High Sporozoite Antibody Titers in Conjunction with Microscopically Detectable Blood Infection Display Signatures of Protection from Clinical Malaria

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Immuonepidemiological studies typically reveal slow, age-dependent acquisition of immune responses against Plasmodium falciparum sporozoites. Naturally acquired immunity against preerythrocytic stages is considered inadequate to confer protection against clinical malaria. To explore previously unrecognized antisporozoite responses, we measured serum levels of naturally acquired antibodies to whole Plasmodium falciparum sporozoites (Pfspz) and the immunodominant (NANP)5 repeats of the major sporozoite surface protein, circumsporozoite protein, in a well-characterized Kenyan cohort. Sera were sampled at the start of the malaria transmission season, and all subjects were prospectively monitored for uncomplicated clinical malaria in the ensuing 6 months. We used Kaplan–Meier analysis and multivariable regression to investigate the association of antisporozoite immunity with incidence of clinical malaria. Although naturally acquired humoral responses against Pfspz and (NANP)5 were strongly correlated (p < 0.0001), 37% of Pfspz responders did not recognize (NANP)5. The prevalence and magnitude of antisporozoite responses increased with age, although some high Pfspz responders were identified among children. Survival analysis revealed a reduced risk of and increased time to first or only episode of clinical malaria among Pfspz or (NANP)5 responders carrying microscopically detectable Plasmodium falciparum (Pf) parasitemia at the start of the transmission season (p < 0.03). Our Cox regression interaction models indicated a potentially protective interaction between high anti-Pfspz (p = 0.002) or anti-(NANP)5 (p = 0.001) antibody levels and microscopically detectable Pf parasitemia on the risk of subsequent clinical malaria. Our findings indicate that robust antisporozoite immune responses can be naturally acquired already at an early age. A potentially protective interaction between high anti-Pfspz and anti-(NANP)5 antibody levels and microscopically detectable Pf parasitemia on the risk of subsequent clinical malaria.

Abbreviations: Pf, Plasmodium falciparum; Pfspz, whole Plasmodium falciparum sporozoites; CSP, circumsporozoite protein (major sporozoite surface protein); (NANP)5, immunodominant icosapeptide repeats of Pf circumsporozoite protein; CRR, central repeat region of circumsporozoite protein; MSP1-3, merozoite surface proteins 1-3; AMA1, apical membrane antigen-1; EBA-175, erythrocyte binding antigen 175.
INTRODUCTION

Malaria transmission by Plasmodium-infected Anopheles mosquitoes results in at least 200 million malaria cases every year, and the majority of disease burden is caused by Plasmodium falciparum (Pf) infections in children (1).

During a blood meal, an infected mosquito typically injects fewer than 40 motile sporozoites into the human skin (2). After injection, a proportion of sporozoites reaches a blood capillary by active intradermal migration, breaches the endothelial cells of the blood vessel, and is passively transported to the liver through the blood circulation. Following a massive replication phase in hepatocytes, tens of thousands of liver merozoites are released to initiate asexual parasite propagation in erythrocytes, the only phase in the parasite life cycle causing malaria symptoms.

When experimentally inoculated in repeated high doses, arrested, but metabolically active, Plasmodium sporozoites are able to elicit protective and often lasting and sterile immune responses against homologous challenge in humans (3–5). Because the preerythrocytic phase of the life cycle is clinically silent, humans can be inoculated with high doses of sporozoites, providing a tantalizing rationale for preerythrocytic malaria vaccine approaches (6). Plasmodium sporozoites are considered immunologically silent in natural infections, because they are mostly inoculated in small numbers and are exposed to the immune system only for a brief period (7, 8).

Naturally acquired immune responses against whole Pf sporozoites (Pfspz) typically reveal age- and transmission-dependent increases in prevalence (9–11). Strong humoral responses are directed against the central repeat region (CRR) of the major sporozoite surface protein, circumsporozoite protein (CSP) (12–17). The CRR is composed of a varying number of NANP repeats, and a single Pf sporozoite inoculation is sufficient to trigger anti-NANP antibody responses (15, 18). However, in virtually all populations studied, a varying proportion of naturally exposed individuals remains unresponsive to NANP repeats, even after repeated exposures (19).

In addition to NANP repeats, immune responses can also target regions in the amino- or carboxy-termini of CSP (20–22) or additional sporozoite antigens, such as thrombospondin-related anonymous protein (23–25) or sporozoite threonine–asparagine-rich protein (26, 27). However, no single preerythrocytic antigen has yet been identified that serves as a correlate of naturally acquired protection against infection or clinical disease. Rather, immunoenumerological studies show that the breadth of preerythrocytic antigens recognized by an individual is associated with a lower risk of and an extended time to reinfection (28, 29).

