

THE RISK OF MALARIAL INFECTIONS AND DISEASE IN PAPUA NEW GUINEAN CHILDREN

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Abstract. In a treatment re-infection study of 206 Papua New Guinean school children, we examined risk of re-infection and symptomatic malaria caused by different *Plasmodium* species. Although children acquired a similar number of polymerase chain reaction–detectable *Plasmodium falciparum* and *P. vivax* infections in six months of active follow-up (*P. falciparum* = 5.00, *P. vivax* = 5.28), they were 21 times more likely to develop symptomatic *P. falciparum* malaria (1.17/year) than *P. vivax* malaria (0.06/year). Children greater than nine years of age had a reduced risk of acquiring *P. vivax* infections of low-to-moderate (>150/μL) density (adjusted hazard rate [AHR] = 0.65 and 0.42), whereas similar reductions in risk with age of *P. falciparum* infection was only seen for parasitemias > 5,000/μL (AHR = 0.49) and symptomatic episodes (AHR = 0.51). Infection and symptomatic episodes with *P. malariae* and *P. ovale* were rare. By nine years of age, children have thus acquired almost complete clinical immunity to *P. vivax* characterized by a very tight control of parasite density, whereas the acquisition of immunity to symptomatic *P. falciparum* malaria remained incomplete. These observations suggest that different mechanisms of immunity may be important for protection from these malaria species.

INTRODUCTION

The epidemiology of *Plasmodium falciparum* malaria suggests children first acquire immunity against severe disease after relatively few infections.^{1,2} However, uncomplicated *P. falciparum* malaria remains common throughout most of childhood, and a significant decrease in risk of infection is only seen in adolescence and early adulthood.¹ Similar patterns have also been described in areas of Papua New Guinea highly endemic for malaria.^{3,4} It has therefore been argued that the mechanisms responsible for protection against severe disease may be distinct from those that protect against infections *per se* and mild episodes of disease,^{1,5} and that immunity might be acquired in stages. Although many potential targets and mechanisms of protective immunity have been identified,^{1,5–8} we still know little about mechanisms involved in the acquisition of protective immunity against *P. falciparum*.

Even less is known about the acquisition of immunity to non-*P. falciparum* malarias. In highly endemic areas such as Papua New Guinea where the different species co-occur, prevalence of infection with *P. vivax* peaks at younger ages^{3,9,10} and contributes proportionally less to the burden of febrile illness¹¹ than *P. falciparum*. Conversely, *P. malariae* reaches maximum prevalence only in adolescents.^{9,10} These data indicate that immunity to *P. vivax* may be acquired more quickly than immunity to *P. falciparum* despite lower transmission rates.¹² Although a number of potential targets and mechanisms for immunity have been identified for *P. vivax*,^{13–15} little is known about acquisition of immunity to *P. malariae* and *P. ovale*.

A better understanding of the incidence of infection and disease caused by different *Plasmodium* species in areas endemic for all species is needed to properly assess differences in the acquisition of clinical immunity to different species. Because mixed infections are common in malaria-endemic areas, but often remain undetected by light

microscopy,^{10,16} polymerase chain reaction (PCR)–based diagnostic methods are needed for quantifying risk of infection and morbidity reliably.

To determine epidemiologic patterns of infections and disease with *P. falciparum* and *P. vivax* and investigate possible mechanisms of immune protection, we conducted a longitudinal treatment re-infection study of 206 Papua New Guinean elementary school children that combines repeated blood sampling and molecular detection of parasitemia with a large array of classic and functional immune assays. We describe the general study design and report patterns of incidence of infection and disease with all four human malaria parasite species. Detailed investigations of immunity to *P. falciparum* and *P. vivax* malaria will be the topic of future reports.

MATERIALS AND METHODS

Field study. This study was conducted between June and December 2004 at the Mugil and Megiar elementary schools situated on the northern coast of Papua New Guinea, 50 km north of Madang. The catchment area of both schools is serviced by a single health center at Mugil (Figure 1) run by the Catholic Health Services. Although the Mugil school is within easy walking distance of the health center, the Megiar schools are 4 km away along a sealed road but with frequent transport available. Bed net use in the study area is limited, with re-treatment of bed nets virtually absent. This study was reviewed and approved by institutional review boards of the Papua New Guinea Medical Research Advisory Council, the Walter and Eliza Hall Institute, and the Veteran's Affairs Medical Center (Cleveland, OH).

After obtaining community support and written parental consent, children from all three grades in Mugil and grades 1 and 2 in Megiar were enrolled. Demographic information was collected from all participating children; the location of each child's home was recorded using a hand-held global positioning system (GPS) receiver (GPS 315; Magellan, Santa Clara, CA).

Before starting treatment, each child was clinically examined: axillary temperature was measured using digital thermometers, the spleen was palpated, and a standard question-

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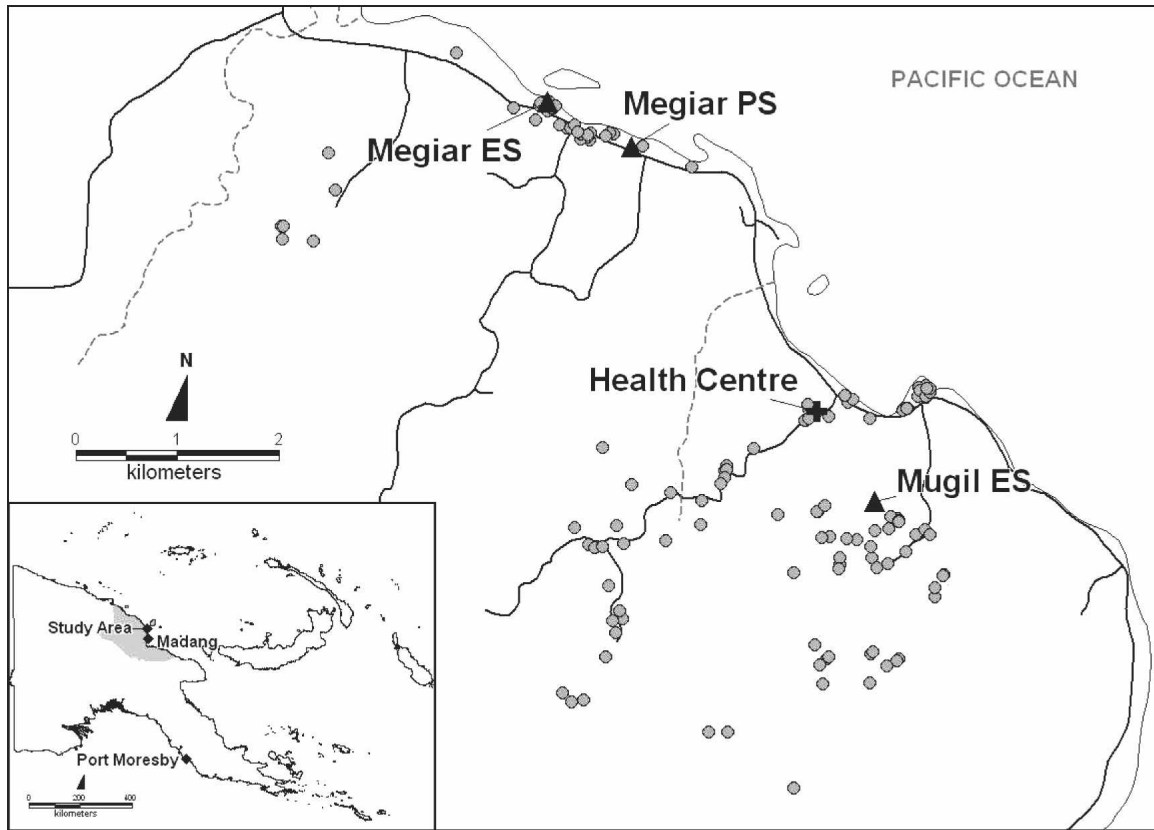


