Naturally Acquired Immune Responses to *Plasmodium falciparum* Sexual Stage Antigens

Pfs48/45 and Pfs230 in an Area of Seasonal Transmission

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Abstract

Acquisition of immunity to *P. falciparum* sexual stages is a key determinant for reducing man-mosquito transmission by preventing the fertilisation and the development of the parasite in the mosquito midgut. Naturally acquired immunity against sexual stages may therefore form the basis for the development of transmission blocking vaccines, but studies conducted to date offer little in the way of consistent findings. Here, we describe the acquisition of anti-gametocyte immune responses in malaria exposed individuals in Burkina Faso. A total of 719 blood samples were collected in a series of three cross-sectional surveys at the start, peak and the end of the wet season. The seroprevalence of antibodies with specificity for the sexual stage antigens Pfs48/45 and Pfs230 was twofold lower (22-28%) than that for an asexual blood stage antigen GLURP (65%) or for the pre-erythrocytic stage antigen CSP (54%). The youngest children responded at similar frequencies to all four antigens but, in contrast with the immune responses to GLURP and CSP that increased with age independently of season and area of residence, there was no evidence for a clear age-dependence of responses to Pfs48/45 and Pfs230. Anti-Pfs230 antibodies were most prevalent at the peak of the wet season (p<0.001). Our findings suggest that naturally acquired immunity against Pfs48/45 and Pfs230 is a function of recent rather than cumulative exposure to gametocytes.

Keywords: Malaria, *Plasmodium falciparum*, gametocyte, immunity
Introduction

Malaria transmission depends on the presence of infectious sexual stage parasites in human peripheral blood, and naturally acquired immune responses to these or other stages can affect malaria transmission in several ways. An important indirect manner in which they influence malaria transmission is by reducing the number of asexual parasites that are the source of gametocytes. Immune responses can also influence malaria transmission more directly. Antibodies with specificity for sexual stages have been associated with a reduction of *P. falciparum* gametocyte prevalence in semi-immune individuals living in a hyper-endemic area of Irian Jaya, Indonesia (1). Early stage (stage I and IIa) gametocytes express the parasite protein PfEMP1 on the erythrocyte surface (38) while recently identified proteins may be expressed in later developmental stages (38, 46). Immune responses against these gametocyte-derived surface antigens may be related to direct clearance of gametocytes (47), and may explain why the duration of gametocyte carriage appears to decrease with age (8). A third way in which antibody responses can affect transmission is by reducing the infectiousness of gametocytes once ingested by mosquitoes.

Gametocytes in infected erythrocytes and gametes that emerge from erythrocytes inside the mosquito midgut express stage-specific antigens on their surfaces (27, 50). These antigens have a role in the fertilization or sporogonic development of malaria parasites in mosquitoes (43, 44). A proportion of gametocytes die in the human host without being passed on to a mosquito, thereby exposing sexual stage antigens to the human immune system. Sexual stage-specific antibodies may be elicited against these antigens (31) and may play a role in transmission-blocking immunity by preventing fertilization or the development of sporogonic stage parasites.
in the mosquito (4, 7, 18, 20, 21, 42, 44, 49). These antibody responses may reduce the spread of malaria in human populations. A better understanding of naturally acquired sexual stage immunity is thus relevant to malaria control as it may form the basis for the development of malaria transmission blocking vaccines.

Pfs230 and Pfs48/45 are major gametocyte and gamete surface antigens that induce antibody responses in naturally exposed individuals (7, 18, 21, 42, 44) that are associated with functional transmission reducing immunity (21, 25, 42, 44).

Here, we describe the profiles of naturally acquired sexual stage immune responses to Pfs230 and Pfs48/45 in individuals from an area of intense seasonal malaria transmission in Burkina Faso.
Methods

Study site and population
The study was conducted in a region close to Ouagadougou, the capital of Burkina Faso (West Africa), where malaria is endemic. Two areas of different endemicity (16) were included in the study to test the effect of transmission intensity on sexual stage immunity. *Plasmodium falciparum* is the predominant malaria species in the region, accounting for 90% of the infections; the remaining 10% being attributed to *P. malariae* and *P. ovale* (24).

The study’s participants are members of subsistence farming communities and all are permanent residents in the area. Participants were explained the procedures, risks and benefits involved in the study and their consent was obtained. The study protocol was viewed and received a written approval of the ministry of health of Burkina Faso (Research authorization number 2000/3174/MS/SG/DEP).

Measurement of transmission intensity
Repeated CDC light trap captures were carried out in both areas to estimate transmission intensity according to both area and season. Each area consisted of 3 grouped villages. Indoor mosquito captures extended from June to November 2002. The CDC light trap was placed close to the bed of the sleeper in randomly selected houses and mosquito capture was done from 7:00 pm to 6:00 am. Mosquito species were identified morphologically, counted and stored in tubes with silica gel. A representative sample of *Anopheles gambiae* mosquito thoraces and heads were examined for *P. falciparum* circumsporozoite protein (CSP) positivity index using routine CSP ELISA (2). The monthly sporozoite rate was estimated, testing representative samples (approximately 50% of all caught mosquitoes from each trap) of randomly selected specimens of
mosquitoes from each village. The entomological inoculation rate (EIR) was calculated as the product of the sporozoite rate by the biting rate.

