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# RAPID ONSET OF TRANSMISSION- REDUCING ANTIBODIES IN JAVANESE MIGRANTS EXPOSED TO MALARIA IN PAPUA, INDONESIA

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## RAPID ONSET OF TRANSMISSION-REDUCING ANTIBODIES IN JAVANESE MIGRANTS EXPOSED TO MALARIA IN PAPUA, INDONESIA

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**Abstract.** Transmission of *Plasmodium falciparum* malaria is initiated by sexual stages in the mosquito. Anti-Pfs48/45 and anti-Pfs230 sexual stage antibodies that are ingested together with parasites can reduce parasite development and subsequently malaria transmission. Acquisition of sexual stage immunity was studied in a cohort of 102 non-immune Javanese individuals migrating to hyperendemic Papua Indonesia. Seroprevalence of antibodies against Pfs48/45 and Pfs230 and functional transmission-reducing activity (TRA) were measured upon arrival and at 6, 12, and 24 months. Asexual parasitemia and gametocytemia were assessed every two weeks. The TRA and seroreactivity increased with the number of *P. falciparum* infections. The longitudinally sustained association between TRA and antibodies against Pfs48/45 (odds ratio [OR] = 3.74, 95% confidence interval [CI] = 1.51–9.29) and Pfs230 (OR = 3.72, 95% CI = 1.36–10.17) suggests that functional transmission reducing immunity is acquired after limited exposure to infection.

### INTRODUCTION

Successful transmission of *Plasmodium falciparum* parasites from humans to mosquitoes depends on the presence of infectious gametocytes in the circulation, which are derived from a small fraction of asexual parasites. After ingestion of the blood meal by mosquitoes, fertilization takes place in the midgut, which will ultimately result in the generation of infectious sporozoites in the salivary glands. Specific antibodies against sexual stages can interfere with fertilization and subsequent sporogonic development when co-ingested with the blood meal, as shown in natural and experimental infections.<sup>1–4</sup> This transmission-reducing activity (TRA) is associated with antibodies directed against the sexual stage-specific antigens Pfs48/45 and Pfs230.<sup>5–8</sup> The rate of development and persistence of TRA is largely unknown. Although it was suggested to be low grade and rapidly transient,<sup>9</sup> persistent TRA has also been observed.<sup>10</sup> Acquisition of immunity directed against asexual blood stage parasites has been intensively studied in people migrating from a non-endemic to a malaria-endemic area.<sup>11–15</sup> In these transmigrants, clinical and parasitologic immunity developed quickly after exposure<sup>13</sup> with inconsistent evidence for an independent role of age.<sup>11–16</sup>

The objective of this study was to examine the development of sexual stage-specific immunity in a longitudinal cohort of Javanese children and adults from non-endemic Java after migration to malaria endemic Papua. Antibody reactivity against sexual stage-specific antigens Pfs230 and Pfs48/45 was determined and compared with antibodies against asexual stage antigen glutamate-rich protein (GLURP) and whole parasite extracts. The GLURP-specific antibodies have previously been associated with protection against high levels of parasitemia<sup>17</sup> and clinical disease.<sup>18–20</sup> The development of sexual stage-specific immunity was evaluated by detecting circulating antibodies against Pfs230 and Pfs48/45 and by assessing functional TRA in the standard membrane-feeding assay.

### MATERIALS AND METHODS

**Study population.** The study site was a newly created transmigration village (designated SP2) located near the northeastern coast of Papua, Indonesia. After informed consent was obtained, healthy volunteer adults between 20 and 40 years of age and children between 6 and 12 years of age were included in the study. The study was reviewed and approved by the institutional ethical review boards of the United States Navy and the Indonesian Ministry of Health under U.S. Department of Defense Protocol #30820. The included human subjects were treated according to relevant regulations of the Indonesian Ministry of Health and the United States government (code 32 of Federal Regulation, Part 219, Protection of Human Subjects; U.S. Navy, SECNAVINST 3900.39B). Subjects were excluded if admitting a history of residence in a malaria-endemic area in the previous five years. Details on recruitment, follow-up, and the epidemiology of malaria in this cohort have been described elsewhere.<sup>16</sup> Enrollment started in 1996 and follow-up ended in 1999. The subjects originated from Java and were enrolled in the study within 48 hours of arrival in Papua, Indonesia. Malaria transmission in Java has been very low for many decades, typically around 0.01 malaria cases/1,000 person-years after the exclusion of the few foci of hypoendemic transmission.<sup>21</sup> In contrast, malaria transmission in Papua is perennial and often hyperendemic to holoendemic, with incidence rates from 500 to 5,000 infections/1,000 person-years.<sup>22</sup>