In this study, we analyzed humoral immune responses against Pfspz in a cohort of naturally exposed individuals in Kenya. We compared prevalence and titers of antibodies against whole Pfspz and NANP repeats of CSP and assessed potentially protective contributions of antisporozoite immunity against clinical malaria. We show previously unrecognized high anti-Pfspz responses in children, which are not captured by (NANP)5–enzyme-linked immunosorbent assay (ELISA), and demonstrate potentially protective interactions between high levels of anti-Pfspz responses and asymptomatic Pf slide positivity at the time of serum collection on the risk of clinical malaria.

MATERIALS AND METHODS

Study Design and Cohort

This was a follow-up study from seroepidemiological cohort studies previously conducted in Chonyi village (Kilifi County, Kenya) (30–34). At the time the study was conducted, this region experienced year-round moderate malaria transmission (~20–100 infective bites/person/year), with a short rainy season from November to December and an extended one from June to August causing two seasonal peaks in transmission (35). Details of the cohort and study area have been published previously (36). For this study, serum samples of 514 individuals aged 1.6 months–82 years were analyzed from the cross-sectional bleed conducted in October 2000, immediately prior to the start of the malaria transmission season. At the time of this cross-sectional bleed, no individual experienced clinical malaria episodes, but 37% carried a microscopically detectable asymptomatic Pf blood stage infection, as assessed by thick and thin blood films. Asymptomatic infection was defined as any microscopically detectable parasitemia in an afebrile individual. We measured serum levels of antibodies to (i) whole Pfspz and (ii) the (NANP)5 repeats of CSP. Active weekly surveillance and passive case detection for development of clinical malaria during a 26-week follow-up permitted a retrospective analysis of potential associations of humoral antisporozoite immune responses with incidence of clinical malaria, which was defined according to the following age-specific criteria: for infants <1-year old and for older children and adults (≥15 years old), an axillary temperature of ≥37.5°C plus any parasitemia; for children aged 1–14 years, an axillary temperature of ≥37.5°C, combined with a parasitemia of ≥2,500 parasites/μl (36).

Generation of Pf Sporozoites

Plasmodium falciparum gametocyte cultures were derived from high parasitemic (5–8%) asexual PfNF54 parasites, cultured in pooled human O+ red blood cells and RPMI-HEPES medium, supplemented with hypoxanthine, sodium carbonate, t-glutamine, and pooled, non-immune, heat-inactivated human A+ serum. The asexual Pf culture was diluted to 1% parasitemia into culture flasks containing 6 ml prewarmed medium, resulting

Keywords: malaria, Plasmodium falciparum, sporozoites, humoral immunity, clinical malaria, protective immunity
Detection of Anti-Pfspz Antibodies by Immunofluorescence Assay (IFA)

For fixation, sporozoite slides were dipped in cold acetone for 1 min and rehydrated in PBS for 30 min. All incubation steps were done in a dark humid chamber for 1 h at 37°C. Blocking was done with PBS/3% BSA. After washing with PBS/0.5% BSA, human sera were added in serial dilutions ranging from 1:50 to 1:24,300 in PBS/0.5% BSA, followed by extensive washing with PBS/0.5% BSA. AlexaFluor488 goat anti-human IgG (1:500; Invitrogen) and Hoechst (1:1,000; Invitrogen) were used as secondary fluorescent antibody and nuclear stain, respectively. Slides were sealed after addition of Fluoromount (Southern Biotech) and analyzed by fluorescence microscopy. For the negative control, one well per slide was stained with pooled sera from 20 unexposed individuals from the United Kingdom. The pooled negative control sera did not deliver any IFA signal detectable by visual examination even at the lowest dilution. Cohort sera displaying a comparable response were considered to be IFA negative or unresponsive to Pfspz. For the positive control, one well per slide was stained with an anti-PfCSP antibody (2A10) (37) or a Kenyan adult hyper immune serum. All IFA slides were randomized prior to microscopic screening to avoid biased evaluation, and arbitrary sample batches were repeated to exclude day-to-day variation of the assay. The reciprocal antisporozoite end titer was defined as the last dilution at which immunofluorescence was detectable by microscopy.