Blood sample collection

The seasonally-spaced cross-sectional surveys for parasitological and serological data collection coincided with entomological data collection in 2002. At each survey, approximately 300 individuals (~150 from each area of residence) were randomly selected from village census lists aiming to include 60 individuals (10 per village) from each of five pre-defined age-groups: 1-4, 5-9, 10-14, 15-29 and ≥30 years of age. Individually selected individuals were invited to a sampling point and were systematically included until the required sample sizes were reached.

For parasites counts, a blood slide film was made from finger prick blood of each individual. For specific anti-plasmodial antibody measurements, 500 μL finger prick blood samples were drawn. Plasma was separated by centrifugation and stored at −20°C before use. Subjects with fever (body temperature ≥37.5°C) were treated with an anti-malarial drug (CQ) following the national policy for malaria treatment in the year of 2002.

Microscopical detection of P. falciparum parasites.

Slides were read independently by two microscopists, each examining 100 microscopic fields, and the mean density was used. A third reader was involved when the first two readers disagreed about the prevalence or estimated densities differed ≥30%. In these cases the mean density of the two closest readings was used. Asexual stage and gametocyte densities were simultaneously assessed by counting against 1000 leucocytes in the thick smear. The lower limit of microscopy for gametocyte quantification was estimated at 5 parasites/μL of blood. Parasite counts were
converted to numbers of parasites per µL by assuming a standard count of 8000 leucocytes/µL of blood.

Antigens

Plasma IgG with specificity for Pfs48/45 and Pfs230 antigens derived from an extract of mature *P. falciparum* NF54 strain gametocytes were measured by ELISA (see below). For this purpose, mature gametocytes were produced in an automated static culture system in red blood cells of blood group O⁺ and non immune AB serum (39) and harvested after 13-14 days. Gametocyte purification was previously described (50). Briefly, mature gametocytes were isolated at 37°C to prevent their activation. Parasite culture was loaded on a cushion of 63% Percoll (GE-Healthcare:17-0891-01) and centrifuged for 30 min at 1,500g. The purified gametocytes were then aliquoted and stored at −70°C before use. For extraction of Pfs48/45 and Pfs230 enriched proteins, gametocytes were re-suspended in 1% sodium desoxycholate / TrisNaCl and 1mM phenylmethylsulfonly fluoride (PMSF), incubated for 10 min at room temperature and spin 13,000 rpm for 10 min. The supernatant was collected as Pfs48/45 and Pfs230-enriched antigens extract and diluted in 0.25% PBSTM (2.5% milk and 0.05% tween20 in PBS) for use. The synthetic peptide NANP₆ corresponding to the repeat region of the circumsporozoïte protein and the synthetic peptide GLURP GMP₇₅-₂₁₃ LR67 (48) were used in standardized ELISA for sporozoite- and asexual blood stage-specific antibody detection respectively (see below).

**Pfs48/45 and Pfs230 IgG Enzyme Linked Immunosorbent Assays (ELISA)**

Samples selection
Although samples for serology were available from almost all participants, it was not possible to screen all samples for the presence of sexual stage antibodies due to the labour intensiveness and costs involved in the necessary antigen preparation. ELISA was therefore performed on a representative sub-sample of randomly selected samples. For this purpose, samples were randomly selected from the list of available samples for each age-group and season separately. Initially, a number of 150 samples per season i.e. 25 in age groups 1-4 and 5-9; 50 samples in age groups 10-19 and ≥20 years of age were randomly selected for testing both Pfs48/45 and Pfs230 antigens. Samples sizes were approximately similar for both antigens tests at the start of the wet season (137 for Pfs48/45 and 136 for Pfs230), peak of the wet season (149 and 148 respectively) and end of the wet season (125 and 129 respectively). To exclude possible variation in gametocyte antigen preparations, only samples that were concurrently tested for both Pfs48/45 and Pfs230 antigens extracted from the same batch of gametocytes were used for data analysis. This resulted in an overall 130 samples at the start and end of the wet season and 150 at the peak of the wet season explaining the slight inconsistencies in the sample sizes between groups of seasons, age groups and areas of different endemicity.