**Follow-up.** Malaria episodes were actively detected over a period of 24 months by periodic clinical assessments and blood smears. Blood smears were collected at two-week intervals or at any time a subject presented to the clinic with symptoms (chills, fever, etc.) suggesting malaria infection. Blood was microscopically examined for *P. falciparum* and *P. vivax* asexual parasites and gametocytes by Giemsa-stained blood films. Parasites were counted against the number of white blood cells. A slide was considered negative after examination of 200 microscopic fields, corresponding to 1,600–2,400 white blood cells and an estimated diagnostic threshold of five parasites per microliter. Parasite densities were calculated using a normal density of 8,000 white blood cells per

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microliter of blood. The first episode of symptomatic or asymptomatic *P. falciparum* malaria was recorded as first infection. Each subsequent *P. falciparum* parasitemia was classified as a new infection if it occurred more than 28 days after directly observed chemotherapy of the previous infection. Sera were collected on the day of arrival and after 6, 12, and 24 months at SP2.<sup>12,14,16</sup> To study the acquisition of immunity in time, we selected samples from subjects for whom at least three samples were available. We selected 51 subjects 6–12 years of age and 51 subjects 20–40 years of age. The incidence of malaria infection in these individuals was not different from that of the general population.

**Enzyme-linked immunosorbent assays (ELISAs).** *Antigen preparation.* Mature gametocytes of *P. falciparum* (NF54 strain) were produced in an automated static culture system as described by Ponnudurai and others,<sup>23</sup> isolated,<sup>4</sup> and stored at  $-70^{\circ}\text{C}$  until used. The NF54 gametocytes were extracted in 25 mM Tris-HCl, pH 8.0, supplemented with 150 mM NaCl, 1.0% sodium desoxycholate, and 1 mM phenylmethylsulfonyl fluoride. Samples were centrifuged at  $13,000 \times g$  for 5 minutes at room temperature to remove insoluble debris and the supernatant was used as source of whole-parasite antigen (Pfs48/45 and Pfs230).

*Pfs230 and Pfs48/45 capture ELISA.* IgG antibodies to Pfs230 were assayed by coating Sterilin® ELISA plates (no. 53011; International Medical Products B.V., Zutphen, The Netherlands) with mouse monoclonal antibody (MAB) 63F6D7-F(ab)<sub>2</sub> fragments (10  $\mu\text{g}/\text{mL}$ ) in phosphate-buffered saline (PBS), pH 7.4. For Pfs48/45, the plates were coated with rat MAb 85RF45.3. Plates were blocked with 5% (w/v) low-fat dry milk (Marvel, Premier International Foods Ltd., Spalding, United Kingdom) in PBS. For Pfs230 or Pfs48/45 antigen capture, plates were incubated with gametocyte extract (250,000 parasite equivalents/well). Serum samples (1:100 dilution) were added to the wells and incubated for two hours. Bound IgG antibodies were detected by horseradish peroxidase-labeled goat anti-human IgG (31412; Pierce Biotechnology, Inc., Rockford, IL). The wells were washed three times with PBS and incubated with tetramethylbenzidine substrate solution for 20 minutes. The color reaction was stopped with 4 N H<sub>2</sub>SO<sub>4</sub>, and optical density (OD) was read at 450 nm (Anthos 2001 microplate reader; Labtec BV, Heerhugowaard, The Netherlands). All incubations were carried out at room temperature. All serum samples were tested in duplicate with a concurrent positive control and a minimum of four negative (Dutch blood bank donor) controls per plate. Sera were considered positive if the OD was 3 SD above the mean of negative controls.