Detection of Serum Antibodies by ELISA

To quantify serum antibody titers against the CRR of CSP, a standard ELISA was performed using the (NANP)5-icosapeptide (Peptides and Elephants, Potsdam, Germany). A peptide stock solution (1 µg/µl) was prepared in sodium carbonate buffer (pH 9.3) and stored at −80°C. For experiments, the stock solution was diluted to a concentration of 400 ng/100 µl. F96 MaxiSorp plates (Nunc) were coated with peptide overnight at 4°C (400 ng/well). After extensive washing with PBS/0.05% Tween, unspecific binding was blocked with 100 µl casein (Thermo Scientific Blocker). After an additional wash, human sera (1:200 in 100 µl casein) were incubated for 1 h at 37°C. After extensive washing, plates were incubated for 1 h at 37°C with horseradish peroxidase-conjugated rabbit anti-human IgG (Dako Ltd.) at a 1:3,000 dilution in 100 µl casein. Following washing, bound human IgG was measured with OPD substrate (Sigmafast). The reactions were stopped with 50 µl 2 M H2SO4, and absorbance was read in an ELISA plate reader at 492 nm. To assess the variability of the ELISA assay, all sera were tested in duplicates, and the coefficient of variation was calculated for each paired serum sample. Samples that varied by ≥20% were tested again. A hyper immune Kenyan adult serum was used in duplicates on each ELISA plate as a positive control. The ratio of mean optical densities (ODs) of the positive serum samples was computed to assess plate to plate and day-to-day variability. For ratios ranging between 1.1 and 1.3 or 0.7 and 0.89, OD values were multiplied to adjust the data to a reference plate. For ratios <0.7 or >1.3, the experiment was repeated. Twenty sera from unexposed individuals from the United Kingdom were used as negative controls. The mean OD plus three SDs from all negative controls was determined as a cutoff for (NANP)5 seropositivity. ELISAs to measure serum levels of antibodies against Pf schizont extract and blood stage merozoite antigens were described previously (31).

Statistical Analysis

A Spearman’s correlation was run to assess the relationship between ELISA ODs and IFA end titers. The Kruskal–Wallis test was applied for non-parametric analyses of variance in levels of antisporozoite antibodies across age groups. The Pearson’s χ2 test was used to determine the statistical significance of differences between proportions. The Mann–Whitney test determined statistically significant differences in antibody titer between two subsets of individuals. A binomial general linear model was used to calculate risk ratios in univariate analysis. Kaplan–Meier survival analysis and the log-rank Mantel–Cox test were used to compare the incidence of clinical malaria in distinct subsets of the cohort.

For multivariable analyses, we used a fractional polynomial Cox regression model that best predicts the time to first or only episode of clinical malaria for individuals with different levels of antisporozoite antibodies and predicted the log relative hazard against increasing levels of antibodies. Stepwise regression was conducted to select the most parsimonious set of covariates that are best predictors of clinical malaria. The variables with least significant coefficients (p > 0.2) were dropped one at a time until the final model was obtained. Likelihood ratio tests were run to compare the fit of the models with and without each variable. The final model was adjusted for the following confounders: age, plasma levels of antibodies against blood stage antigens [merozoite surface proteins 1-3 (MSP1-3), apical membrane antigen-1 (AMA1), erythrocyte binding antigen 175 (EBA-175), and Pf schizont extract], and malaria exposure, or unweighted local malaria prevalence index, as measured by the proportion of infected individuals within a 1 km radius of the index case. The malaria exposure index was calculated over the 6-month follow-up period using weekly slide data for acute episodes only (active case detection) and using the cross-sectional slide data (38).

To examine the effect of interactions between baseline antisporozoite antibody levels and microscopically detectable Pf parasitemia on malaria risk, hazard ratios were computed through a Cox regression model with and without one-, two-, or three-way interactions between antisporozoite antibody levels,
microscopically detectable *Pf* infection, and malaria exposure index. Models with and without interactions were compared using the log likelihood ratio test.

The fractional polynomial Cox regression model and the interaction model are presented using anti-(NANP) ODs as a continuous variable. To reduce the skewness while maintaining the direction of association, anti-(NANP) OD values were transformed to negative inverse values before running the models.

Data were compared for statistically significant differences at a significance level of $\alpha = 0.05$. The Bonferroni correction was applied to control for the increasing familywise error rate through multiple comparisons. Four families of statistical tests were defined based on the common dependent variable and null hypothesis tested. For each family composed of $k$ significance tests, a new critical value was defined as $\alpha/k$. Individual tests within these families were considered significant at $\alpha$ tests, a new critical value was defined as $\alpha / k$.

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RESULTS

Incidence of Clinical Malaria

Overall, the risk of developing at least one episode of clinical malaria during 6 months of follow-up decreased with age ($p < 0.0001$), with an overall rate of 0.4 episodes/person/year (Table S1 in Supplementary Material).