FIGURE 1. Location of study site, schools, and participants' houses in Papua New Guinea.

naire of common signs and symptoms of malarial illness was administered. Hemoglobin (Hb) levels were measured using a portable device (HemoCue, Ångholm, Sweden). A 10-mL venous blood sample was collected using EDTA-Vacutainer® tubes (Becton Dickinson, Franklin Lakes, NJ) and two blood slides (thick and thin films) were made for determination of malarial infection. All children were subsequently treated with a seven-day course of artesunate monotherapy according to Papua New Guinea National treatment guidelines (i.e., 4 mg/kg on day 1 and 2 mg on days 2–7). All children received all seven doses with at least five of them directly observed.

After treatment, children were actively followed-up at the schools every two weeks for new infections and febrile illness with the first visit taking place two weeks after receiving the first artesunate treatment. A total of 12 follow-up visits were conducted. Because of national holidays that prevented field work, the eighth follow-up period had to be extended to three weeks, which resulted in a total length of active follow-up of 25 week. Follow-up visits were conducted on a class-by-class basis with one class checked every day. Children that did not attend school on the day of scheduled follow-up were checked the next day or at their homes at the earliest possible time within the next week. During school holidays (follow-up time points weeks 4 and 14), special clinics that included the screening of children's videos were set up at the schools or in the villages that regrouped most students. Children who did not come to these clinics were individually followed-up at their homes, when possible.

At each active follow-up, all children were clinically examined, their axillary temperatures were taken, their health

books were checked for recent antimalarial treatments, and they were questioned for recent bed net use. Hemoglobin levels were measured every four weeks and spleens were palpated every eight weeks. At the same time, two malarial blood films were made, a rapid diagnostic test (ICT Diagnostics, Brookvale, New South Wales, Australia) was conducted and a 250- μ L blood sample was collected from each child into an K+-EDTA Microtainer® tube (Becton Dickinson) by finger prick using a retractable lancet (Genie® Lancet; Becton Dickinson). Children with symptoms of clinical malaria were transported to the Mugil health center after a third blood slide was made for parasitologic assessment, and treatment was given at the health center according to blood slide results. The scheduled active follow-ups, together with collection of additional venous blood samples, resulted in the study team visiting the Mugil school five times and the Megiar schools three times over each two-week period.

For six months after treatment, a passive case-detection system was maintained at the Mugil health center. In addition, children, their parents, and teachers were encouraged to report any illness to the study team at any time the team was visiting the school. All study children attending the outpatient clinic at Mugil health center with any illness, as well as those diagnosed by the study teams during their school visit, were referred to specific study staff based at the Mugil health center for clinical and parasitologic assessment. After a detailed clinical assessment, a 250- μ L finger prick sample was collected, 3 blood slides were made, and Hb levels were measured. One of the slides was stained using rapid Giemsa staining (10% Giemsa for 10 minutes) and read immediately for

diagnostic purposes. The other two slides were stained for research readings (5% Giemsa for 30 minutes). If children had signs of a febrile illness and a *P. falciparum* parasitemia > 500/ μ L or a *P. vivax* parasitemia > 250/ μ L, an additional 5-mL venous blood sample was collected within 24 hours. All children with clinical signs of malaria and a positive blood slide (irrespective of parasite density) were treated by the study team according to Papua New Guinea national treatment guidelines with chloroquine (three days) and sulfadoxine-pyrimethamine (single dose). A concurrent *in vivo* efficacy study at Mugil health center showed an overall 28-day failure rate (corrected by polymerase chain reaction [PCR]) of 12% for the treatment of *P. falciparum* infections (4% late clinical failures and 8% parasitologic failures) (Marfurt J, Mueller I, Genton B, unpublished data). Any other medical conditions were referred to the health center for appropriate treatment.

During the study period, monthly mosquito sampling was conducted in two villages using the all-night landing catch method¹⁷ and specimens were identified morphologically as described by Belkin.¹⁸ A subset of morphologically identified mosquitoes was processed by PCR to assess species identity.¹⁹ Mosquitoes were tested by enzyme-linked immunosorbent assay (ELISA)²⁰ to detect circumsporozoite antigens using monoclonal antibodies specific for *P. falciparum*, *P. vivax* (PK210), and its variant (PK240).

Laboratory methods. All venous blood samples were separated into plasma, peripheral mononuclear cells, and remaining blood cells, centrifuged, and aliquoted accordingly. Finger prick blood samples were separated into plasma and cell pellets. DNA was extracted using the QIAamp 96 DNA Blood kit (Qiagen, Valencia, CA) from the cell pellet fraction of all samples.

All research blood films were read by two expert microscopists independently. Slides with discrepant results were re-read by a third microscopist. Thick blood films were examined by light microscopy (LM) for 100 thick-film fields (under a 100 \times oil-immersion lens) before being declared infection negative. Parasite species in positive films were identified and densities were recorded as the number of parasites per 200 white blood cells (WBCs). Densities were converted to the number of parasites per microliter of blood assuming 8,000 WBCs/ μ L (population average WBC count³). Slides were scored as LM positive for an individual *Plasmodium* species if the species was detected independently by at least two microscopists and subsequent PCR-based analysis confirmed the presence of the species. Densities were calculated as the geometric mean densities of all positive results.

Infection by each of the four human malaria species was assessed in all blood samples collected using a semi-quantitative post-PCR, ligase detection reaction-fluorescent microsphere assay (LDR-FMA).²¹ This assay combines PCR amplification of the small subunit (SSU) ribosomal RNA gene (491–500-basepair fragments) using genus specific primers, followed by a multiplex species-specific LDR. The LDR products are hybridized to FlexMAP™ classification bead sets (5') (Luminex, Austin, TX) and receive reporter labeling after incubation with streptavidin-R-phycoerythrin that binds to biotin (3'). Double-labeled species-specific LDR complexes are detected using a Bio-Plex array reader (Bio-Rad Laboratories, Hercules, CA). Species-specific fluorescence

data were collected with Bio-Plex Manager version 3.0 software (Bio-Rad Laboratories). To ensure maximum sensitivity for the detection of *Plasmodium* infections, the PCR cycle number was set at 35. Differentiation of negative from positive fluorescent signals was performed by comparing median fluorescent intensity from study participants with values obtained from uninfected North American controls. Cut-off values for positivity were set at the 99% quantile of signals in controls as reported by Kasehagen and others.¹⁰ The design and sensitivity of this assay has been previously described.^{10,21,22} In these studies, the assay demonstrated high sensitivity compared with LM and real-time PCR. Nevertheless, we are not able to exclude the possibility that sequence polymorphisms in the *Plasmodium* species SSU ribosomal RNA gene sequences may contribute to false-negative results. However, to date, we have observed no direct evidence identifying specific variants of the *P. falciparum*, *P. vivax*, *P. malariae*, or *P. ovale* target sequences in our malaria prevalence studies in Papua New Guinea (Zimmerman PA, unpublished data).