*Whole parasite and GLURP ELISA.* Antibodies against whole parasite extract and GLURP served as markers for general anti-parasite and specific asexual stage immunity, respectively. Sterilin® ELISA plates (International Medical Products B.V.) were coated overnight with 250,000 parasites per well with a diluted stock that contained  $30 \times 10^6$  parasites/mL in extraction buffer plus PBS. After blocking with 5% milk/PBS, serum samples were incubated for two hours at room temperature (1:100 dilutions in 0.1% milk, Tween 20, PBS). Anti-GLURP<sub>85-213</sub> antibodies were measured in plasma samples by ELISA.<sup>24</sup> Microtiter plates (Nunc Maxisorp; Nalge Nunc International Corp., Roskilde, Denmark) were coated overnight at  $4^{\circ}\text{C}$  with 50  $\mu\text{L}$  of 0.2  $\mu\text{g}$  of GLURP<sub>85-213</sub> /mL (final concentration) in 0.05 M carbonate

buffer, pH 9.6. The wells were washed Tween 20, PBS, and incubated with 2.5% (w/v) milk in PBS. Plasma samples were diluted in 0.05% PBS, Tween 20, 1.25% milk. For the detection of IgG antibodies to GLURP<sub>85-213</sub>, rabbit anti-human IgG peroxidase (P-214; Dako, Glostrup, Denmark) was used at a dilution of 1:10,000 in 0.05% PBS, Tween 20, 1.25% milk. These ELISAs were carried out as for Pfs230 and Pfs48/45.

**Standard membrane feeding assay (SMFA).** Experimental infections of mosquitoes were carried out as previously described.<sup>25,26</sup> Three-to-five-day-old colony-reared *Anopheles stephensi* were allowed to membrane feed on freshly cultured mature NF-54 *P. falciparum* gametocytes in batches of 50 mosquitoes each in the presence of serum. A maximum of 17 transmigrant serum samples were compared with three batches of a single (Dutch bloodbank donor) control serum. Immediately after the feed, non-fed and partially fed mosquitoes were removed. Blood-fed mosquitoes were kept at a temperature of  $26^{\circ}\text{C}$  and a relative humidity of 80% for seven days. Surviving mosquitoes (> 90%) were dissected and oocysts were counted in extracted midguts. Twenty mosquitoes per batch were examined for oocysts. An SMFA experiment was considered valid when the percentage of infected mosquitoes was  $\geq 90\%$  in all three control batches. The observed TRA of serum was determined as the percentage reduction in oocyst numbers in test samples compared with controls.<sup>27</sup> Functional TRA was defined as a minimum of 50% reduction in oocysts numbers in the standard SMFA. Not all sera were analyzed in the SMFA because of resource limitations. A random sample of individuals was therefore selected and all available serum samples were tested in the same SMFA experiment.

**Statistical analyses.** Analyses focused on the relation between the development of (functional) sexual stage-specific immunity and cumulative exposure to *P. falciparum*, which was quantified as the cumulative number of infections with asexual stage *P. falciparum* parasites. Statistical analyses were carried out using SPSS for Windows version 10 (SPSS Inc., Chicago, IL) and Stata version 7.0 (Stata Corporation, College Station, TX). Prevalence of antibodies and functional TRA (dichotomous variables) as a function of exposure to asexual *P. falciparum* parasites were tested using multiple logistic regression models with generalized estimating equations (GEEs). Regression  $\beta$  coefficients were calculated with 95% confidence intervals (CIs). Prevalence was logit-linked analyzed. The number of infections was included as a continuous variable and a random effect was included in the models to allow for correlations within individuals. Age group (20–40 years versus 6–12 years) and number of *P. vivax* infections were included in the models as covariates. Interaction terms were added to the models to assess a possible modifying effect of age on the relation between exposure to infections and the development of immunity.