Asymptomatic *Pf* blood stage infection at the beginning of the malaria transmission season was detected in 37% of individuals, although this proportion was higher in younger age groups (3–15 years) (Table S1 in Supplementary Material). One or more malaria episodes were reported in 22% of blood film-positive individuals (Table S1 in Supplementary Material). This proportion was significantly lower (11%) among subjects who were blood film negative at the beginning of the observation period ($p = 0.001$) (Table S1 in Supplementary Material). This group may include parasite-free individuals and subjects carrying *Pf* parasites below the detection threshold by microscopy.

Naturally Acquired Antibody Responses against *Pf* Sporozoites

A substantial fraction (63%) of the Chonyi sera recognized air-dried, whole *Pf*spz by IFA (Figure 1). The proportion of anti-(NANP) responders was substantially lower (27%), and all (NANP)5-reactive individuals except three were also classified as *Pf*spz responders (Figure 1). A large subset (36%) of sera recognized *Pf*spz but not (NANP)5. We observed an overall correlation of increased intensity of (NANP)5 responses in individuals with high anti-*Pf*spz titers ($p < 0.0001$) (Figure 1). However, even among the sera that produced the highest *Pf*spz reactivities by IFA, some remained (NANP)5-negative (Figure 1).

Stratification of the Chonyi cohort revealed age-dependent acquisition of anti-*Pf*spz antibodies ($p < 0.0001$). The age-related increase in prevalence was particularly accentuated among blood film-negative individuals ($p < 0.001$) (Figure 2A). This was in marked contrast with blood film-positive individuals ($p = 0.72$), where prevalence of anti-*Pf*spz responses reached 68% in children younger than 6 years (Figure 2A). In individual age groups, the proportion of *Pf*spz responders was consistently higher among blood film-positive individuals, although this difference was only significant among individuals aged 7–8 years ($p = 0.001$) (Figure 2A).

Despite the presence of several high responders among children, anti-*Pf*spz antibody titers significantly correlated with age ($p < 0.001$) (Figure 2B). This was significant among blood film-negative individuals ($p = 0.001$), but not blood film-positive individuals ($p = 0.08$). Differences in median end titer between the two cohort subsets were non-significant in all age groups.

Acquisition of antibodies against the *P*CS*P* repetitive region (NANP5) occurred in an age-dependent manner ($p < 0.0001$). Anti-(NANP5)5 antibody acquisition correlated with age among blood film-negative individuals ($p < 0.001$) and blood film-positive individuals ($p = 0.002$) (Figure 2C). The proportion of (NANP5)5-reactive sera was comparable between blood film-positive individuals and blood film-negative individuals.
There was an age-dependent increase in levels of anti-(NANP)$_3$ antibodies, but this increase was not statistically significant ($p = 0.19$) (Figure 2D). Levels of anti-(NANP)$_3$ antibodies were comparable between blood film-negative and blood film-positive individuals.

**Correlation of Antibodies against Pf Sporozoites with Malaria Incidence**

**Univariate Analysis**

In univariate analysis, there was no marked difference in the cumulative risk of clinical malaria depending on $P$fspz responder status ($p = 0.19$) (Table S2 in Supplementary Material). However, the risk of clinical malaria was significantly reduced among $P$fspz responders who were blood film positive at the beginning of follow-up ($p = 0.015$). This association was not observed among blood film-negative individuals.

Overall, individuals with a positive antibody response to (NANP)$_3$ displayed a significantly lower risk of clinical malaria during follow-up ($p = 0.006$), although this association was only significant among blood film-positive individuals ($p = 0.03$) (Table S2 in Supplementary Material).

**Survival Analysis**

According to Kaplan–Meier analysis, antibodies against whole $P$fspz did not have any impact on clinical malaria incidence
among individuals who were blood film negative at the time of serum collection \((p = 0.85)\) \(\text{(Figure 3A)}\). In marked contrast, low \((1:50–1:100)\), moderate \((1:300–1:2,700)\), and high \((≥1:8,100)\) anti-Pfspz titers correlated with a reduced risk of and increased time to first (or only) episode of clinical malaria among blood film-positive individuals \((p = 0.014)\) \(\text{(Figure 3B)}\).

\((\text{NANP})_5\) responses did not correlate with the incidence of febrile malaria among blood film-negative individuals \((p = 0.1)\) \(\text{(Figure 3C)}\), but \((\text{NANP})_5\) seropositivity was significantly associated with the reduced risk of and increased time to clinical malaria among blood film-positive individuals \((p = 0.025)\) \(\text{(Figure 3D)}\).