To differentiate between treatment failures and newly established blood stage infections, all infections with *P. falciparum* or *P. vivax* (by PCR or LM) that occurred between the pre-treatment time point and six weeks post-treatment were genotyped to identify individual *P. falciparum* merozoite surface protein 2 alleles (*P. falciparum* infections^{23,24}) or the highly polymorphic *P. vivax* Duffy binding protein alleles (*P. vivax* infections²⁵).

Statistical analysis. Children were monitored for acquiring new infections until they withdrew from the study, did not provide two consecutive bi-weekly blood samples, or were re-treated with antimalarial drugs. Time-to-first infection with each species was calculated as the time between the date of first treatment and an infection-positive result by PCR or LM by either active or passive case detection. In addition, we calculated time-to-first infections with > 500 and > 5,000 parasites/ μ L for *P. falciparum* and > 150 parasites/ μ L for *P. vivax*. Children that remained negative for a particular species and/or density cut-off until the end of active follow-up were censored after the last active follow-up time point (after 169–181 days of follow-up). Prevalence of infection during follow-up was calculated for only those children who had not been re-treated.

The risk of symptomatic malarial illness was assessed as time-to-first clinical episode and incidence of clinical episodes during follow-up. Clinical malaria was defined as a measured fever (axillary temperature $\geq 37.5^{\circ}$ C) or history of febrile illness during the 48 hours preceding examination in conjunction with any malaria infection. The cut-off value for clinical disease was set at 5,000/ μ L for *P. falciparum*²⁶ and 1,000/ μ L for all other species (Mueller I, unpublished data). Children were monitored for clinical disease until they either withdrew from the study or were re-treated with antimalarial drugs. Children without a clinical episode were excluded after the last bi-weekly follow-up time point.

For the calculation of incidence rates, all disease episodes observed during active follow-up and passive morbidity surveillance were considered. In contrast to analyses for risk of infections, a child was considered at risk until he or she withdrew or reached the end of the study with the exception of the four weeks after further antimalarial treatments for the analy-

ses of difference in incidence rates. If a child was recorded as ill with the same species twice within a two-week period, this was considered a single episode.

Time-to-event (infection or clinical episode) data were analyzed using standard survival analysis techniques. A log-rank test was used to evaluate the difference in non-parametric survival curves. Because hazards for the different explanatory variables were generally proportional over the follow-up study, Cox regression was used to test for univariate and multivariate risk factors. Poisson regression was used for univariate and multivariate analyses of factors associated with the incidence of clinical *P. falciparum* disease. In all multivariate analyses, backwards selection and likelihood ratio tests were used to identify the best fitting models. All statistical analyses were performed using STATA 8 statistical analysis software (Stata Corporation, College Station, TX).

RESULTS

Enrollment and baseline characteristics. After obtaining informed consent and baseline health assessment, 206 children 5–14 years of age (inter-quartile range = 8.1–9.3 years) were enrolled into the study. Of these, 152 attended Mugil Elementary School, 44 attended Megiar Elementary School, and 10 attended Megiar Primary School. Of these children, 51.5% were girls and 35.9% reported having slept under a bed net the previous night.

At the time of enrollment, 92 children (44.7%) were positive for *Plasmodium* trophozoites by LM with *P. falciparum* the most common infection followed by *P. vivax* and *P. malariae* (Table 1). An additional 12 children were positive for *P. falciparum* gametocytes only. Geometric mean parasite densities for all species were generally low (Table 1). Densities of infections with *P. vivax* were significantly lower than those with *P. falciparum* ($P < 0.001$) or mixed infections ($P < 0.001$). The prevalence of infection by all species increased significantly when infections were diagnosed by LDR-FMA. Overall, 166 children (80.6%) were positive for any malarial infection with 54 (26.2%) having mixed species infections (Table 1). There was no significant association with prevalence between any species (by LDR-FMA) and bed net use.

Ninety-eight children (47.6%) had an enlarged spleen that

was associated with a concurrent malarial infection by either LM (odds ratio [OR] = 2.49, $P = 0.001$) or LDR-FMA (OR = 2.51, $P = 0.01$). Six children (2.9%) had moderate-to-severe anemia (Hb level = 5–8 g/dL) and 74 (35.9%) had mild anemia (Hb level = 8–11 g/dL) with no significant difference between boys and girls. All five children who had an axillary temperature $\geq 37.5^\circ\text{C}$ were positive for *P. falciparum* by both LM and LDR-FMA.

Initial antimalarial treatment. Of the 139 children with an LDR-FMA-positive *P. falciparum* infection at baseline, only 12 (8.6%) showed evidence of infection with the same merozoite surface protein 2 genotype in the six weeks after the seven-day treatment with artesunate. In one child, the genotype of the first re-infection could not be ascertained and the infection was thus considered as a suspected treatment failure. Genotyping for *P. vivax* showed that only one PCR-positive infection observed within the first six weeks after treatment had the same DBP genotype as the infection at baseline. Among *P. malariae* infections, there was one infection that remained PCR-positive at day 14. Because of the lack of a genotyping assay for *P. malariae*, this case was therefore also treated as a suspected treatment failure. No treatment failure was observed for *P. ovale*. Overall, the artesunate treatment had an efficacy of 91.4% for treating infections with *P. falciparum*, 92.9% for infections with *P. malariae*, and 98.6% for infections with *P. vivax*. All treatment failure children were excluded from further analysis of re-infection with the homologous *Plasmodium* species. However, they were included in analyses for heterologous *Plasmodium* infections.

Risk of re-infections during active follow-up. When treatment failure cases were excluded, only one child was positive for *P. falciparum* and none for any other species by LM two weeks after treatment. However, when we used LDR-FMA, 17 (8.9%) of 192 children were positive for *P. falciparum*, 12 (5.9%) of 204 were positive for *P. vivax*, and 9 (4.4%) of 203 were positive for *P. malariae* two weeks after treatment. Prevalence of infection increased steadily over the active follow-up period (Figure 2), with prevalence rates for *P. vivax* and *P. malariae* reaching pre-treatment levels 6 and 10 weeks after treatment, respectively. The prevalence of *P. falciparum* reached pre-treatment levels after 16 weeks. The comparatively slower increase in *P. falciparum* prevalence may be partly due to the large number of *P. falciparum* infections that became symptomatic (see below), which needed to be treated and were thus excluded from the analyses of prevalence rates after re-treatment time points (Figure 2).

The risk of acquiring blood stage *Plasmodium* infection was thus better estimated by the time to re-infection (Table 2 and Figure 3). When diagnosed by LDR-FMA, re-infection rates for *P. falciparum* and *P. vivax* were identical (incidence of 5.00 new infections with *P. falciparum* and 5.28 with *P. vivax*/child/year; $P = 0.29$, by log-rank test). However, the relative incidence of infections with either species changed over the course of follow-up. A significantly higher incidence of new *P. vivax* infections compared with *P. falciparum* was observed during the first 90 days of follow-up (Figure 3) (0–90 days: 4.33 *P. falciparum* and 5.28 *P. vivax* infections/child/year; $P = 0.04$), whereas the incidence of *P. falciparum* was significantly higher in the second 90 days (90–180 days: 9.04 *P. falciparum* and 5.23 *P. vivax* infections/child/year; $P = 0.03$). Infections with *P. malariae* (0.98/child/year) and *P. ovale* (0.42/child/

TABLE 1
Parasitologic and clinical assessment prior to treatment*

Infection species†	PCR-LDR-FMA		Light microscopy		
	No. pos	%	No. pos	%	Geometric mean density‡
Pf	85	41.3	68	33.0	361 (240, 544)
Pv	25	12.1	17	8.3	102 (74, 142)
Pm	2	1.0	4	1.9	421 (38, 4,646)
Pf and Pv	40	19.4	3	1.5	315 (183, 540)
Pf and Pm	8	3.9	0	0.0	–
Pf and Po	1	0.5	0	0.0	–
Pf, Pv, and Pm	4	1.9	0	0.0	–
Pf, Pv, and Po	1	0.5	0	0.0	–
Pf gametocyte only	–	–	12	5.8	68 (51, 90)
all positive	166	80.6%	104	50.5%	–

* PCR = polymerase chain reaction; LDR = ligation detection reaction; FMA = fluorescent microsphere assay; Pf = *Plasmodium falciparum*; Pv = *P. vivax*; Pm = *P. malariae*; Po = *P. ovale*.

