of males and females between the protected and the susceptible groups ($P = 0.99$). Children in the protected group were significantly older (8.7 years) than those in the susceptible group (6.7 years, $P < 0.0001$). Of the 129 susceptible children, 116 (89.9%), 12 (9.3%) and 1 (0.8%) had one, two and three episodes of malaria, respectively (Supplementary Table 1). The cumulative incidence of malaria was higher in children who were 5 years and below compared to those above 5 years (Table 1).

Higher antibody levels are associated with age, protection and reduced number of clinical malaria episodes

To identify the molecular targets of naturally acquired immunity, levels of IgG antibodies against a panel of 10 recombinant *P. falciparum* antigens were measured at baseline by ELISA. In general, levels of total IgG against all the antigens increased with age. However, when the study population was categorized into two, all antigens tested significantly increased with age in the susceptible group, while only MSP3, GLURP-R0, GLURP-R2 GMZ2, C-MSP3 and N-MSP3 increased significantly with age in the protected group (Supplementary Figure 1). Age was grouped into two (those 5 years and below and those above 5 years) the mean levels of log-transformed antibody titres in the participants above 5 years were significantly higher in protected individuals compared to the susceptible ones, except for C-MSP3, GMZ2, SERA5, CSP and MSP1-19 K ($P > 0.05$). Only levels to MSPDBL2 in those 5 years and below showed higher levels in the protected children (Figure 1).

Table 2
Association between antibody levels and protection from febrile malaria.

Antigens	aOR	95% CI	*P*-value
MSP3	0.60	0.46-0.79	0.0002
GMZ2	0.88	0.67-1.15	0.36
GLURP-R2	0.76	0.63-0.91	**0.0026**
GLURP-R0	0.88	0.76-1.01	0.072
C-MSP3	0.83	0.62-1.12	0.23
MSPDBL2	0.54	0.36-0.82	**0.004**
CSP	0.79	0.52-121	0.27
SERA5	0.85	0.61-1.19	0.35
MSP1-19K	0.76	0.52-1.10	0.14
N-MSP3	0.56	0.38-0.82	**0.002**

Odds ratios (aOR), 95% confidence intervals (95% CI) and *P*-values for each antigen were obtained with multiple logistic regression models adjusting for age. Significant *P*-values are in bold font.

Further, to adjust for the potential confounding effect of age on antibody responses and protection against febrile malaria, a logistic regression for each antibody response, adjusting for age was fitted to determine responses associated with protection from febrile malaria. We found higher antibody responses to MSP3 (adjusted *P*-value [aP] = 0.0002), GLURP-R2 (aP = 0.0026), MSPDBL2 (aP = 0.004) and N-MSP3 (aP = 0.002) were associated with protection from febrile malaria, indicating their role in immune protection (Table 2).

Concomitant baseline parasitaemia is negatively correlated with antibody levels

Since baseline parasitaemia was different between protected and susceptible children, we determined the association between concomitant baseline parasitaemia and antibody levels in each febrile malaria status group (protected and susceptible respectively) in a correlation analysis. In the protected group, higher antibodies to MSP3, GMZ2, GLURP-R2 and MSPDBL2 negatively correlated with parasite density ($P < 0.05$ in all cases). Antibodies to the other antigens tested did not show a significant correlation with parasite density. In the susceptible group, none of the antigens tested significantly correlated with parasite density (Figure 2).

Antibody co-acquisition patterns

Some merozoite antigens are targets of immune responses and antibodies to these antigens may be co-acquired due to the similarity of their functions, their location on merozoite surface, and the complexes they form. A correlation matrix of antibody co-acquisition pattern was thus generated for the antigens tested. This was to determine which antibody levels correlated with other anti-