### Multivariate Analysis
To minimize bias through potential confounders, we developed a multivariable polynomial Cox regression model including the three critical covariates (i) age, (ii) malaria exposure, and (iii) plasma levels of antimerozoite stage antibodies. Increasing levels of anti-Pfspz antibodies were independently associated with a significantly increased risk of experiencing clinical malaria during follow-up among blood film-negative individuals \((p = 0.006)\) \(\text{(Figure 4A)}\). The model showed a similar trend among anti-(NANP)\(_5\) responders, with a significantly increased hazard of clinical malaria among blood film-negative individuals \((p = 0.007)\) \(\text{(Figure 4C)}\). Among blood film-positive individuals, increasing levels of anti-Pfspz antibodies were associated with a decreased risk of clinical malaria during follow-up, although this association was not significant after the Bonferroni correction was applied \((p = 0.035)\) \(\text{(Figure 4B)}\). Anti-(NANP)\(_5\) antibody levels were not associated with risk of clinical malaria during follow-up among blood film-positive individuals \((p = 0.99)\) \(\text{(Figure 4D)}\).

### Interaction Analysis
The relationship between antisporozoite antibodies, microscopically detectable \(Pf\) parasitemia, and the incidence of clinical malaria was further analyzed through a Cox regression interaction model assessing (i) the effect of antisporozoite antibodies among individuals who were blood film negative at the time of serum collection \((p = 0.85)\) \(\text{(Figure 3A)}\). In marked contrast, low \((1:50–1:100)\), moderate \((1:300–1:2,700)\), and high \((≥1:8,100)\) anti-Pfspz titers correlated with a reduced risk of and increased time to first (or only) episode of clinical malaria among blood film-positive individuals \((p = 0.014)\) \(\text{(Figure 3B)}\).

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blood film-negative individuals, (ii) the effect of microscopically detectable \( Pf \) parasitemia among sporozoite non-responders, and (iii) the effect of interactions between antisporozoite antibody levels and microscopically detectable \( Pf \) parasitemia on the time to first or only clinical malaria episode during follow-up, independent of age and malaria exposure.

Among blood film-negative individuals, the model indicated an increased hazard of clinical malaria in high \( Pf/spz \) responders, although this association was not significant after the Bonferroni correction \( (p = 0.014) \) (Table 1). Microscopically detectable \( Pf \) parasitemia significantly increased the hazard of clinical malaria among individuals who did not react to \( Pf/spz \) \( (p < 0.001) \). Increasing titers of anti-\( Pf/spz \) antibodies among blood film-positive individuals conferred a strong protection against clinical malaria, independent of age and malaria exposure \( (p = 0.002) \) (Table 1). Hence, the model indicated a strong interaction between the effect of anti-\( Pf/spz \) antibody levels and \( Pf \) parasitemia on the risk of clinical malaria. The effect of anti-\( Pf/spz \) antibody levels was protective in blood film-positive individuals and predicted increased risk of first or only clinical episode in blood film-negative individuals. There was a significant difference between the model with and without the interaction term (likelihood ratio test: \( \chi^2 = 26.05; \ p = 0.0005 \)). A similar model identified an association between increasing anti-(NANP) \(_5\) antibody levels and microscopically detectable \( Pf \) parasitemia on the risk of clinical malaria \( (p = 0.001) \) (Table 2). Again, there was a significant difference between the model with and without interaction term (likelihood ratio test: \( \chi^2 = 24.38; \ p < 0.0001 \)).
TABLE 1 | Effect of interactions between anti-Pfspz antibody levels and microscopically detectable Pf parasitemia on the risk of clinical malaria.

| Pfspz antibody end titer among blood film-negative individuals | Hazard ratio (95% CI) | p Value |
|---------------------------------------------------------------|-----------------------|---------|
| 0                                                             | 1                     |         |
| 50–100                                                        | 1.54 (0.63–3.76)      | 0.339   |
| 300–2,700                                                     | 1.39 (0.51–3.77)      | 0.520   |
| 8,100–24,300                                                  | 4.27 (1.34–13.56)     | 0.014   |

| Microscopically detectable Pf parasitemia in Pfspz non-responders | Hazard ratio (95% CI) | p Value |
|------------------------------------------------------------------|-----------------------|---------|
| bf (−)                                                           | 1                     |         |
| bf (+)                                                          | 5.12 (2.34–11.19)     | <0.001* |

Interaction between anti-Pfspz antibody levels and microscopically detectable Pf parasitemia

| Interaction | Hazard ratio (95% CI) | p Value |
|-------------|-----------------------|---------|
| bf (+)* Pfspz (0–100) | 0.43 (0.14–1.34)      | 0.146   |
| bf (+)* Pfspz (300–2,700) | 0.24 (0.06–0.90)      | 0.035   |
| bf (+)* Pfspz (8,100–24,300) | 0.07 (0.01–0.37)      | 0.002*  |
| Age (years)                                                   | 0.85 (0.76–0.95)      | 0.005   |
| Exposure indexa                                                | 4.42 (0.89–21.81)     | 0.068   |