† Only species combinations that were observed are shown.

‡ Mean number of parasites per microliter (95% confidence interval).

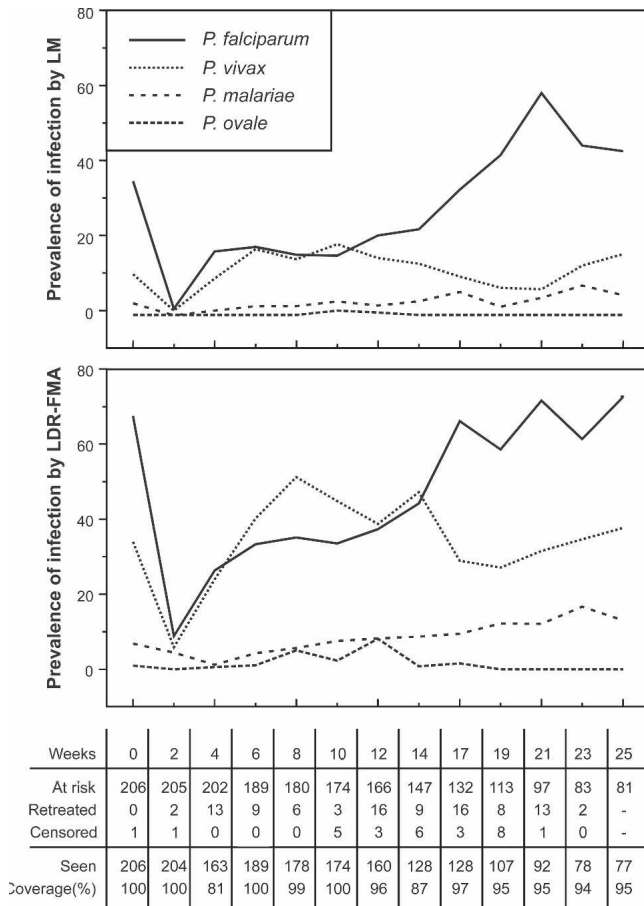


FIGURE 2. Prevalence of malarial infections at baseline and at active bi-weekly follow-up obtaining of blood samples. **Top**, Diagnosis by light microscopy (LM). **Bottom**, Diagnosis using post-polymerase chain reaction and ligation detection reaction-fluorescent microsphere assay (LDR-FMA). The number of children at risk, treated, and withdrawn during each two-week period and number of children seen during each follow-up are shown below the bottom panel.

year) were significantly less frequent than those with the two other species ($P < 0.001$).

Re-infection rates decreased significantly in all *Plasmodium* species when infections were diagnosed by LM ($P < 0.001$), with children acquiring 3.25 LM-detectable *P. falciparum* infections, 2.03 *P. vivax* infections, 0.24 *P. malariae* infections, and 0.04 *P. ovale* infections/child/year. Although children had significantly less new *P. vivax* infections than *P. falciparum* infections ($P < 0.001$), this effect was almost exclusively observed in the second half of follow-up (Figure 3: 0–90 days: 2.23 *P. falciparum* and 1.98 *P. vivax* infections/child/year; $P = 0.46$; after 90 days, 6.63 *P. falciparum* and 2.19 *P. vivax* infections/child/year; $P < 0.001$). The difference in incidence between these two species increased when only new moderate (*P. falciparum* $> 500/\mu\text{L}$ and *P. vivax* $> 150/\mu\text{L}$) and high-density (*P. falciparum* $> 5,000/\mu\text{L}$ and *P. vivax* $> 1,000/\mu\text{L}$) infections were considered (Figure 4). Although 78.9% and 47.8% of the children were re-infected with *P. falciparum* infections $> 500/\mu\text{L}$ and $> 5,000/\mu\text{L}$, respectively, only 25.1% and 5.6% had *P. vivax* infections $> 150/\mu\text{L}$ and $> 1,000/\mu\text{L}$, respectively ($P < 0.001$, by log-rank test).

For all *Plasmodium* species except *P. vivax*, some geo-

graphic variation in infection rates was observed, with children in more remote areas of the study area (those further away from health center and schools) having a significantly higher risk of acquiring new infections (Table 2). No significant associations were found between frequency of bed net use (average use = 35.9%) and risk of re-infections with any *Plasmodium* species. However, effects were small and not consistent between LDR-FMA- and LM-positive infections. Although some significant associations were observed between infection risk during the active follow-up period and infection status prior to artesunate treatment, no consistent relationships were detected. Time-to-first LM-positive *P. falciparum* infection was decreased in children who were PCR positive for *P. falciparum* at baseline but not in those who were only LM positive (Table 2). Infection status at baseline was also not significantly associated with time-to-first PCR-positive infections. Similarly, no clear association of infection status at baseline with risk of infection during follow-up was found for *P. vivax*. Although the risk of acquiring a new PCR-positive *P. vivax* infection was associated with an increased risk of having a *P. vivax*-positive blood slide at baseline, this association was not found with PCR positivity at baseline or with a risk of acquiring new LM-positive infections (Table 2). Although having a PCR-positive *P. malariae* infection was associated with a decreased risk for later infections with *P. ovale*, no association in infection status was observed for risk of infection with *P. malariae*.

A decrease in risk of acquiring infections with increasing age was only observed in LM-detectable *P. vivax* (adjusted hazard ratio [AHR] = 0.65, $P = 0.028$) and PCR-diagnosed *P. ovale* (AHR = 0.41, $P = 0.038$) infections. However, progressively larger decreases in risk with age were observed not only for increasing density cut-offs of *P. vivax* infections ($> 150/\mu\text{L}$, AHR = 0.42, $P = 0.01$) but also for *P. falciparum* infections (Figure 5). However, this effect only reached statistical significance for moderate and high-density infections (i.e. *P. falciparum* $> 500/\mu\text{L}$, AHR = 0.71, $P = 0.053$ and *P. falciparum* $> 5,000/\mu\text{L}$, AHR = 0.48, $P = 0.002$).

Incidence and risk factors for symptomatic malarial infections.