## RESULTS

The malarimetric indices of the study population are shown in Table 1. Most individuals experienced at least one episode of malaria, with a median number of three infections during the 24-month period of follow-up. Although prevalence of infection was similar for adults and children, the latter were exposed to higher densities of asexual parasites ( $P = 0.02$ ; Table 1). Gametocytes were detected in 26% of

TABLE 1

Characteristics and *Plasmodium falciparum* malariometric indices of 102 study subjects from the SP2 cohort during 24 months of follow-up in Papua, Indonesia\*

	Children	Adults	<i>P</i>
No.	51	51	
Age, median (IQR)	9 (8–11)	32 (28–37)	
No. (%) of individuals infected	49 (96.0)	49 (96.0)	1.0
No. of infections per person, median (IQR)	3 (1–3)	3 (2–4)	0.53
Asexual parasite density, GM (IQR)	2,792 (879–11,962)	1,697 (520–5,920)	0.02
No. (%) of individuals gametocytemic	13 (25.5)	15 (29.4)	0.66
Gametocyte density, GM (IQR)	112 (40–280)	176 (48–579)	0.21

\* IQR = interquartile range; GM = geometric mean parasite density in parasites/ $\mu$ L for *P. falciparum* asexual parasite or gametocyte carriers only.

the children and 29% of the adults with no statistically significant difference in gametocyte density.

The cumulative number of infections per six months is shown in Figure 1. By six months, 20% of the 102 migrants had experienced  $\geq 2$  infections; by 24 months, this proportion had increased to 73%. The cumulative number of subjects in whom gametocytes were observed in peripheral blood films was 0, 2, 8, and 18 for time points 0, 6, 12, and 24 months post-migration, respectively.

Seroreactivity against whole *P. falciparum* extracts and asexual stage-specific antigens increased with cumulative exposure (Figure 2A). Antibody prevalence in the whole parasite ELISA increased to 98% after two infections and was maintained after further infections. The prevalences of antibodies against sexual stage-specific antigens Pfs48/45 and Pfs230 were also increased after two infections (Figure 2B). The proportion of serum samples with TRA gradually increased from 10% (3 of 29) prior to migration to 27% (3 of 11) after more than four infections. There was a clear association between the presence of gametocytes at the time of sampling and TRA (odds ratio [OR] = 5.22, 95% CI = 1.87–14.56). Nevertheless, only 31.3% (10 of 32) of sera with TRA were derived from individuals with patent gametocytemia at the time of sampling ( $n = 9$ ) or prior to sampling ( $n = 1$ ).

After adjustment for the number of infections, TRA was significantly associated with the prevalence of antibodies to Pfs48/45 (OR = 3.74, 95% CI = 1.51–9.29) and antibodies to Pfs230 (OR = 3.72, 95% CI = 1.36–10.17). No statistically significant association was observed between TRA and the prevalence of antibodies to whole parasites (OR = 4.21, 95% CI = 0.53–33.18) or antibodies to GLURP (OR = 1.90, 95% CI = 0.83–4.34).

To adjust for a correlation between observations from the same individual and for potential confounders, a GEE model was used for statistical analyses of the data shown in Figure 2. There was a statistically significant increase in positive response after *P. falciparum* infections in all ELISAs, which was strongest for the whole parasite assay. There also was a statistically significant positive association between the prevalence of TRA and the number of infections (Table 2).

Seroprevalence of antibodies against Pfs230 (Figure 3), whole parasite, and GLURP were consistently higher in adults. In adults, 23.8% (10 of 42) of individuals had antibodies to Pfs230 prior to exposure compared with 0% (0 of 32) of the children. This higher seroprevalence for adults remained apparent throughout follow-up. Although the intercept of the trend lines was higher in adults ( $P = 0.001$ ), the slope of both lines was not different ( $P = 0.62$ ), which indicated a similar