DISCUSSION

In this study, we analyzed the association of naturally acquired antibody responses against whole Pfspz and the PfCSP CRR (NANP)5 with microscopically detectable Pf parasitemia and clinical malaria in the longitudinally monitored Chonyi cohort. The data indicate that robust antisporozoite immune responses can be naturally acquired early in life and reveal a potentially protective interaction between the effect of anti-Pfspz antibodies and microscopically detectable Pf parasitemia on the risk of uncomplicated malaria.

TABLE 2 | Effect of interactions between anti-(NANP)5 antibody levels and microscopically detectable Pf parasitemia on the risk of clinical malaria.

| (NANP)5 antibody levels among blood film-negative individuals | Hazard ratio (95% CI) | p Value |
|----------------------------------------------------------------|-----------------------|---------|
| <0.001*                                                        |                       |         |

Microscopically detectable Pf parasitemia per OD increase in anti-(NANP)5 antibody level

| Interaction | Hazard ratio (95% CI) | p Value |
|-------------|-----------------------|---------|
| bf (+) (NANP)5 | 0.77 (0.66–0.89)      | 0.001*  |
| Age (years)   | 0.83 (0.74–0.95)      | 0.004*  |

Exposure indexa

| Exposure indexa | Hazard ratio (95% CI) | p Value |
|----------------|-----------------------|---------|
| 3.57 (0.68–18.8) | 0.132                |         |

Hazard ratios were computed with a logistic Cox regression model with and without one-, two-, or three-way interactions between Pfspz antibodies, microscopically detectable blood stage Pf infection at the time of serum collection prior to the start of the malaria transmission season, and malaria exposure. The model assesses (i) the effect of Pfspz antibody levels among blood film-negative individuals, (ii) the effect of microscopically detectable Pf parasitemia per OD increase in anti-(NANP)5 antibody level, and (iii) the effect of interactions between anti-(NANP)5 antibody levels and microscopically detectable Pf parasitemia on the time to first malaria episode during follow-up, independent of age and malaria exposure index. (NANP)5, immunodominant icosaspeptide repeats of Pf circumsporozoite protein; OD, optical density; bf (−), blood film negatives at the time of serum collection; bf (+), blood film positives at the time of serum collection; Pf, Plasmodium falciparum.

*Significant at α = 0.05 after Bonferroni correction (critical value α = 0.004).

Interaction terms.

aExposure index: proportion of infected individuals within 1 km of the index case, calculated using weekly slide data for acute episodes only, and using the cross-sectional slide data.

Antisporozoite Antibodies and Malaria present internally or on the sporozoite surface (40), suggesting that exclusive quantification of anti-(NANP)5 titers may largely underestimate the prevalence and breadth of naturally acquired preerythrocytic humoral immune responses. This discrepancy may be, at least partly, explained by different sensitivities of the assays used. In particular, the use of air-dried, acetone fixed sporozoites may inactivate some epitopes on the sporozoite surface and instead expose others that are usually inaccessible to protective antibodies. However, since our serology data show that fixed sporozoites are well recognized in IFA, we anticipate that live sporozoites tested in follow-up studies will likely display comparable activity.

In this study, we confirmed an age-dependent pattern of anti-Pfspz antibody acquisition (9–11), although we also identified several high responders among children, whose sera reacted to Pfspz at dilutions of up to 1:24,000. This is remarkable, considering that virtually all sera from adult volunteers immunized five times intravenously with ~135,000 metabolically active, irradiation-attenuated Pf sporozoites recognized Pfspz at ≤1:7,000 in a similar IFA assay (41). This would indicate that naturally acquired immune recognition of Pfspz fundamentally differs from syringe-injected, purified, and cryopreserved parasites.

Especially among younger age groups, the prevalence of antisporozoite responders was consistently higher among blood film-positive subjects. Whether the increased reactivity against sporozoites in this group could be attributable to recent or recurrent exposure to Pf could not be inferred based on the available
data. Since the presence of asymptomatic Pf infection was determined by microscopy, we cannot exclude that a significant proportion of blood film-negative individuals may have carried blood stage parasites below the microscopy detection level. In future studies, more sensitive methodologies, such as quantitative polymerase chain reaction or loop-mediated isothermal amplification, may provide a more accurate estimate of the prevalence of asymptomatic Pf infection among study participants.