Overall, 162 febrile episodes with concurrent parasitemia were observed in 109 children (52.9%, Table 3). Of these, 111 (65.7%) exceeded species-specific pyrogenic thresholds of $5,000/\mu\text{L}$ for *P. falciparum* and $> 1,000/\mu\text{L}$ for non-*P. falciparum*. Most of these malaria-attributable illness episodes (92.8%) were caused by *P. falciparum*, with only five episodes caused by *P. vivax* (4.5%), two each by *P. malariae* and *P. ovale* (1.8%), and one by a *P. falciparum/P. vivax* mixed infection (0.9%). The distribution of febrile illness episodes with any concurrent parasitemia and malaria-attributable episodes was consistent with a Poisson distribution both overall and for *P. falciparum* episodes ($P > 0.5$, by Kolmogorov-Smirnov test). Although 20 children (9.7%) had more than one *P. falciparum*-attributable malaria episode, none had more than one non-*P. falciparum* malaria episode. With an incidence rate of 1.17 episodes/child/year, *P. falciparum*-attributable illness episodes were 21 times more frequent than those caused by *P. vivax* (0.06/child/year) and 52 times more frequent than *P. malariae* or *P. ovale* (0.02/child/year) ($P < 0.001$).

The incidence of febrile illness with any *P. falciparum* density was significantly lower in children greater than nine years of age (adjusted incidence rate ratio [IRR] = 0.67, 95% CI =

TABLE 2

Median time to first infections, incidence of new infections, and multivariate risk factors for acquiring new infections with different *Plasmodium* species diagnosed by LM and LDR-FMA*

	<i>P. falciparum</i>		<i>P. vivax</i>		<i>P. malariae</i>		<i>P. ovale</i>	
	PCR	LM	PCR	LM	PCR	LM	PCR	LM
% Re-infected†	95.3	87.6	82.0	49.5	29.3	8.3	13.6	1.5
Median time to re-infections (days)	55	99	54	118	–‡	–‡	–‡	–‡
Incidence rate (years)	5.00 (4.30, 5.80)	3.23 (2.76, 3.78)	5.28 (4.54, 6.14)	2.03 (1.68, 2.46)	0.98 (0.76, 1.26)	0.24 (0.15, 0.39)	0.42 (0.29, 0.61)	0.04 (0.01, 0.13)
Multivariate predictors§	AHR	AHR	AHR	AHR	AHR	AHR	AHR	–¶
Age > 9 years				0.67 (0.44, 0.96)			0.41 (0.17, 0.95)	
Distance from Mugil HC#	1.14 (1.02, 1.27)					1.44 (1.03, 2.01)		
Distance from school#		0.76 (0.64, 0.91)				1.68 (1.16, 2.45)	1.54 (1.08, 2.19)	
Enrolled at Megiar Elementary School							5.68 (2.45, 13.2)	
Pf LDR-FMA + at baseline		1.67 (1.18, 2.35)						
Pf LM + at baseline							3.79 (1.75, 8.18)	
Pv LM + at baseline			1.70 (1.03, 2.83)					

* LM = light microscopy; AHR = adjusted hazard ratio. For definitions other abbreviations, see Table 1. Values in parentheses are 95% confidence intervals.

† Proportion of children with at least one positive sample while at risk. Total children at risk: Pf: n = 194, Pv: n = 206; Pm: n = 205; Po: n = 206.

‡ Not estimated because less than 50% of children re-infected.

§ Only significant factors ($p < 0.05$) from multivariate Cox regression analyses are given. Other factors included sex, distance from seacoast, presence of enlarged spleen and/or mild anemia (hemoglobin < 11 g/dL), reported bed net use (slept > 50% of nights under bed net), and infection status with any infection by PCR and LM at baseline.

¶ Not estimated due to very low re-infection rate (n = 3).

Distances calculated as straight line distance (km) from child's residence to Mugil Health Center (HC) or school.

0.48–0.93, $P = 0.019$) and in children with an LM-positive *P. falciparum* infection at baseline (IRR = 0.66, 95% CI = 0.45, 0.97, $P = 0.032$). In addition, children living within one kilometer of the coastline more frequently had a fever and concurrent parasitemia (IRR = 1.57, 95% CI = 1.11–2.23, $P = 0.011$).

The decrease in incidence of *P. falciparum* illness with increasing age was more pronounced when only febrile episodes with a *P. falciparum* parasitemia greater than 5,000/ μ L were considered (IRR = 0.55, 95% CI = 0.37–0.83, $P = 0.005$). However, the incidence of high *P. falciparum* density episodes was no longer significantly associated with infection status at baseline ($P > 0.1$). As for fevers with any *P. falciparum* density, spatial differences in incidence of high *P. falciparum* density episodes were observed with those children living near the coastline (IRR = 1.61, 95% CI = 1.05, 2.46, $P = 0.030$) and/or with those attending Mugil Elementary School (IRR = 2.02, 95% CI = 1.20, 3.40, $P = 0.008$) who had an increased risk. A tendency for lower incidence of high-density clinical *P. falciparum* infections was observed in mildly anemic children (Hb level < 11 g/dL, IRR = 0.69, 95% CI = 0.45–1.05, $P = 0.084$).

Of the 103 children with ≥ 1 febrile illness episodes and concurrent *P. falciparum* parasitemia of any density, five (4.9%) were re-treated with antimalarials prior to the episode and were thus excluded from time-to-event analyses. Because all febrile episodes with any concurrent parasitaemia were treated, 12 (15.0%) of 80 children who had ≥ 1 episode with a *P. falciparum* parasitemia > 5,000/ μ L were excluded because of earlier re-treatment. Similar to our preceding analysis, the hazard of having a *P. falciparum* episode, both overall and for high-density infections, was higher in children attending the Mugil school and/or coastal hamlets, but lower in

children greater than nine years of age (Table 4 and Figure 6). In addition, female children tended to present more quickly with *P. falciparum* illness than males. Children with mild anemia (Hb level < 11 g/dL) had a significantly a lower risk of having a febrile illness (all episodes: AHR = 0.59, $P = 0.020$ and *P. falciparum* > 5,000/ μ L: AHR = 0.52, $P = 0.016$).

Mosquito transmission dynamics. The biting rates of *Anopheles* sp. in the coastal and inland (> 2.5 km) villages were 1.2 bites/person/night and 5.81 bites/person/night, respectively. A sampling effort of 188 person-nights over a 12-month period, identified 659 mosquitoes belonging to the *An. punctulatus* group. These included *An. punctulatus* (34.1%), *An. koliensis* (51.6%), and *An. farauti* (14.3%). *Anopheles farauti* (41.6%) and *An. koliensis* (41.6%) were equally abundant in coastal villages where *An. punctulatus* accounted for only 16% of mosquitoes caught. In inland villages, *An. punctulatus* (37.3%) and *An. koliensis* (51.9%) were notably more prevalent than *An. farauti* (8.6%).

Only 113 *Anopheles* mosquitoes were caught in the coastal village and none of the 53 processed by ELISA was positive for malaria parasites. In inland villages, the *P. falciparum* and *P. vivax* sporozoite rates for *An. punctulatus* s.l were 1.89% and 1.26%, respectively. The corresponding entomologic inoculation rates were 36.56 and 24.38 infective bites/person/year for *P. falciparum* and *P. vivax*, respectively.