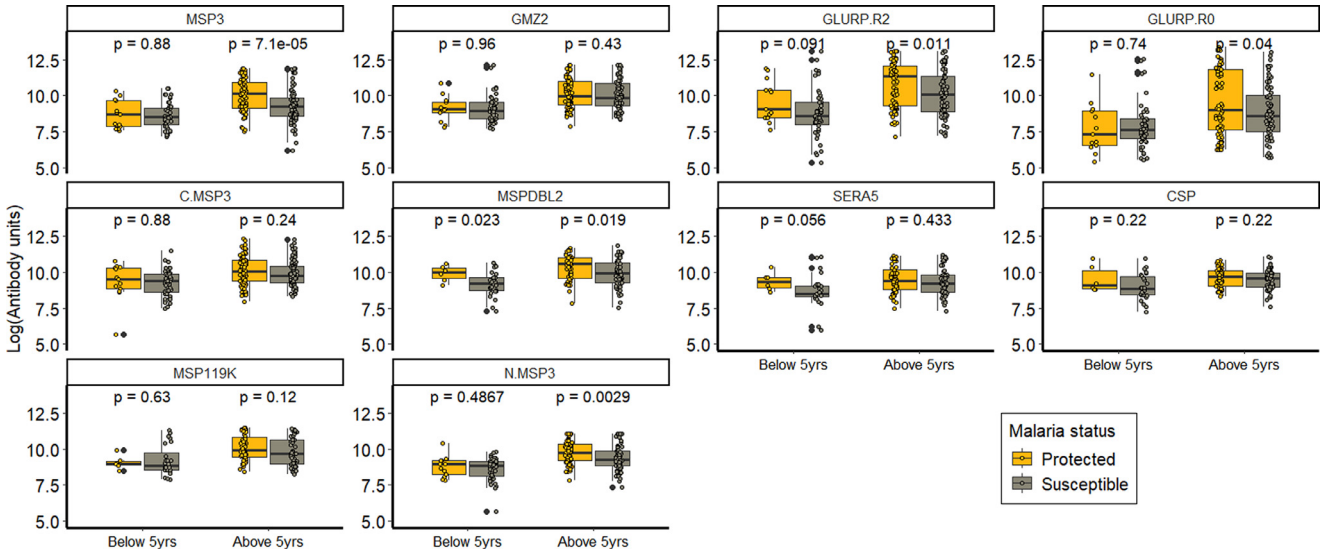

Figure 1. Antibody levels to malaria multiple antigens. The differences in antibody levels to the 10 antigens tested between protected and susceptible children. Antibody levels were log-transformed and the mean differences between protected (gold boxes) and susceptible (grey boxes) were compared using the Student's *t*-test. The round dots show the number of participants in each group. Statistical significance is indicated by asterisks, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

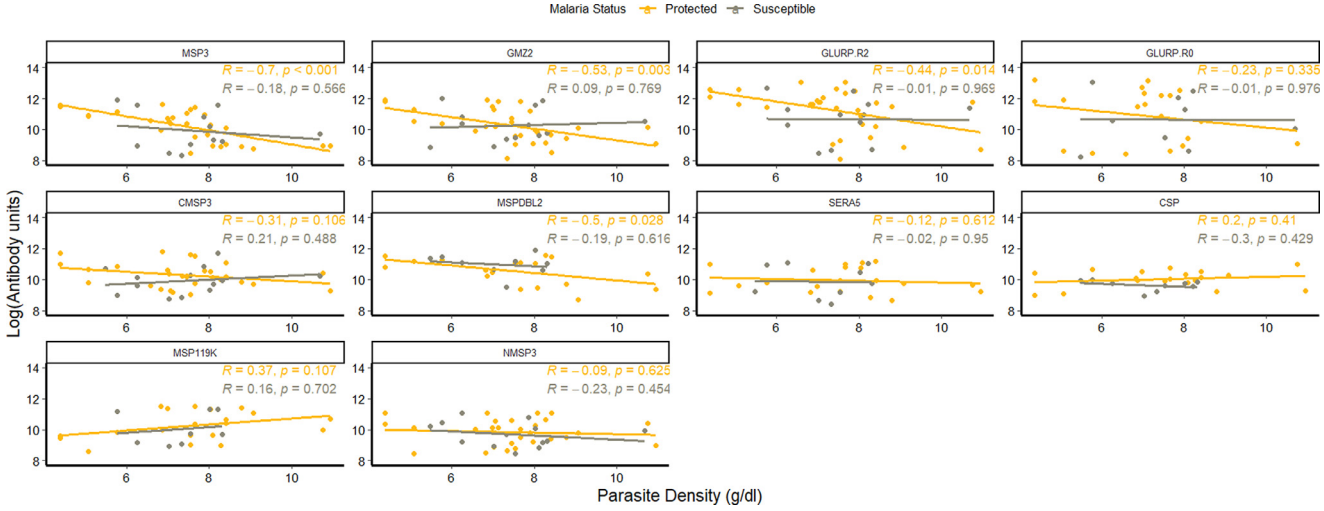

Figure 2. Infection status and antibody responses. Children were categorized as protected and susceptible and antibody responses were correlated with *Plasmodium falciparum* infection status. A correlation plot was made between the protected and susceptible groups, with correlation coefficient and *P*-values calculated for each plot. Baseline parasitaemia is presented in this graph. The *Y*-axis represents the log-transformed antibody units and the *X*-axis represents log-transformed baseline parasitaemia. The gold lines and dots represent children in the protected group while the grey lines and dots represent the susceptible groups.