Despite the overall lower reactivity, an age distribution of the prevalence of anti-(NANP)5 antibodies was detectable in this cohort, confirming previous findings (14, 15, 42). Microscopically detectable Pf parasitemia did not seem to affect acquisition of the anti-(NANP)5 antibody repertoire, corroborating previous observations that anti-(NANP)5 antibodies do not necessarily correlate with exposure (43, 44). On the other hand, several studies showed that anti-(NANP)5 antibody responses fluctuate with seasonal or geographical transmission variation (13, 16), and outbreak investigations showed that a single sporozoite inoculation is able to induce humoral immunity against (NANP)5 (15, 18). These conflicting reports may reflect epidemiological differences between the geographically distinct study areas.

Together, these observations support the notion of naturally induced acquisition of antibodies against the whole sporozoite and, to a lesser extent, the dominant repeat region of its main surface antigen. Whether the prevalence and intensity of anti-Pf-spz responses may represent a valuable marker for recent and/or repeated exposure remains to be elucidated in further longitudinal cohort studies, which should include entomological data to estimate the temporospatial dynamics of sporozoite transmission. Additional immune markers are needed to ultimately disentangle recent and past Plasmodium exposures.

**Potentially Protective Associations of Antisporozoite Antibodies with Clinical Malaria**

Previous investigations in the Chonyi cohort have shown that the correlation of naturally acquired antibodies to blood stage antigens with the risk of disease varies depending on the presence of microscopically detectable Pf infection (31, 34, 45, 46). Therefore, the relationship of antisporozoite antibodies with malaria incidence was analyzed separately in individuals who were blood film positive or blood film negative at sampling.

Our analysis revealed dual features of antisporozoite antibodies. Among individuals who were parasite free or carrying Pf parasites below the level of detection by microscopy at the start of the malaria transmission season, high anti-Pf-spz and anti-(NANP)5 antibody titers were significantly correlated with increased hazard of clinical malaria during follow-up. Acute antibody responses against the sporozoite are most likely elicited upon Pf inoculation. Blood film-negative individuals displaying higher antisporozoite antibody titers may have been recently exposed to a strong inoculation dose and, as a consequence, may be at higher risk of clinical malaria. Since our study only assessed humoral immune responses against the NF54 Pf strain, we cannot formally exclude that these individuals may have been infected by novel Pf strains to which they were immunologically unresponsive.

In marked contrast, higher levels of anti-Pf-spz antibodies predicted a lower risk of and delayed progression to clinical malaria among those individuals who were blood film positive at the start of the malaria transmission season. Notably, we detected a strong and potentially protective interaction between the effect of both anti-Pf-spz and anti-(NANP)5 antibody levels and microscopically detectable Pf parasitemia at the start of the transmission season on the risk of clinical malaria, which was independent of age and Pf exposure. The interaction analysis showed that the potentially protective effect of antisporozoite antibodies was greater among individuals with detectable baseline Pf parasitemia than those who were blood film negative at the beginning of the malaria transmission season. Because of this interaction, blood film-negative and -positive individuals displaying high antibody titers against sporozoites may experience different risks of clinical malaria. In contrast, the difference in risk of disease may be comparatively similar among blood film-negative or -positive individuals who display low antisporozoite titers. At higher antisporozoite antibody levels, blood film-positive individuals had significantly lower risk of malaria compared with blood film-negative individuals. This correlation was previously unrecognized and provides a rationale for future studies on the role(s) of preerythrocytic immunity in partial protection against malaria. Although highly speculative, one possibility is that members of the Chonyi cohort may have been infected by a diverse range of Pf strains, which in turn could have different immunological and pathogenic properties, thus influencing both individual immune responses and the patterns of clinical malaria incidence during the follow-up period. In such a scenario, selection through protective effects of antisporozoite antibodies could favor alternative strains.

One major limitation of this analysis is that data on participants’ Pf carrier status were only available from a cross-sectional measurement at the start of the malaria transmission season. It is plausible that the status of single individuals may have changed during the follow-up. Such changes may have affected the patterns of malaria incidence in the cohort over time, but this could not be taken into account in this analysis.

Although naturally induced antisporozoite immunity does not translate into sterile protection, partial preerythrocytic immunity might play a significant role in protection from clinical symptoms (28, 29). However, frequent inoculations over a longer time period might be required to induce short-lived, potentially protective anti-Pf-spz antibodies. While such requirements might not have been met in the blood film-negative subset, the identification of children with high Pf-spz antibody titers among the blood film-positive individuals indicates that high and potentially protective threshold levels may be acquired early in life. These hypotheses are based on the findings from this data set, but do not take into account the variability of the data. It is possible that other serologic data sets based on different cohorts or transmission settings may deliver contrasting results.