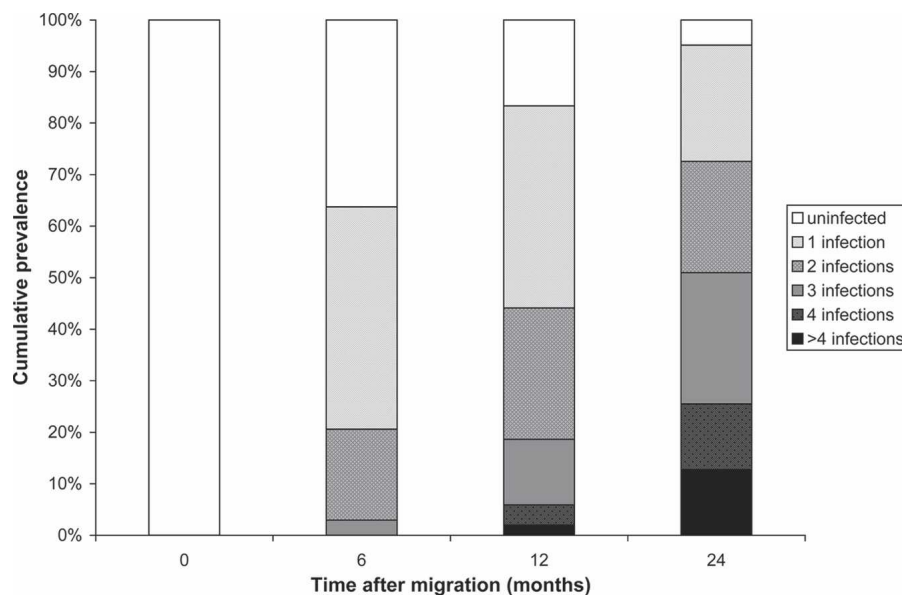


FIGURE 1. Cumulative prevalence of asexual stage infections in a cohort of 102 non-immune migrants from Java to Papua, Indonesia experiencing 1–4 infections with *Plasmodium falciparum* during two years of follow-up in the malaria-endemic area.