ELISA experiment

The presence of anti-Pfs48/45 and anti-Pfs230 IgG antibodies in plasma samples was determined by coating 10µg/mL of anti-Pfs48/45 rat monoclonal antibody (mAb) 85RF45.3 (45) or anti-Pfs230 rat mAb 63F6D7-F(ab)_2 (41), diluted in PBS, in the wells of 96-well polystyrene U-bottom ELISA-hard plates (Sterilin®, International Medical Products B.V., Zutphen, the Netherlands). Free sites were blocked with 5% milk (Marvel, Premier International Foods Ltd, Spalding, Lincs, United Kingdom) in PBS and Pfs48/45 and Pfs230 antigens contained in 50 µL gametocyte extract (250,000 gametocytes equivalents/well) were captured by overnight
incubation at 4°C. The buffer (0.25% PBSTM) used for dilution of gametocyte extract was added to control wells (i.e. no extract added) for background measurement. A dilution (1:100) in 0.25% PBS/TweenMilk of the test plasma was added to the wells prepared with and without antigen (control wells); and incubated for 2 hours at room temperature. The plate was washed and bound IgG antibodies were detected by addition of 100 µL of 1:30,000 diluted Goat-anti Human IgG-PO (H+L; Pierce) for 1h 30 min at room temperature. Wells were washed with PBS and subsequently incubated with tetramethyl benzidine (TMB) substrate solution for 20 min. The colour reaction was stopped with 4N H2SO4, and the optical density (OD) was read at 450 nm in an Anthos 2001 Microplate Reader (Labtec BV). All plasma samples were tested in duplicate. Three non-immune plasmas from Dutch blood bank donors as negative controls and one positive control plasma of a Dutch man that have been exposed to malaria for almost 30 years in sub-Saharan Africa were included per plate. The value for IgG titer (OD) of a sample was expressed as the difference in OD between the antigen and control wells. The cutoff was calculated as the mean OD of negative controls plus two standard deviations. A sample was considered positive if its background-adjusted OD was above the cutoff.

**GLURP and NANP6 IgG ELISA**

To evaluate anti-asexual blood stage or anti-sporozoite antibody responses, 0.2µg/mL of GLURP85-213 in 0.05M carbonate buffer (50 µL/well) or 1µg/mL of NANP6 in PBS (50 µL/well) were coated in flat bottom high binding microtiter 96 well plates (NUNC™ Maxisorp, Nalge Nunc International Corp, Life Techn, The Netherlands) at 4°C. Coated plates were incubated overnight at 4°C and washed with PBST. Free sites were blocked with 150 µL/well of 2.5% milk/PBS (Marvel). Subsequently, the blocker was washed off and plates incubated 1h at room
temperature with 50 µL of 1:200 plasma diluted in PBSTM for GLURP ELISA or with 50 µL of
1:100 diluted plasma for anti-sporozoite ELISA. Plates were washed and incubated for 1 hour
with rabbit anti-human IgG-Peroxidase (Dako, P-214) diluted 1:10,000 in PMSTM before
reaction with the substrate (TMB). The staining was stopped after 15 min of reaction by 4N
H2SO4 and the plate was read as described in Pfs48/45-Pfs230 ELISA. Plasma was considered
positive if the OD value was greater than two standard deviations above the mean of the negative
control plasmas from Dutch blood bank donors.

Statistical analysis

Study participants were categorized into groups by age (1-4 years, 5-9 years, 10-19 years and
≥20 years). The statistical analysis was performed using SPSS version 14.0 (SPSS Inc., Chicago,
IL, USA). The influence of age on antibody prevalence was tested using logistic regression
analyses using age in categories. Multivariate regression models allowed for confounding effects
of age, season and area of residence. The Pearson χ2 test was used for comparing proportions
and trends in dichotomous variables. Spearman’s rank correlation test was used to assess
association between antibody levels of tested antigens. The association between Pfs230 and
Pfs48/45 antibody responses and functional transmission reducing activity (TRA) in the Standard
Membrane Feeding Assay in previous studies (9, 11, 18) was presented after categorization of
TRA as >50% and >90% reduction (9, 11, 18) and calculating odds ratios (OR) with 95%
confidence intervals (95% CI). The level of significance was set at a two-tailed P < 0.05.
Results

Entomology
A representative sample of 4525 mosquitoes was tested for transmission intensity estimates. EIR details per season and area of residence are summarized in Table 1. Overall, the mean EIR was estimated to be 28.5 infective bites/person/month (ib/p/m) in the whole study area and this varied by season (1.47; 69.57; and 14.67 ib/p/m at start, peak and end-wet season, respectively) and area of residence (52.4 ib/p/m versus 3.05 ib/p/m).

Parasitology
A total of 719 blood slide samples were collected over the three cross-sectional surveys. At the site with the highest endemicity, 147, 101 and 130 samples were collected at the start, peak and end of the wet season, respectively. At the site of lower endemicity, these figures were 149, 50 and 142, respectively. The overall proportion of individuals harbouring asexual parasites was 63.4% (456/709); while 18.9% (136/709) carried gametocytes. There was a significant age-dependent decrease in both prevalence of asexual parasites (OR = 0.54, p < 0.001, 95% CI 0.46-0.63) and gametocytes (OR = 0.61, p < 0.001, 95% CI 0.51-0.74) and in the density of asexual parasites (β = -0.027, se(β) = 0.003, p < 0.001) and gametocytes (β = -0.009, se(β) = 0.022, p = 0.004) after adjustment for season and area of residence.