bodies. We define a strong co-acquisition with a correlation coefficient of 0.7 or higher. A strong positive correlation was observed between MSP3—GMZ2 (0.76) MSP—GLURP-R2 ($r = 0.72$), MSP3-GLURP-R0 (0.70), GMZ2—GLURP-R0 ($r = 0.74$), GLURP-R2—GLURP-R0 ($r = 0.79$), indicating these antibodies may be co-acquired (Figure 3).

Antibody breadth and immunity to clinical malaria

The breadth of antibody response, which is defined here as the number of different antigens recognized by an individual, is associated with protection from clinical malaria [7,13]. We derived a score for each antibody for each study participant based on quantiles derived from the 10 antigens tested. Antibody breadth in this study was between zero (0) and 30 with a breadth of 30 being the highest. Generally, higher antibody breadth was associated with increasing age ($\beta = 13.41$, CI 95% = 9.33-17.49, $P < 0.0001$). Other covariates like gender, and bed net usage did not influence antibody breadth. Notably, higher antibody breadth to the studied antigens was significantly associated with protection from febrile malaria (Figure 4a) and this was more often observed in older children having higher breadth. Children who had no episode of malaria during the follow-up period had significantly higher antibody breadth compared with those with one or multiple malaria episodes ($P = 0.003$) (Figure 4b) indicating the importance of higher antibody breadth in immunity to malaria.

Discussion

This study investigated the association between the level and breadth of antibody responses to selected pre-erythrocytic and blood-stage malaria antigens and protection, and antibody co-acquisition patterns to multiple malaria antigens in hotspots of transmission in southern Ghana. The study found that higher antibody levels and breadth were associated with protection from

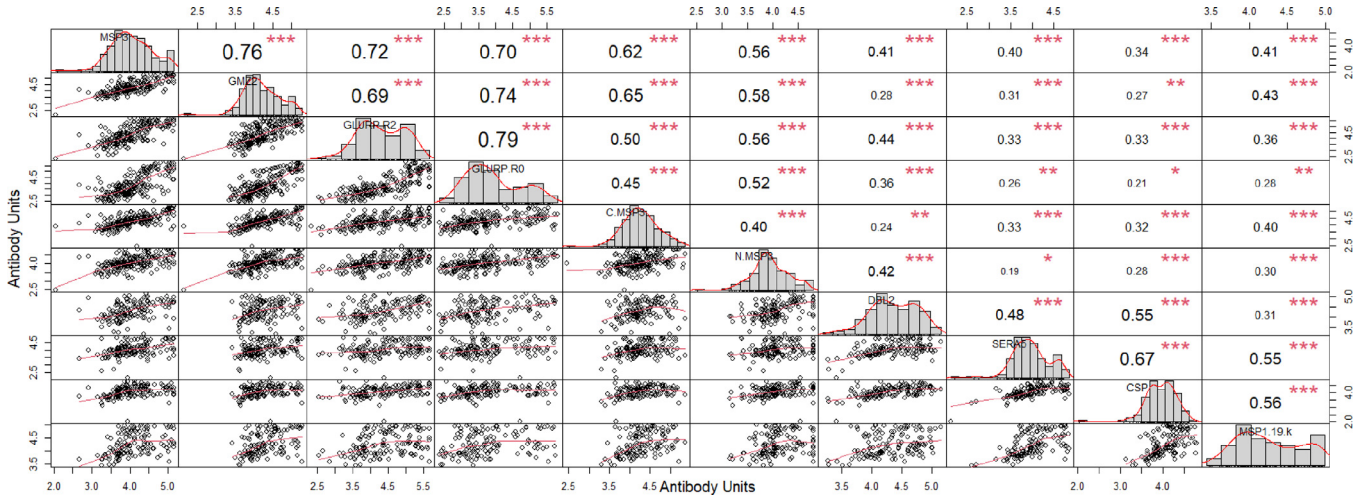

Figure 3. Correlation matrix for antigen-specific antibody co-acquisition in younger and older children. A correlation matrix showing antibody co-acquisition pattern in 10 antigens tested. The figure shows correlation plots in the bottom left with the distribution of antibody levels in the histogram in the middle diagonal and their corresponding coefficient of correlation *P*-values as stars (Spearman correlation test) in the upper right part. *X* and *Y* axes are antibody levels to the 10 antigens correlating against each other.