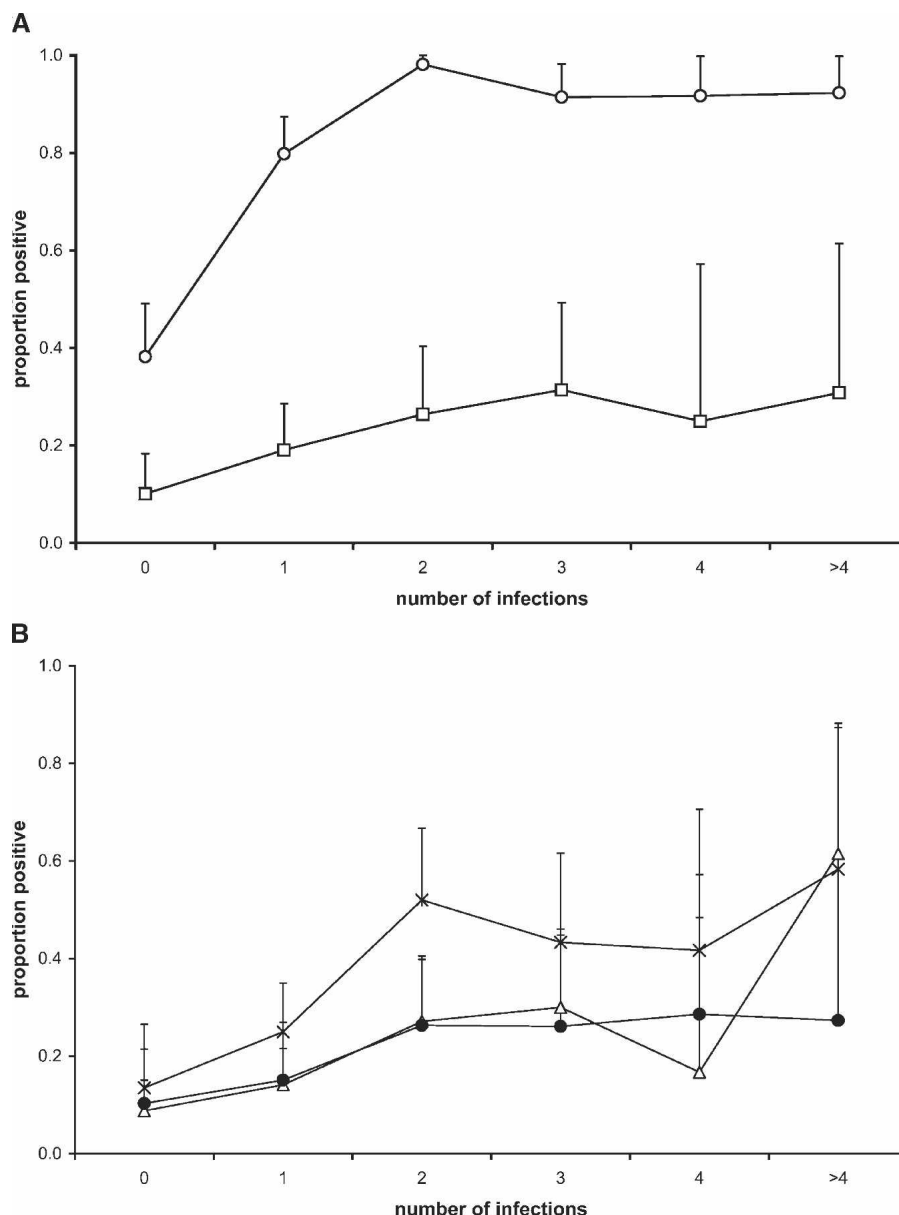


FIGURE 2. Proportion of serum samples with *Plasmodium falciparum*-specific antibodies as a function of the number of prior *P. falciparum* infections. **A**, Seroreactivity for whole parasite (○) and asexual stage-specific glutamate-rich protein (□). **B**, Seroreactivity for sexual-stage specific Pfs230 (×) and Pfs48/45 (△), and functional transmission-reducing activity (TRA) > 50% (●). For TRA, 20 mosquitoes were dissected per serum sample included in the standard membrane feeding assay. The control samples resulted in a median number of 21 oocysts (interquartile range = 7–46) per mosquito. Error bars indicate the upper limit of the 95% confidence interval.

TABLE 2

Regression coefficient of the number of prior *Plasmodium falciparum* infections as a predictor of seroreactivity or transmission reducing activity\*

	No.†	Positive response (95% CI)‡	P
Whole parasite	102 (296)	1.30 (0.64–1.96)§	< 0.001
GLURP	102 (296)	0.49 (0.21–0.77)§¶	0.001
Pfs48/45	95 (268)	0.40 (0.24–0.57)	< 0.001
Pfs230	89 (252)	0.46 (0.25–0.67)§	< 0.001
TRA	76 (161)	0.39 (0.07–0.71)¶	0.016

\* GLURP = glutamate-rich protein; TRA = transmission-reducing activity.

† No. of individuals (no. of measurements).

‡ Regression coefficient β (95% confidence interval [CI]).

§ Estimate adjusted for age group.

¶ Estimate adjusted for number of *P. vivax* infections.

relationship between number of infections and Pfs230 seroreactivity for adults and children. In the GEE models (Table 2), the relationship between number of infections and whole parasite, GLURP, and Pfs230 seroreactivity was not different between children and adults.

DISCUSSION

This study showed that sexual stage-specific seroreactivity develops after a limited number of *P. falciparum* infections in a cohort of non-immune transmigrants. The prevalence of antibodies to Pfs48/45 and Pfs230 and functional TRA increase with the number of infections experienced.