Parasite prevalence and density showed seasonal fluctuations. The prevalence of asexual parasites was significantly higher at the peak compared to the start (p < 0.001) and the end of the wet season (p = 0.004). Similarly we observed a higher asexual parasite density at the peak compared to the start (p < 0.001) and the end of the wet season (p = 0.01). Details on gametocyte prevalence are presented in Table 1. Similar to asexual parasites, gametocytes were more
prevalent at the peak (28.5%, 43/151) compared to the start of the wet season (22%, 65/296) ($p = 0.1$) and were least prevalent at the end of the wet season (10.3%, 28/272) ($p < 0.001$). The median density of gametocytes/μL blood was higher at the peak (24, IQR 24-72) compared to the start (24, IQR 16-40) ($p=0.03$) and, at a borderline level of significance, lower compared to the end of the wet season (40, IQR 40-57) ($p = 0.09$). Despite a substantial difference in EIR between them, there was no significant variation in asexual parasite and gametocyte prevalence between the study areas. The prevalence of asexual parasites was 61% (208/341) in the low transmission area and 65.6% (248/378) in the high transmission area ($p = 0.2$). The distribution of gametocytes in both areas is shown in Table 1. The proportion of gametocytes carriers was 17% (60/341) in the low transmission area and 20.1% (76/378) in the high transmission area ($p = 0.4$). This equality in parasite prevalence could reflect the high endemicity of malaria across the entire area (16).

**Serology**

Plasma samples were screened for antibody response profiles in the population and related to age (Figure 1), season (Figure 2) and area of residence (Figure 3). The average prevalence of IgG antibodies was 54.3% (367/676) for the sporozoite antigen NANP6 and 64.9% (460/709) for the asexual blood stage antigen GLURP. In contrast to sporozoite and asexual blood stage antigens, sexual stage-specific antibody responses were only detected in a minority of the samples: 22.2% (92/415) contained anti-Pfs48/45 IgG and 28.6% (117/409) anti-Pfs230 IgG.

**Effect of age on antibody responses**
Among children below 5 years, the prevalence of antibodies with specificity for NANP$_6$ (23.6%) and GLURP (32.4%) was broadly similar to those for Pfs48/45 (27.1%) and Pfs230 (38.1%). As expected, the prevalence of asexual stage antibodies to NANP$_6$ and GLURP increased significantly with age (Adjusted OR = 2.35, p < 0.001, 95% CI 1.98-2.78; Adjusted OR = 1.79, p < 0.001, 95% CI 1.53-2.08 respectively) reflecting cumulative exposure to infection, while no evidence of an age-dependent increase in sexual stage-specific antibody responses was observed (Table 2). The seroprevalences for Pfs48/45 and Pfs230 in the youngest children (27.1% and 38.1% respectively) were comparable to those in adult (38% and 28.6% respectively). However, there was a significant decline of Pfs48/45- and Pfs230-specific antibody prevalence from 1-4 year-olds to 5-9 year-olds ($\chi^2 = 13.61$, p = 0.001 and $\chi^2 = 12.02$, p < 0.001 respectively) followed subsequently by a significant increase with increasing age (OR = 3.09, p < 0.001, 95% CI 2.02-4.71 and OR = 1.41, p = 0.04, 95% CI 1.01-1.97 respectively) (Figure 1).

Differences between seasons and areas

GLURP antibody responses showed no variation according to season or area whilst, conversely, the prevalence of NANP$_6$ antibodies differed by both season (Figure 2) and area of residence (Figure 3). Antibodies with specificity for NANP$_6$ were detected in 83.9% of individuals at the peak wet season while this proportion was significantly lower at the start (44.2%) and at the end (48.5%) of the wet season. Thus the prevalence of NANP$_6$ antibodies at the peak wet season was significantly higher than either at the start ($\chi^2 = 60.34$, p < 0.001) or the end of the wet season ($\chi^2 = 48.91$, p < 0.001). There was also a difference in NANP$_6$ antibody prevalence between the area of high transmission (60.9%) and the area of lower transmission (47.2%; $\chi^2 = 12.61$, p < 0.001).
The prevalence of sexual stage-specific antibodies in relation to season is presented in Figure 2. Pfs48/45 antibody prevalence increased at the peak of the wet season (27.5%) but did not significantly differ from the prevalence at either the start (21.9%) or the end of the wet season (16.3%). The variation in Pfs230-specific antibody prevalence was significant, reaching 51.4% at the peak compared to 17.6% at the start ($\chi^2 = 35.29, p < 0.001$) and 13.6% at the end of the wet season ($\chi^2 = 42.99, p < 0.001$). This increased Pfs230 antibody prevalence at the peak of the wet season remained significant after adjustment for age (Adjusted OR $= 4.93, p < 0.001$, 95% CI 2.85-8.52, Table 2). Both antigens are on the surface of the gametocyte and were expected to have shown similar patterns of immune response. The difference observed in their immune responses may be dependent on the difference in their immunogenicity, Pfs230 being more immunogenic than Pfs48/45 (22).

In terms of area of residence, there was no difference in the prevalence of antibodies with specificity for either Pfs48/45 or Pfs230 (Table 2).

The relationship between antibody responses and parasite carriage is shown in Table 2. Both anti-NANP6 and anti-GLURP immune responses were significantly higher in individuals negative to gametocytes (Adjusted OR $= 0.62$, 95% CI 0.38-1.00, $p = 0.04$ for NANP6 and Adjusted OR $= 0.58$, $p = 0.01$, 95% CI 0.38-0.90 for GLURP). The seroprevalences for Pfs48/45 and Pfs230 antibodies were not influenced by the concurrent presence of asexual parasites or gametocytes.