DISCUSSION

The present study shows that Papua New Guinean children 5–13 years of age have acquired significantly different levels of immunity against *P. falciparum* and *P. vivax*. Despite comparable number of PCR-detectable new blood infections, children were 21 times more likely to have illness from *P.*

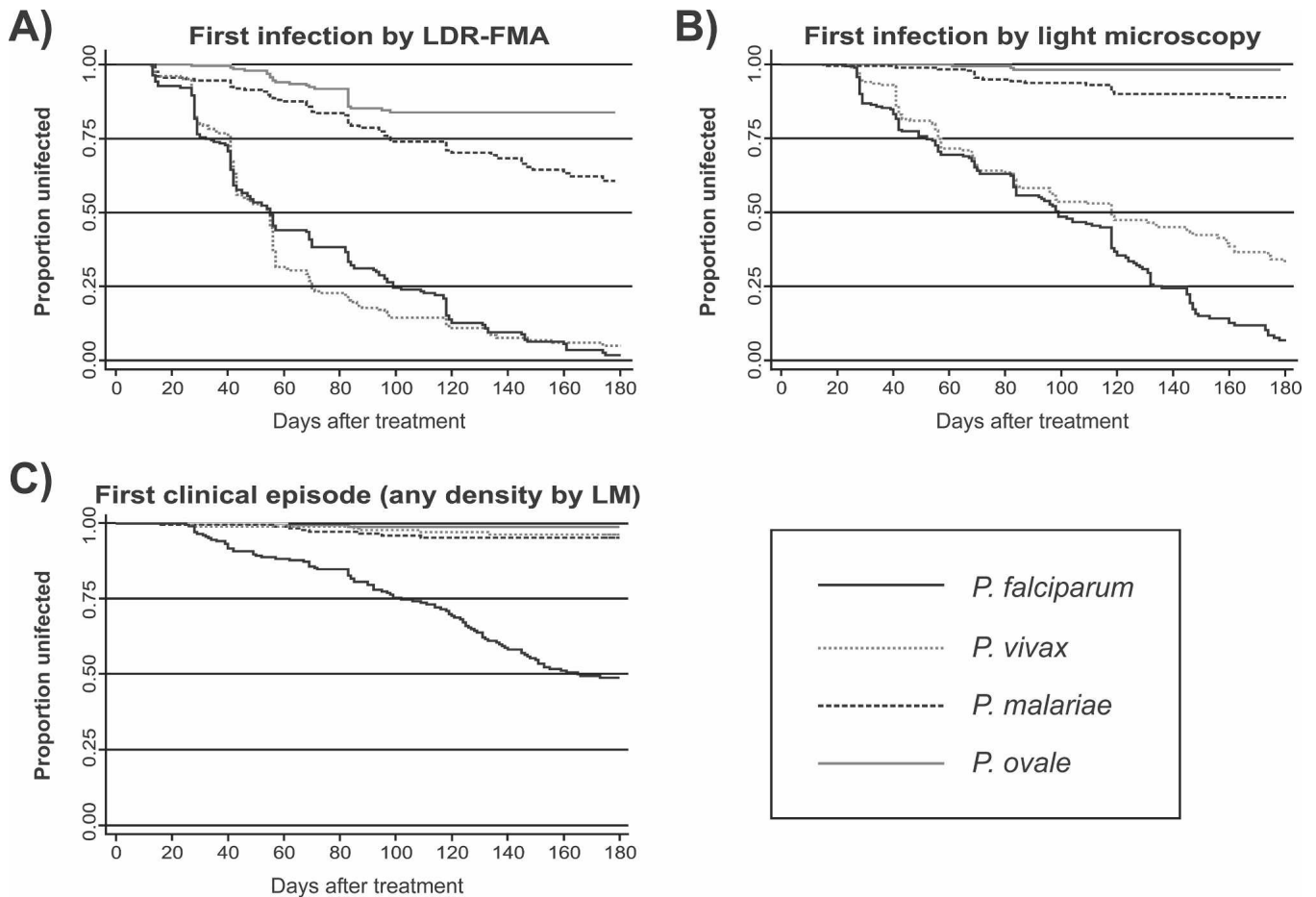


FIGURE 3. Risk of acquiring new infections after treatment of blood-stage infections. Kaplan-Meier curves for **A**, Asymptomatic infections diagnosed by post-polymerase chain reaction and ligation detection reaction-fluorescent microsphere assay (LDR-FMA), **B**, Asymptomatic infections diagnosed by light microscopy (LM), and **C**, Episodes of febrile illness with concurrent malarial infection of any density (by LM).

falciparum than from *P. vivax* infections. By the time they reach elementary school age, children in the study appeared to have acquired almost complete clinical immunity to *P. vivax*, whereas the acquisition of clinical immunity against *P. falciparum* was ongoing, with older children showing reduced risk.

After treatment with a very short half-life drug, we saw rapid re-infection of children. In particular, prevalence of *P. vivax* increased quickly and parasite prevalence (by LDR-FMA and LM) exceeded those observed prior to treatment. Although by the end of follow-up virtually all children had been infected at least once with both parasites, it took significantly longer for *P. falciparum* prevalence rates to reach the levels seen at baseline. The fact that we did not use a drug with activity against liver stages may have influenced the rapid recurrence of *P. vivax* infections. However, in an area with year-round, albeit moderately seasonal, transmission such as Madang,²⁷ the rates of acquiring new liver stage infections through mosquito bites and establishing new blood stage infections from liver stages are likely to be comparable. The clearance of liver stages by drug treatment may lead to an underestimation of the incidence of *P. vivax* blood stage infections, particularly early in the follow-up period when new liver stage infections are acquired, but no new blood stage infections are established from existing liver stages. The high

number of blood stage infections observed is thus likely to be reflective of the true burden of infection with *P. vivax* in the study community.

The varying delay between time of initial infection by mosquito bite and appearance of blood stages in *P. vivax* may mean that seasonality of blood stage infections is partially uncoupled from seasonality of transmission. Some evidence for this is seen in this study cohort. The study was started and treatment was given in June, at the end of the high transmission season with the first half of the follow-up coinciding with the period of lowest transmission (July–September).^{11,27} Although the incidence of new *P. falciparum* infections (by LDR-FMA and LM) was lower during the first half of follow-up, the incidence of *P. vivax* was relatively constant over time. Relapses from long-lasting liver stages may be an important contributor to the more limited seasonality of *P. vivax* that was also observed in earlier population surveys in the Madang area.^{11,27}

It was evident that the children in the cohort were significantly better at controlling moderate and high-density *P. vivax* infections than *P. falciparum* infections (Figure 4). A considerable number of LDR-FMA-detectable *P. vivax* infections never became detectable by LM. Of those that did, few reached the pyrogenic threshold of 1,000 parasite/ μ L and only five children had a symptomatic *P. vivax* episode. This