We cannot formally exclude that additional factors may have confounded this analysis. For instance, multiclonoality of asymptomatic Pf infections correlates with a reduced risk of subsequent clinical malaria (47–49), and this association is dependent on the intensity of transmission (50). In children, sickle cell trait
is independently associated with a delayed time to first clinical episode (51). Since these factors were not accounted for in our analysis, residual confounding may have occurred.

In addition, it is possible that the anti-Pfspz antibodies detected by IFA may actually recognize antigens that are shared between sporozoites and erythrocytic stages (52). Potentially protective anti-Pfspz antibodies detected in this study may be (i) antibodies that have been generated by blood stage infection but are also effective against sporozoites or (ii) antibodies that are effective against blood stages, in which case the IFA assay may have served as a proxy of protective blood stage responses.

Although there is currently scarce evidence that reducing the sporozoite load may reduce clinical malaria, possible scenarios for the contribution of antisporozoite antibodies to protection from clinical malaria include (i) reduction of sporozoite numbers by opsonization and subsequent phagocytosis through innate immune cells and (ii) blocking of essential parasite ligands for liver invasion receptors, as suggested by reduced sporozoite invasion of human liver cell cultures in vitro following the addition of endemic sera (53). Both effects may ultimately limit the scale of liver infection and, consequently, delay or mitigate the emergence of liver merozoites that initiate blood stage infection. Although the NANN repeats of CSP represent an immunodominant B-cell epitope (37), it is tempting to speculate that only broad recognition of multiple sporozoite antigens may represent a good marker for recent exposure and elicit protection against subsequent malaria incidence.

**CONCLUSION**

Observations in Kenya and other endemic areas indicated that only a very small fraction of mosquito inoculations results in reappearance of malaria after drug treatment (54–56). While these observations might be partly attributed to potent blood stage immune responses or inefficient sporozoite inoculation by infected mosquitoes, they could also suggest that malaria-exposed individuals are able to develop partially protective immunity against sporozoites. In this study, whole Pf sporozoites were employed for the first time as target antigen in a longitudinal study to assess the role(s) of naturally acquired antibodies in protective immunity against clinical malaria. A potentially protective contribution of antisporozoite antibodies was detected, suggesting that preerythrocytic immunity might indeed contribute to naturally acquired protection and that antibodies against several sporozoite antigens might be superior than responses to single antigens. Several obstacles complicate the interpretation of naturally acquired antibodies against sporozoites and their potentially protective roles. First, assessment of the relationship between disease and immune responses that never reach sterile protection is complex. In addition, endpoints in Pf malaria include microscopic detection of blood stage infection, while preerythrocytic stages remain undetectable. Additional functional assays, such as sporozoite invasion inhibition and antibody-dependent, cell-mediated immunity, will add further confidence to whether high antisporozoite titers can effectively reduce the burden of this vector-borne parasitic disease and exert a hitherto neglected benefit in preventing clinical malaria episodes.

**ETHICS STATEMENT**

Ethical approval was obtained from the Kenya National Research Ethics Committee (study protocol number REF CTMDR/SCC/1340). All participants (or parents/guardians of children aged ≤14 years) gave written informed consent (31, 34, 36). The local dialect was used to explain the study protocol to the participants, and a copy of the consent form was left in each household, so that all family members could review it prior to consenting.

**AUTHOR CONTRIBUTIONS**

All authors delivered substantial contributions to the conception or design of the work or the acquisition, analysis, or interpretation of data for the work; contributed to drafting the work or revising it critically for important intellectual content; gave final approval of the version to be published; agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

**ACKNOWLEDGMENTS**

We thank Philip Bejon and Steffen Borrmann for discussions and critical reading of this manuscript.

**FUNDING**

This work was supported by the Max Planck Society, the Kenya Medical Research Institute Center for Geographic Medicine Research-Coast (KEMRI-CGMRC), and the Wellcome Trust (grant numbers 092741 and 077092 to KEMRI-CGMRC). VT was also supported by a Wellcome Trust Intermediate Fellowship in Public Health and Tropical Medicine (grant number 084378/Z/07/A). This article is published with the permission of the director of KEMRI.

**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fimmu.2017.00488/full#supplementary-material.

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**Conflict of Interest Statement:** This research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest. The authors did not at any time receive payment or services from a third party for any aspect of the submitted work. There are no financial relationships with entities that could be perceived to influence or that give the appearance of potentially influencing, what is written in the submitted work. There are no patents and copyrights, whether pending, issued, licensed, and/or receiving royalties that are relevant to this work. All the authors declare no conflict of interest.