Correlation of antibody responses between the different antigens

We also examined the correlation between antibody responses at the individual level. As presented in Table 3, the levels of antibody directed to NANP6 and Pfs230 and those to Pfs230
and Pfs48/45 were strongly correlated ($p \leq 0.001$.) Antibody levels to GLURP were associated with those to NANP.

Functionality of sexual stage immune responses

The volume of plasma that remained from this study was insufficient to study functional transmission reducing activity in the standard membrane feeding assay (SMFA). To aid interpretation of the current results, paired ELISA-SMFA data from three studies (9, 11, 18) were combined and presented in Table 4. Sexual stage antibody responses were determined using exactly the same ELISA methodology as used in the current study. TRA of $>50\%$ and TRA of $>90\%$ were significantly associated with antibodies to Pfs48/45 ($p < 0.001$ and $<0.001$, respectively) and Pfs230 ($p < 0.007$ and $<0.002$, respectively).
Discussion

As in previous studies (6, 9, 18, 23, 26, 43, 44), we found evidence for naturally acquired immunity against *P. falciparum* sexual stages. Overall, 22 to 28% of our samples were positive for sexual stage-specific antibodies while 54 to 65% were positive for pre-erythrocytic- and asexual blood stage-specific antibodies. Unlike immune responses to NANP₆ and GLURP, we found no evidence for age as a predictor for anti-Pfs48/45 and anti-Pfs230 antibody responses. Our data do reveal that sexual stage-specific antibody responses increase markedly during the transmission season. This would indicate a role for recent exposure to parasite antigens in the short-term boosting of sexual stage-specific immunity, although this requires confirmation in longitudinal studies.

The lower prevalence of sexual stage-specific antibodies compared with either pre-erythrocytic- or asexual stage-specific antibodies in the same study population confirms previous findings (6, 13, 18). In contrast to antibodies directed to sexual stage antigens, those directed to an asexual blood stage antigen (GLURP) increased with age, as described before (14, 15, 17, 19, 32), with no apparent season-dependent changes (17, 19). This would suggest that anti-GLURP antibody responses may be long-lived and stable even when antigen exposure decreases in the low transmission season and/or with increasing age. If sexual stage-specific antibody responses persisted in the absence of boosting, we would expect to see a similar pattern of increasing antibody prevalence with increasing age, but, despite a significant increase in the prevalence of sexual stage-specific antibodies from 5-9 years of age onward in our population, the close similarity of the prevalences in the youngest children and in adults above 20 years old of age effectively obscured any such association.
A single recent study reported an age-dependent increase of sexual stage-specific antibody responses, although it should be noted that that study concerned pre- and post-treatment determinations in residents of an area with a very low rate of transmission and is therefore not directly comparable with the study we report here (11). The results of other studies of the age-dependence of such responses are inconsistent (6, 18, 30). In Cameroon, the level of transmission blocking immunity, as measured in membrane feeding experiments, was not related to age (3, 5). The lack of age-dependence of sexual stage-specific antibody responses has often been attributed to their short-lived nature and hence a requirement for frequent boosting (30, 40). Cumulative immune memory, likely the result of repeated exposure to infections, is thought to form the basis of the age-dependent increase of asexual blood stage-specific immune responses (51). Immature and/or short-term memory for responses directed to sexual stage antigens is plausible (30) and possibly explained by a predominantly T-cell independent induction of the immune response (29).

Interestingly, the prevalence of sexual stage-specific IgG responses in the youngest children in our study group was similar to that for the asexual (GLURP) and pre-erythrocytic (NANP) antigens, suggesting that sexual stage-specific immune responses may readily develop in response to antigen exposure early in life. Since age-related cumulative exposure seems to have little effect on sexual stage immune responses, we speculate that the greater prevalence in young children may reflect the initial immune response to gametocytes as observed in individuals after a single or limited number of exposures to infection with either *P. falciparum* (6, 22, 33) or *P. vivax* (31). The remarkable nadir in both Pfs48/45 and Pfs230 seroprevalences in children aged 5-9 years old was previously observed in populations exposed to endemic malaria. The studies found that sexual stage immune responses decreased with age in the younger age groups but...
there was no further decline in older age groups (6, 18). Sexual stage antibody responses in young children are likely to be the result of high gametocyte exposure in this age group. In older children, gametocyte exposure decreases, possibly explaining the reduction in antibody prevalence. In adults gametocyte exposure may be lowest but sexual stage commitment during infections may be relatively increased (34) and antibody responses to sexual stage antigens may become more long-lived, reflecting a maturation of the immune response.