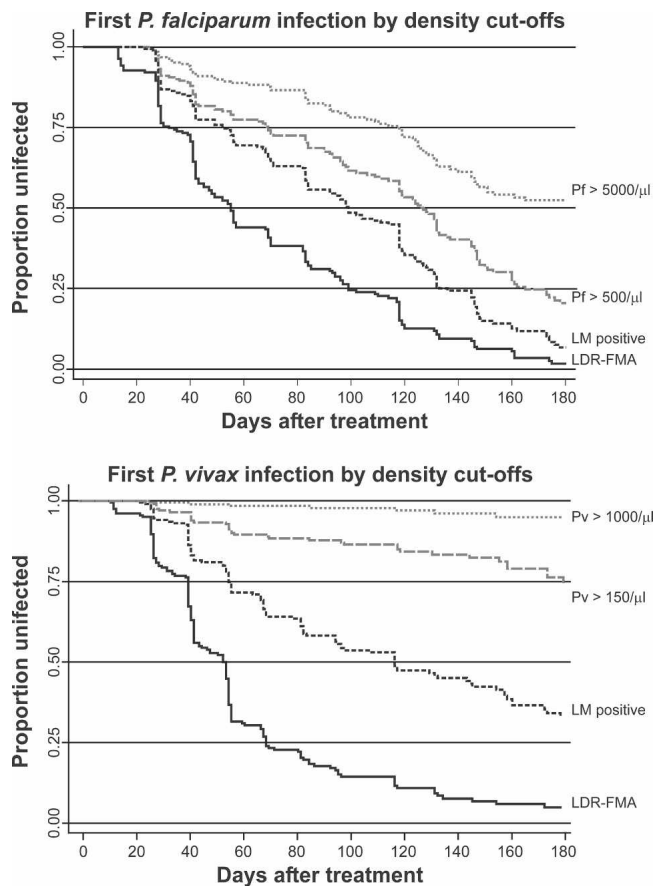


FIGURE 4. Kaplan-Meier curves of time to first *Plasmodium falciparum* (Pf) and *P. vivax* (Pv) infections exceeding increasing density thresholds. LM = positive by light microscopy; LDR-FMA = positive by post-ligation polymerase chain reaction ligation detection reaction-fluorescent microsphere assay. Shown are species-specific density cut-offs: higher cut-offs are set at species-specific pyrogenic thresholds and lower cut-offs are set at approximately 1.5 times the geometric mean density observed at baseline.

low incidence of clinical *P. vivax* disease in children 5–13 years of age is consistent with data from a passive morbidity surveillance system in another community in Papua New Guinea,⁴ which showed that the incidence of *P. vivax* illness peaks in children 1–2 years of age and becomes rare in children greater than five years of age (Mueller I, Genton B, unpublished data). Conversely, almost half of all children had at least one *P. falciparum* infection > 5,000/ μ L and 80 (39%) children were diagnosed with 1–3 episodes of *P. falciparum* illness during the six months of follow-up. In a similar treatment re-infection study conducted in northern Ghana,²⁸ the incidence of *P. falciparum* disease after radical treatment for blood stage infections was higher than that usually observed for the same age group. On the basis of evidence that in older children asymptomatic infections of high multiplicities offered protection against subsequent morbidity,²⁹ Smith and others reported that the initial treatment of asymptomatic infections increased the children's risk of clinical illness. Although the incidence of *P. falciparum* illness in our study was high, it was similar to that observed in an earlier study in a neighboring population that measured malarial associated fever rates in children 2–15 years of age using a weekly morbidity surveillance program.¹¹ Because this earlier study did

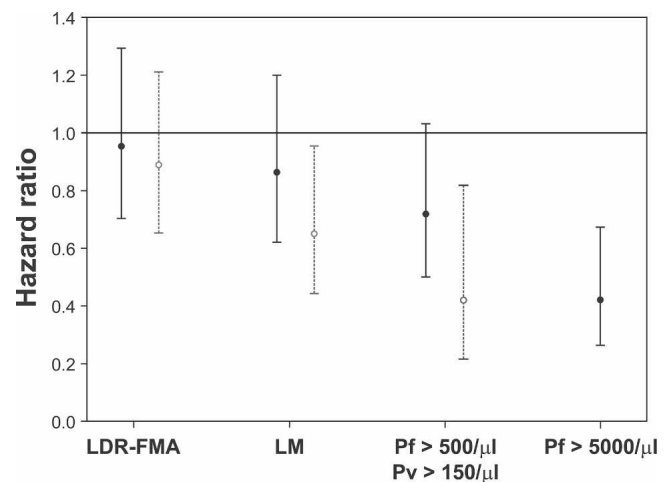


FIGURE 5. Risk of acquiring new infections in relations to age and increasing density cut-offs. Values are adjusted hazard ratios and 95% confidence intervals. ● = *Plasmodium falciparum* (Pf); ○ = *P. vivax* (Pv). Because of low number of infections, no age effect for Pv parasitemias > 1,000/mL was estimated. LDR-FMA = positive by post-polymerase chain reaction ligation detection reaction-fluorescent microsphere assay; LM = positive by light microscopy. Shown are species-specific density cut-offs: higher cut-offs for Pf are set at species-specific pyrogenic thresholds and lower cut-offs set at approximately 1.5 times the geometric mean density observed at baseline.

not treat children prior to follow-up and overall *P. falciparum* prevalence in asymptomatic individuals (39%) was comparable to what we observed at baseline, we believe it is unlikely that the initial treatment led to an increase in incidence of *P. falciparum* disease.

The relatively limited heterogeneity in the cohort in re-infection risk in relation to sex and place of residence indicates that age is likely to be closely correlated with lifetime exposure and thus is a good proxy for overall immune status. Comparing differences in risk of infections with different parasite densities not only shows that high-density *P. vivax* infections are rare, but that despite a similar number of new blood stage infections (as detected by LDR-FMA), the risk of acquiring even moderate and low-density *P. vivax* infections is significantly lower in older children (i.e. > 9 years of age). This indicates that by nine years of age children have acquired an ability to control *P. vivax* densities at levels well below pyrogenic thresholds with many infections not becoming patent by LM. In contrast, control of *P. falciparum* densities is less developed. Most blood stage *P. falciparum* infections do eventually become LM positive, and only at moderate-to-high densities is a reduction in risk apparent in older children. Nevertheless, some older children still have frequent high-density *P. falciparum* infections and clinical illness. These differences in ability to control parasite densities may explain why in most studies in areas with moderate-to-high endemicity for malaria, *P. vivax* prevalence^{3,10,11,27} and incidence rates³⁰ peak in younger age groups than *P. falciparum* prevalence and incidence rates.

The observed difference in rates of immune acquisition for *P. falciparum* and *P. vivax* is remarkable, given that entomologic inoculation rates, as well as re-infection rates (by PCR), for both species are comparable. By the time children reach elementary school age, i.e. 6–7 years, they are likely to have

TABLE 3
Incidence of symptomatic malaria episodes by species and case definition*

	Any episode		<i>P. falciparum</i>		<i>P. vivax</i>		<i>P. malariae</i>		<i>P. ovale</i>	
	Any	Threshold	Any	> 5,000/ μ L	Any	< 1,000/ μ L	Any	> 1,000/ μ L	Any	> 1,000/ μ L
No. episodes	162	111	148	103	10	5	8	2	3	2
No. mixed	7	1	5	1	4	1	5	0	0	0
No. of children with										
0 episodes	97	123	103	126	196	201	198	204	203	204
1 episode	62	57	64	60	10	5	8	2	3	2
2 episodes	36	23	33	17						
3 episodes	9	3	6	3						
4 episodes	2	0	0	0						
% children with ≥ 1 episode	52.9	40.3	50.0	38.8	4.9	2.4	3.9	1.0	1.5	1.0
Incidence rate [†] (/child/year)	1.92	1.27	1.68	1.17	0.11	0.06	0.09	0.02	0.03	0.02
	(1.65, 2.23)	(1.06, 1.53)	(1.43, 1.98)	(0.97, 1.42)	(0.06, 0.21)	(0.02, 0.14)	(0.05, 0.18)	(0.01, 0.09)	(0.01, 0.11)	(0.01, 0.09)

* Values in parentheses are 95% confidence intervals.
[†] Estimated from Poisson regression model. Total time at risk = 32,125 person days.