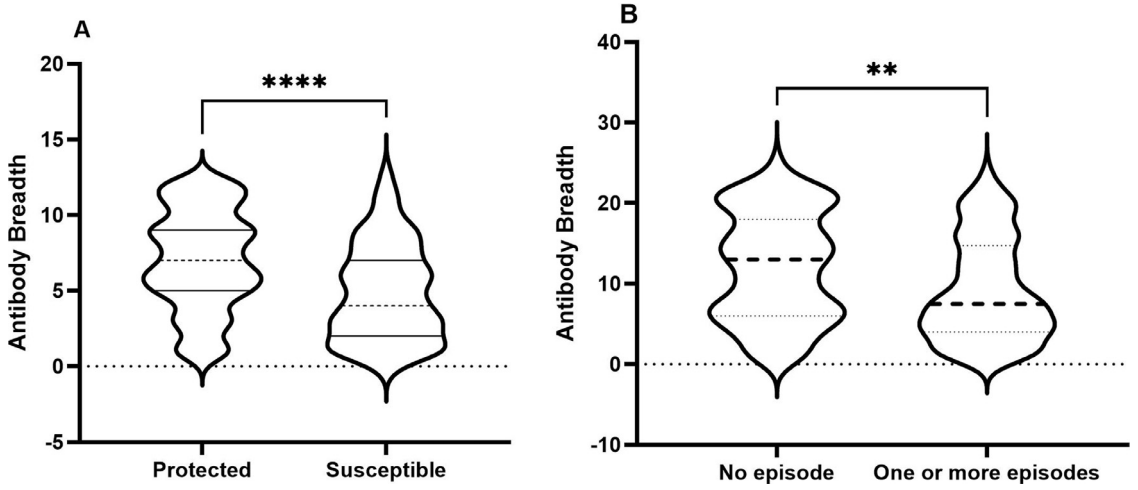

Figure 4. Antibody breadth is associated with protection from febrile malaria. The *Y*-axis of the violin plots represents antibody breadth, and the *X*-axis represents (a) protection status and (b) the number of clinical malaria episodes. *P*-values were was by student *t*-test.

febrile malaria and that multiple malaria episodes were significantly associated with reduced antibody breadth.

The antigens used in this study: (MSP3, NMSP3, CMSP3, MSPDBL2, SERA5, GLURP-R0, GLURP-R2, GMZ2, CSP and MSP1-19k) have been described as important targets associated with protective immunity in different epidemiological settings [19–22] and some have undergone clinical trials.

Our data showed a significantly higher cumulative malaria incidence (the probability of having malaria-specific infection throughout the follow-up period) in children under 5 years. Children above 5 years had a significantly reduced risk of malaria. Though bed net usage among the younger and older children was not different, the reduced risk of contracting malaria in the older children could be due to their matured immune system, and the quality of antibodies over time since antibodies are acquired with age.

This study found significantly high baseline parasitaemia in the protected children compared to susceptible children, suggesting immune tolerance to circulating parasites. High parasite density has been associated with high polyclonal infection in Senegalese children [23] and may protect from febrile disease. Asymptomatic carriage of multiple clones has been associated with protection from clinical malaria [24], though other studies have linked high parasite density to the pathophysiology of malaria with some authors pointing to its potential use as a marker for malaria morbidity and mortality [25]. Our finding of higher antibodies to MSP3, GMZ2, GLURP-R2 and MSPDBL2 correlating with reduced parasite density [26] in the study participants indicate their importance in reducing parasitaemia and thus increased protection from malaria. This correlation could also result from recent exposure. Antibodies to these antigens, MSP3, GMZ2, GLURP-R0 and MSPDBL2 are all found to be associated with protection using different mechanisms [11,19].