An important finding from the study reported here is that sexual stage-specific antibody responses may vary with season. Pfs230-specific antibody prevalence, in particular, increased during the peak of the transmission season, suggesting that the level of sexual stage-specific antibodies may reflect recent exposure. A possible association between sexual stage immunity and recent antigen exposure is also suggested by the correlation between NANP$_6$ and anti-sexual stage antibodies. In contrast to those directed to GLURP, NANP$_6$-specific antibody responses seemed to be short-lived. Thus the pattern of variation in NANP$_6$ antibody responses closely reflected the seasonality of transmission in our study, indicating that seroreactivity to NANP$_6$ is dependent on recent exposure and therefore highly susceptible to changes in sporozoite exposure. The close association we observed between NANP$_6$-and Pfs230-specific antibodies may be an indication that the immune responses to sexual stage antigens are therefore also related to recent exposure to infection, explaining the seasonality of the anti-Pfs230 antibody response. Our results showed that many infections with asexual parasites are accompanied by gametocytes (35, 37) and this proportion will be further increased if sub-microscopic gametocyte densities are considered (10). Since asexual parasite carriage increases during the transmission season (36), exposure to gametocytes increases in parallel, possibly explaining the seasonality of
anti-sexual stage IgG responses. However, the seasonality of sexual stage-specific immune responses (28) has been rarely studied, making any comparison difficult. The importance of recent exposure to infection for the acquisition of sexual stage-specific immunity has been reported from previous studies in which the prevalence of anti-Pfs48/45 and anti-Pfs230 antibodies increased in migrants from a non-endemic to an endemic area (9) and where functional transmission reducing immunity was shown to increase during the transmission season (12). We did not determine functional transmission reducing immune responses in the current dataset. Three studies that used the same methodology for determining total IgG antibody responses to Pfs48/45 and Pfs230 did relate these responses to TRA in the standard membrane feeding assay (9, 11, 18). When combined, these data show a strong association between Pfs48/45 and Pfs230 antibody prevalence and different levels of TRA. These data support the current findings on sexual stage antibody responses that plausibly reflect associations between functional TRA and age, season and recent exposure to malaria antigens.

We further tried to explore the relevance of recent exposure for the acquisition of parasite stage-specific immunity by relating antibody responses to concurrent asexual parasite and gametocyte carriage. Our results showed that NANP6 and GLURP–specific antibodies appear to negatively associate with gametocytes suggesting that immune responders to these two antigens are more likely to have a shorter period of asexual parasite carriage that will translate in a lower likelihood of gametocyte carriage. However, we found no effect of anti-Pfs48/45 or anti-Pfs230 antibodies on gametocyte carriage. Concurrent gametocytes may be positively related to development of sexual stage-specific immune responses (11) although a straightforward relationship is not always evident (6, 18). Immune responses to gametocyte antigens induced
prior to sampling may persist for several weeks after gametocyte clearance (11) or following
to gametocytes circulating at the time of sampling may take longer e.g. after their
death/destruction and subsequent clearance. Intuitively, then, an antibody response that is
boosted by recent gametocyte antigen exposure can better be determined in studies that
determine gametocyte carriage at both microscopic and submicroscopic level repeatedly and
relate sexual stage-specific antibody responses to previous as well as current gametocyte
exposure.

In summary, plasma samples from malaria exposed individuals were analyzed to provide
indications of the development of naturally-acquired immunity to *P. falciparum* sexual stages.
Our findings indicate that sexual stage-specific immune responses are naturally acquired in the
study population and that they are a function of recent rather than cumulative exposure to
gametocytes. This occurs after limited exposures to gametocytes in the youngest age-groups, but
boosting is also evident during the peak of the transmission season. A next step would be to
determine the functional importance of sexual stage-specific antibody responses in this
population that, despite developing both asexual and sexual stage-specific antibody responses, is
repeatedly exposed to intense transmission (35, 36).
Authors’ contributions ALO and TB analysed the data and wrote the manuscript; ALO, SJV, EIS, IN, NCO, AT, BSS, JPV and RS were responsible for the original study design and data collection; ALO, WR and KT conducted the experiments; SJV and AJFL contributed in data analysis; SJV, AJFL and RS contributed to data interpretation and manuscript preparation.

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Potential conflicts of interest: The authors have declared that no competing interests exist.
References


Table legends

Table 1. Entomological inoculation rates and gametocyte indices in the study population.

Table 2. Factors associated with antibody prevalence to \textit{P. falciparum} pre-erythrocytic and asexual blood stage antigens (2A) and sexual stage antigens (2B).

Table 3. Spearman rank correlation of level of anti-sporozoite and anti-asexual blood stage antibody with level of anti-sexual stage antibody

Table 4. The association between sexual stage antibody prevalence and functional transmission reducing activity in three studies using the same ELISA methodology.

Figure legends

Figure 1. Prevalence of antibodies against pre-erythrocytic stage (NANP\textsubscript{6}), asexual blood stage (GLURP) and sexual stage (Pfs48/45 and Pfs230) in relation to age. The error bars show the ± limits of the 95% confidence intervals. The number of plasma samples tested per age group (1-4, 5-9, 10-19 and ≥20 years of age) are 127, 144, 230 and 175 for NANP\textsubscript{6}; 142, 147, 236 and 184 for GLURP; 85, 84, 138 and 108 for Pfs48/45 and 84, 83, 137 and 105 for Pfs230 respectively.