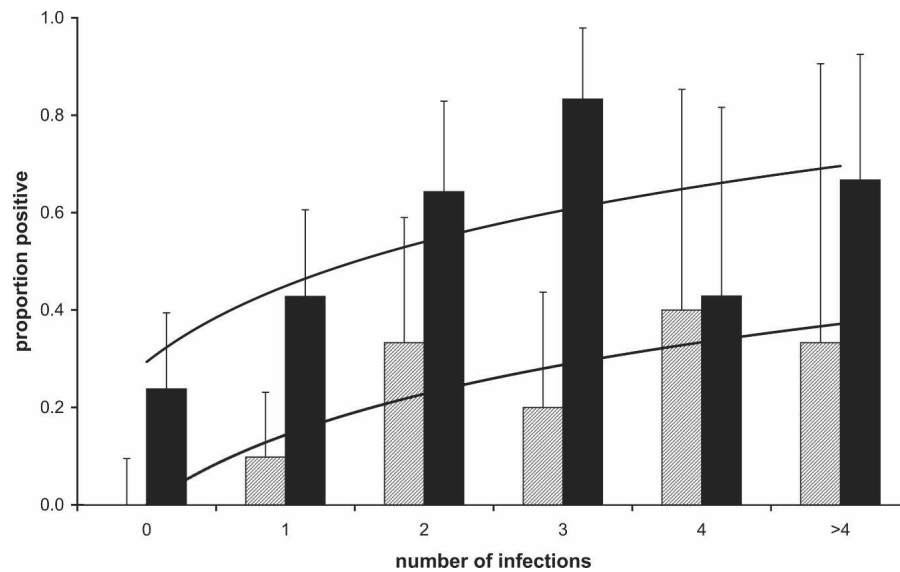


FIGURE 3. Prevalence of Pfs230 seroreactivity as a function of the number of prior *Plasmodium falciparum* infections. The prevalence of Pfs230 antibodies is depicted for children (shaded bars) and adults (black bars). Error bars indicate the upper limit for the 95% confidence interval. The number of observations for children is 0 infections (n = 32); 1 (n = 41); 2 (n = 18); 3 (n = 20); 4 (n = 5); and > 4 (n = 3). For adults, 0 infections (n = 42); 1 (n = 35); 2 (n = 28); 3 (n = 12); 4 (n = 7); and > 4 (n = 9). Logistic trend lines are shown for adults (top line) and children (bottom line).

Development of sexual stage-specific immunity resembles that of immunity against asexual stages with similar patterns for acquisition of antibodies against GLURP, Pfs230, and Pfs48/45. Reactivity against all antigens develops quickly after exposure, with seroprevalence increasing with number of infections. Consistent with previous studies, immune response seems to require recent but not necessarily long-term exposure.<sup>11–13</sup> Sexual stage-specific antibody responses seem to be part of the initial immune response, which corroborates previous studies in individuals after a primary infection with *P. falciparum*.<sup>28,29</sup> The finding is remarkable because exposure to sexual stage parasites is generally considered much lower than to asexual parasites. In this study, less than one-third of the individuals had microscopically detectable gametocytes on any occasion during the two-year follow-up. This apparent low exposure may be the result of poor sensitivity of microscopy in detecting low-density gametocytemia,<sup>30</sup> which can contribute substantially to the total exposure to gametocytes.<sup>31</sup> Sexual stage-specific antibodies may be induced by these low-density gametocytes or by gametocytes that are sequestered in capillary vessels.<sup>32</sup> The combined possibilities may explain the frequently observed reactivity against sexual stage-specific antigens in the absence of microscopically detectable gametocytes. In addition to these antibody responses, we also frequently observed functional TRA without concurrent or prior detection of gametocytes. Although we may have missed some gametocytemic periods as a result of our screening every two weeks, we conclude that submicroscopic levels of gametocytemia play an important role in eliciting sexual stage-specific immune responses.

We found no evidence for age as a key determinant in the development of immune responses in our population of 6–40-year old transmigrants. Although other studies reported an age-dependent acquisition of clinical and parasitic immunity,<sup>12,13</sup> we did not find a similar effect for our selected markers of asexual and sexual stage-specific immunity. The reac-

tivity in the ELISAs was higher in adults but could reflect background reactivity because of the presence of cross-reactive antibodies reacting with common epitopes.<sup>15</sup> We did not detect a difference in the relationship between exposure to infection and the acquisition of (sexual stage-specific) immunity for different age groups.

Our most important finding was the presence of functional TRA in a substantial proportion of the individuals that increased with exposure. This has not been previously shown for *P. falciparum*, but is analogous to transmission-blocking immunity in *P. vivax*, where TRA is more prevalent and efficient after repeated infections.<sup>33–35</sup> In this study, more than 25% of the individuals with more than four *P. falciparum* infections showed TRA, i.e., > 50% reduction in oocysts numbers. This pattern of increasing prevalence of TRA accompanies the occurrence of antibodies to Pfs230 and Pfs48/45, as previously reported.<sup>8,36–39</sup> The rapid development of TRA and associated antibodies is particularly promising because Pfs230 and Pfs48/45 are considered candidates for inclusion in future transmission-blocking vaccines.<sup>28</sup>

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