Immune-epidemiological studies have reported an association between high antibody levels and reduced risk of clinical malaria [19,27]. To determine which antibody responses are associated with reduced number of clinical malaria episodes, a regression analysis correcting for age was done for each antibody response. A significant association between protection from febrile *P. falciparum* malaria and high MSP3, N-MSP3, GLURP-R2 and MSPDBL2 antibody levels was found, suggesting their importance as immune targets in this low malaria transmission cohort. In another study on the same cohort, opsonizing and growth inhibitory (GI) antibodies were found to protect against febrile malaria independently. This suggests that the antibodies associated with protec-

tion in the current study may be exerting their protective roles through mechanisms that include GI and opsonic phagocytosis [28] and other antibody-mediated mechanisms. Indeed, antibodies to MSPDBL1 and MSPDBL have been found to act by inhibiting parasite growth and by opsonization of merozoites in other studies [19]. Also, GLURP-specific antibodies mediate opsonic phagocytosis [11], and MSP3, and the polymorphic N-terminal, NMSP3 elicit an immune response through the antibody-dependent cellular inhibition mechanism [29]. Though antibodies to CSP were not significantly associated with protection in this cohort, antibodies to the N-terminal, central-repeat and C-terminal regions of CSP, have been reported to fix complement to eliminate sporozoites [30].

Most studies on anti-malarial antibody levels and protection from the disease include in their analysis of the "protected" group, all individuals without detectable parasites [6]. What is however not known in this type of analysis is whether these individuals were exposed to the parasite. The strength of this study is because as a longitudinal study, the children in the "protected" group in this cohort were only those who had detectable parasites but no clinical symptoms during the follow-up period.

The pattern of acquisition of anti-malaria antibody with different parasite proteins is influenced by age, transmission intensity, exposure, host immunity, environmental and other host genetic factors [31]. Some merozoite proteins are co-acquired due to the complexes they form, or similarity of their functions. Using a correlation matrix, the study found differential patterns of IgG responses to multiple malaria antigens. The correlations observed in the study may be an indication that these antibodies were similarly produced to fight infection simultaneously. The very similar responses to GMZ2, MSP3, GLURP-R0 could also be explained by the fact that GMZ2 is a fusion protein composed of MSP3 and GLURP [32].

Naturally acquired immunity to malaria is mediated by responses to multiple *Plasmodium* proteins. These responses known as the breadth have been proposed to protect better than responses to single proteins and that a certain threshold of antibody response must be met to achieve this protection [7,33]. Higher antibody breadth was associated with age and protection in this cohort reflecting the accumulation of a repertoire of parasite antigens as one ages. Parasitaemia also contributed to a higher breadth indicating probable carriage of multiple parasites. A limitation is the lack of data on the specific clones the children were carrying, which was not the focus of this work, but it has been shown that antibody breadth increases with the number of clones [13]. One key challenge to vaccine and malaria elimination is finding that individuals may transition between symptomatic and asymptomatic states within a year in areas with low malaria transmission [34].

The data presented in this study supports the multivalent vaccine approach where different antigens are incorporated into a single vaccine, and that more antibodies are co-acquired in children under 5 years than older children.

Acknowledgments

This work was supported by the Ministry of Foreign Affairs of Denmark (DFC file no.14-P01-GHA) and administered by Danida Fellowship Centre. The study sponsors had no involvement in the study design, in the collection, analysis and interpretation of data; in the writing of the manuscript; and in the decision to submit the manuscript for publication. The authors would like to thank the participants, parents/guardians for participating in the study. We also acknowledge the technical assistance of Miss Sophia Eyia-Ampah of the Immunology Department, NMIMR and the Senior Physician Assistant in charge, and the laboratory staff of Danfa Health Centre, respectively, in sampling. We also acknowledge the Functional Immunogenetics field assistants for their support during sampling.

Ethical approval

Ethical approval for this study was granted by the Ghana Health Service Ethics Committee, Ministry of Health, Ghana (GHS-ERC-11/01/15), and the Institutional Review Board of the Noguchi Memorial Institute for Medical Research (NMIMR), University of Ghana, Accra (NMIMR-IRB 022/14-15). Consent was given by the parents and guardians of children before they were enrolled in the study.

Availability of data

The datasets analysed in this study are available from the corresponding author on reasonable request.

Author contributions

Conceived and designed the experiments: EKB, DD, MT, KAK and BA. Performed the experiments: EKB, QAI, EOY and SK. Analysed the data: EKB, KAK, DD and BA. Contributed reagents/materials/analysis tools: SKS and MT. Wrote the manuscript: EKB, KAK, FKNA, BA and MT. All authors contributed to the article and approved the submitted version.

Declarations of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this article.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ijid.2025.107804.

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