Figure 2. Prevalence of antibodies against NANP\textsubscript{6}, GLURP, Pfs48/45 and Pfs230 in relation to season. Bars indicate the proportion of positive individuals in each area of residence. Error bars indicate the upper limit of the 95% confidence interval around the proportion. The number of individuals (with antibodies) during the different seasons was: start-wet season: 119 (269) for NANP\textsubscript{6}, 195 (293) for GLURP, 30 (137) for Pfs48/45, 24 (136) for Pfs230; peak-wet season: 120 (143) for NANP\textsubscript{6}, 89 (148) for GLURP, 41 (149) for Pfs48/45 and 76 (148) for Pfs230; end-wet season: 128 (264) for NANP\textsubscript{6}, 92 (176) for GLURP, 17 (125) for Pfs48/45 and 21 (129) for Pfs230.

Figure 3. Prevalence of antibodies against NANP\textsubscript{6}, GLURP, Pfs48/45 and Pfs230 in relation to the area of residence.
Bars indicate the proportion of positive individuals in each area of residence. Error bars indicate the upper limit of the 95% confidence interval around the proportion. The number of positive individuals in both area of residences was: high transmission area: 213 (350) for NANP₆, 238 (372) for GLURP, 55 (275) for Pfs48/45 and 81 (271) for Pfs230; low transmission area: 154 (326) for NANP₆, 222 (337) for GLURP, 37 (140) for Pfs48/45 and 36 (138) for Pfs230.
<table>
<thead>
<tr>
<th>season</th>
<th>Number of traps</th>
<th>Start wet</th>
<th>Peak wet</th>
<th>End wet</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Low endemic</td>
<td>36</td>
<td>51</td>
<td>54</td>
</tr>
<tr>
<td></td>
<td>High endemic</td>
<td>36</td>
<td>50</td>
<td>53</td>
</tr>
<tr>
<td>EIR, (95% CI)</td>
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<td>0.0</td>
<td>9.16</td>
<td>0.0</td>
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<td>2.9</td>
<td>129.9</td>
<td>29.3</td>
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<tr>
<td>Gametocyte prevalence</td>
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<td>20.0 (6/30)</td>
<td>40.0 (4/10)</td>
<td>24.0 (6/25)</td>
</tr>
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<td>High endemic</td>
<td>43.3 (13/30)</td>
<td>42.9 (9/21)</td>
<td>14.8 (4/27)</td>
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<td>5-9 years, % (n/N)</td>
<td>Low endemic</td>
<td>30.0 (9/30)</td>
<td>40.0 (4/10)</td>
<td>9.7 (3/31)</td>
</tr>
<tr>
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<td>High endemic</td>
<td>34.5 (10/29)</td>
<td>36.8 (7/19)</td>
<td>6.9 (2/29)</td>
</tr>
<tr>
<td>10-19 years, % (n/N)</td>
<td>Low endemic</td>
<td>17.3 (9/52)</td>
<td>42.1 (8/19)</td>
<td>13.7 (7/51)</td>
</tr>
<tr>
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<td>High endemic</td>
<td>26.5 (13/49)</td>
<td>22.9 (8/35)</td>
<td>5.9 (2/34)</td>
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<tr>
<td>20+ years, % (n/N)</td>
<td>Low endemic</td>
<td>2.7 (1/37)</td>
<td>9.1 (1/11)</td>
<td>5.7 (2/35)</td>
</tr>
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<td>High endemic</td>
<td>10.3 (4/39)</td>
<td>7.7 (2/26)</td>
<td>5.0 (2/40)</td>
</tr>
<tr>
<td>Total</td>
<td>Low endemic</td>
<td>22.0 (65/296)</td>
<td>28.5 (43/151)</td>
<td>10.3 (28/272)</td>
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</tbody>
</table>

Low endemic = area with lower endemicity; high endemic = area with higher endemicity
Table 2

2A

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<th></th>
<th>NANNP-6</th>
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<td>Multivariate</td>
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<td>Odd ratio (95% CI)</td>
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<td>1 (ref)</td>
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<td>10-19 yr N</td>
<td>4.05 (2.49-6.59)</td>
<td>4.29 (2.62-7.03)</td>
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<td>20+ yr N</td>
<td>16.27 (9.19-28.82)</td>
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<td>1 (ref) 269</td>
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<td>Peak wet N</td>
<td>6.57 (3.96-10.91)</td>
<td>10.59 (5.91-18.98)</td>
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<td>End wet N</td>
<td>1.18 (0.84-1.66)</td>
<td>1.33 (0.90-1.96)</td>
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<td>Area Low tran. N</td>
<td>1 (ref) 326</td>
<td>1 (ref)</td>
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<td>1.73 (1.27-2.35)</td>
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<td>Asexual Absent N</td>
<td>1 (ref) 244</td>
<td>1 (ref)</td>
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<tr>
<td>Present N</td>
<td>0.72 (0.53-0.99)</td>
<td>1.08 (0.79-1.49)</td>
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<td>Gametocyte Absent N</td>
<td>1 (ref) 544</td>
<td>1 (ref)</td>
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<tr>
<td>Present N</td>
<td>0.57 (0.39-0.84)</td>
<td>0.62 (0.38-1.00)</td>
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</table>