TABLE 4
Multivariate predictors for time to first *Plasmodium falciparum* (Pf) episode: infections with any density versus infections > 5,000/ μ L*

Multivariate predictors [†]	Any Pf density		Pf > 5,000/ μ L	
	AHR	(95% CI)	AHR	(95% CI)
Enrolled at Mugil				
Elementary School	1.62	(0.98, 2.68)	3.35	(1.58, 7.10)
Coastal residence [‡]	2.08	(1.34, 3.21)	1.98	(1.19, 3.30)
Female	1.41	(0.94, 2.12)	1.52	(0.93, 2.40)
Age > 9 years	0.56	(0.37, 0.85)	0.51	(0.31, 0.84)
Hb < 11 g/dL	0.59	(0.38, 0.92)	0.52	(0.30, 0.88)

* AHR = adjusted hazard ratio; CI = confidence interval; Hb = hemoglobin.
[†] Only factors with a *P* value < 0.05 and 0.05–0.10 (**bold**) from multivariate Cox regression analyses are given. Other factors included distance from school, distance from health center, presence of enlarged spleen, reported bed net use (slept > 50% of nights under bed net) and infection status with any *Plasmodium* species diagnosed by PCR and LM at baseline.
[‡] Residence of child situated less than one kilometer from coastline.

been exposed to 150–200 infected bites and had 30 successful blood stage infections with each species. Why a similar amount of exposure can lead to these apparent differences in immunity to the two species is not clear. However, similar differences in rates of immune acquisition have been described in Vanuatu³⁰ and other areas of the world where the species co-existed.^{31,32} These results are consistent with data from malaria therapy patients in whom immunity to *P. vivax* was more rapidly acquired than that to *P. falciparum*.³³ Whereas a single infection with *P. vivax* resulted in a strongly reduced incidence of a febrile episode upon homologous and to a lesser extent heterologous re-infection,³⁴ most secondary *P. falciparum* infections were associated with fever, and in some cases high-density parasitemia, even if re-infected with the same strain.³⁵ Similar patterns were observed under natural conditions in Sri Lankan patients.³⁶

Contrary to the relative homogeneity in re-infection risk in relation to sex and place of residence, there were significant, spatially structured differences in risk of *P. falciparum* disease between children attending the Mugil Elementary School and those living near the coast who more often had a febrile episode of *P. falciparum* parasitemia; girls also tended to be at higher risk of illness. However, these differences are more likely to be related to differences in accessibility and health-seeking behavior rather than reflect true difference in risk of

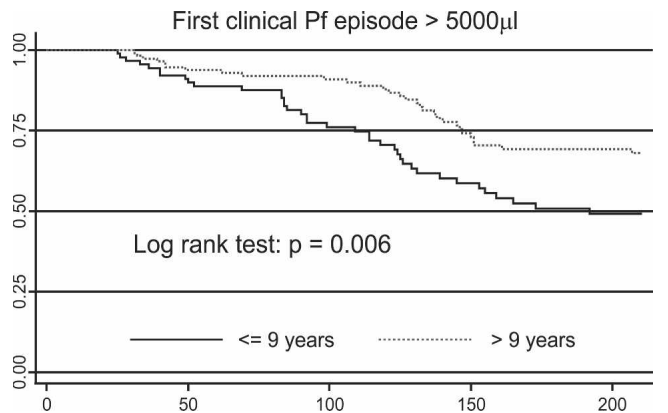


FIGURE 6. Kaplan-Meier curves of risk of experiencing an episode of symptomatic *Plasmodium falciparum* (Pf) malaria with a parasite density greater than 5,000 parasites/ μ L in relation to age.

disease. The higher frequency of study team visits to the Mugil school indicated that children who were sick while attending school were more likely to be reported to the study team on non-surveillance days than at the other schools. Similarly, the vicinity to the schools and the sealed road leading to the health center indicated that it was substantially simpler for children living in coastal hamlets to seek treatment from either study team or health center. Such a decrease in attendance at health centers with increasing distance has been previously observed in Papua New Guinea.³⁷

The observed association of mild anemia (Hb level < 11 g/dL) with protection against clinical *P. falciparum* disease is intriguing because such a mild degree of anemia is by itself unlikely to be an impediment to parasite growth. Because malaria is an important contributor to anemia,^{38–40} Hb levels at baseline may be a marker of recent malarial exposure rather than directly affecting risk of *P. falciparum* illness.

Although almost 40% of children did have at least one LDR-FMA-detectable *P. malariae* infection, most of these infections were not apparent by LM, and as in areas of Africa highly endemic for malaria,^{41,42} episodes of *P. malariae* illness were rare. The low number of episodes precludes drawing firm conclusion with regards to acquisition of immunity to *P. malariae* in this population. However, because seven of eight clinical episodes occurred in children greater than nine years of age, older children are still at least as susceptible as younger children to *P. malariae* and the acquisition of immunity to this species may consequently be rather slow. Similar slow acquisition of immunity to *P. malariae* is also supported by observations by Smith and others⁹ and Kasehagen and others¹⁰ that in another population in Papua New Guinea the prevalence of *P. malariae* infections peaked later than those for *P. vivax* and *P. falciparum*. As observed in earlier cross-sectional surveys^{10,16} infections and illness with *P. ovale* were rare and, as observed in Africa,⁴³ most *P. ovale* infections were of very short duration. In contrast to infections with other species, *P. ovale* infections were clustered in time and space with significantly higher risk of infection between weeks 6 and 12 of follow-up and in children attending Megiar Elementary School.

Since the suggestion by Williams and others that an increased incidence of *P. vivax* infection in early life may protect children with α -thalassemia from later severe *P. falciparum* malaria,⁴⁴ a number of studies have investigated potential cross-species protection. Although clinical studies from Thailand,^{45,46} as well as an epidemiologic study from Papua New Guinea,⁴⁷ provide some support to the idea of cross-species protection by *P. vivax* against *P. falciparum* morbidity, it has been remarkably difficult to find consistent evidence that infection with one species protects against (concurrent or subsequent) heterologous infections.^{16,47–50} With the exception of a possible positive interaction between prior infection with *P. falciparum* and subsequent risk of infection with *P. ovale*, the present study did not find any evidence that the presence of a heterologous infection at baseline provided protection against subsequent re-infections or *P. falciparum* illness. However, persistent, concurrent parasitemia may be required for cross-species protection. Thus, it is possible that the lack of associations between heterologous species is caused by clearance of blood stage infections by the initial treatment rather than truly indicating a lack of cross-species protection. The existence of cross-species protection is thus

better studied in conventional cohort rather than a treatment re-infection design.

The current study clearly demonstrates differences in the acquisition of clinical immunity to *P. falciparum* and *P. vivax* among Papua New Guinean children. Despite similar incidence of infections, *P. vivax* is no longer a consistent source of morbidity by the time children reach school age. Although it continues to decrease with age, the burden of *P. falciparum* illness remains high. In the age group studied (i.e., 5–14 years), clinical immunity seems to be linked closely to the ability to control parasite densities, with children able to control *P. vivax* at much lower densities than *P. falciparum*. Potential reasons for the differential rates in the acquisition of immunity may include key biologic differences between the two species, particularly in relation to red blood cell invasion, adhesion or sequestration in vascular beds, and the nature of variant surface antigens. Understanding these differences may provide key insights into immunity and pathogenesis with *P. falciparum* and *P. vivax* and is the focus of on-going studies. Comparative studies on immune acquisition to both *P. falciparum* and *P. vivax* species may thus provide valuable insight into the important different immune targets and mechanisms underlying acquisition of clinical protection.

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