ref=reference; tran.=transmission intensity; sero-reactivity related to a given variable was adjusted for all others variables that primarily play a significant role in the univariate model.
<table>
<thead>
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<th>Pfs48/45</th>
<th>Pfs230</th>
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<tbody>
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<td>Univariate Odd ratio (95% CI)</td>
<td>Multivariate Odd ratio (95% CI)</td>
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<td><strong>Age</strong></td>
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<tr>
<td>1-4 yr N</td>
<td>1 (ref) 85</td>
<td>1 (ref) 84</td>
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<td>5-9 yr N</td>
<td>0.17 (0.06-0.47)</td>
<td>0.16 (0.06-0.47)</td>
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<td>10-19 yr N</td>
<td>0.53 (0.28-1.03)</td>
<td>0.52 (0.26-1.00)</td>
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<tr>
<td>20+ yr N</td>
<td>1.65 (0.89-3.05)</td>
<td>1.67 (0.89-3.11)</td>
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<tr>
<td><strong>Season</strong></td>
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</tr>
<tr>
<td>Start wet N</td>
<td>1 (ref) 137</td>
<td>1 (ref) 136</td>
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<tr>
<td>Peak wet N</td>
<td>1.35 (0.78-2.32)</td>
<td>4.92 (2.85-8.50)</td>
</tr>
<tr>
<td>End wet N</td>
<td>0.69 (0.37-1.28)</td>
<td>0.73 (0.37-1.44)</td>
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<tr>
<td><strong>Area</strong></td>
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<tr>
<td>Low tran. N</td>
<td>1 (ref) 140</td>
<td>1 (ref) 138</td>
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<tr>
<td>High tran. N</td>
<td>0.69 (0.43-1.12)</td>
<td>1.20 (0.76-1.91)</td>
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<tr>
<td><strong>Asexual</strong></td>
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<tr>
<td>Absent N</td>
<td>1 (ref) 131</td>
<td>1 (ref) 128</td>
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<tr>
<td>Present N</td>
<td>0.65 (0.40-1.05)</td>
<td>0.98 (0.62-1.55)</td>
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<td><strong>Gametocyte</strong></td>
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<tr>
<td>Absent N</td>
<td>1 (ref) 326</td>
<td>1 (ref) 321</td>
</tr>
<tr>
<td>Present N</td>
<td>0.72 (0.40-1.31)</td>
<td>1.06 (0.63-1.78)</td>
</tr>
</tbody>
</table>

ref=reference; tran.=transmission intensity; sero-reactivity related to a given variable was adjusted for all others variables that primarily play a significant role in the univariate model.
Table 3

<table>
<thead>
<tr>
<th></th>
<th>NANP6 $r$ (P-value)</th>
<th>GLURP $r$ (P-value)</th>
<th>Pfs48/45 $r$ (P-value)</th>
<th>Pfs230 $r$ (P-value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NANP6</td>
<td>0.209 (&lt;0.001)</td>
<td>0.53 (0.29)</td>
<td>0.170 (0.001)</td>
<td></td>
</tr>
<tr>
<td>671</td>
<td></td>
<td>390</td>
<td>384</td>
<td></td>
</tr>
<tr>
<td>GLURP</td>
<td></td>
<td>-0.067 (0.17)</td>
<td>-0.039 (0.43)</td>
<td></td>
</tr>
<tr>
<td>413</td>
<td></td>
<td>407</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pfs48/45</td>
<td></td>
<td></td>
<td>0.414 (&lt;0.001)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>409</td>
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<tr>
<td>Pfs230</td>
<td></td>
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</tr>
</tbody>
</table>

$r =$ spearman correlation coefficient; $N =$ number of individuals. Paired NANP-GLURP, NANP6-Pfs230 and Pfs48/45-Pfs230 data showed strong correlations ($p \leq 0.001$).
Table 4

<table>
<thead>
<tr>
<th>Functional transmission reducing activity</th>
<th>Pfs48/45 antibody prevalence (n=350) OR (95% CI)</th>
<th>p-value</th>
<th>Pfs230 antibody prevalence (n=344) OR (95% CI)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>&gt;50% reduction</td>
<td>3.76 (2.27-6.23)</td>
<td>&lt;0.001</td>
<td>1.87 (1.18-2.96)</td>
<td>0.007</td>
</tr>
<tr>
<td>&gt;90% reduction</td>
<td>6.08 (3.21-11.49)</td>
<td>&lt;0.001</td>
<td>2.84 (1.47-5.50)</td>
<td>0.002</td>
</tr>
</tbody>
</table>

This summary table combines data from Tanzania and Indonesia (9, 11, 18) that used identical ELISA protocols and related antibody prevalence to functional transmission reducing activity (i.e. the percentage reduction in mosquito oocyst numbers in test samples compared with controls) in standard membrane feeding assays. OR=odds ratio; 95% CI=95% confidence interval.