Asymptomatic Multiclonal *Plasmodium falciparum* Infections Carried Through the Dry Season Predict Protection Against Subsequent Clinical Malaria

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Background. Immunity to the antigenically diverse parasite *Plasmodium falciparum* is acquired gradually after repeated exposure. Studies in areas of high malaria transmission have shown that asymptomatic individuals infected with multiclonal infections are at reduced risk of febrile malaria during follow-up.

Methods. We assessed the relationship between the genetic diversity of clones in *P. falciparum* infections that persist through the dry season and the subsequent risk of febrile malaria in 225 individuals aged 2–25 years in Mali, where the 6-month malaria and dry seasons are sharply demarcated. Polymerase chain reaction–based genotyping of the highly polymorphic merozoite surface protein 2 gene was performed on blood samples collected at 5 cross-sectional surveys.

Results. In an age-adjusted analysis, individuals with multiclonal *P. falciparum* infections before the rainy season were at reduced risk of febrile malaria, compared with individuals who were uninfected (hazard ratio [HR], 0.28; 95% confidence interval [CI], .11–.69). In contrast, there was no significant association between risk of malaria and having 1 clone at baseline (HR, 0.71; 95% CI, .36–1.40).

Conclusions. The results suggest that persistent multiclonal infections carried through the dry season contribute to protection against subsequent febrile malaria, possibly by maintaining protective immune responses that depend on ongoing parasite infection.

Keywords. immunity; *P. falciparum*; *msp2*; genotyping; malaria; children.

A clear understanding of how and when immunity to *Plasmodium falciparum* is acquired and maintained under different transmission intensities could inform the development and implementation of interventions that contribute to the control and elimination of malaria, such as antimalarial vaccines [1]. Immunity to malaria

develops over time and after repeated exposure in individuals living in areas of endemicity [2]. Antibodies against antigens expressed by the merozoite stage have been associated with protection against malaria [3, 4], rendering these antigens interesting vaccine candidates, but to date, vaccines targeting merozoite antigens have been unsuccessful. *Plasmodium falciparum* infections are often composed of several genetically distinct clones with different composition of merozoite surface proteins and other antigens [5–7]. Thus, the long time required to achieve protection against malaria may be due in part to the need to encounter and thus mount immunity against a wide range of these variants.

In areas of high transmission, multiclonal *P. falciparum* infections in individuals who are asymptomatic at

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baseline have been associated with a reduced risk of subsequent clinical malaria [8–10], whereas in the youngest age groups the inverse relationship has been observed [11, 12], and the associations appear to be transmission dependent [13]. Whether the number of clones is simply a marker of exposure and thus a proxy for immune responses generated by repeated and recent infections is unclear. Circulating low-density parasitemias in individuals with partial immunity might, however, also contribute to the maintenance of protection [14]. Distinguishing between persistent and transient infections in areas of continuous transmission is difficult. To establish the importance of asymptomatic persistent infections for maintenance of immunity, we studied the genetic diversity of *P. falciparum* infections carried through the dry season in a cohort living with seasonal and sharply demarcated malaria transmission in Mali. Cohort participants positive for *P. falciparum* by microscopy at the end of the 6-month dry season have a lower risk of febrile malaria during the ensuing 6-month rainy season [15]. Here, we tested the hypothesis that the genetic diversity of *P. falciparum* infections persisting through the dry season affects the risk of febrile malaria during the following malaria transmission season. In addition, by genotyping the gene encoding merozoite surface protein 2 (*msp2*) with high-resolution capillary electrophoresis for fragment analysis [16, 17], we sought to examine the diversity of *P. falciparum* populations in the setting of intense seasonal malaria transmission.

MATERIAL AND METHODS

Study Site

The study was conducted in Kambila, a rural village with a population of approximately 1500 individuals, situated 20 km north of Bamako, Mali. Transmission of *P. falciparum* is intense and strictly seasonal, beginning in June, peaking during September through November, and ending in December. Details of the study site and cohort have been previously described [15].

Study Population and Sampling

The study cohort consisted of 176 children 2–10 years of age and 49 adults 18–25 years old. Overall in the study population, 52% were female. The HbAS genotype (ie, the sickle cell trait) was present in 10.4% of individuals. From an age-stratified census of the entire village population, a predetermined number of individuals was randomly selected from each age stratum and invited to participate in this study. By design, randomization was skewed to include more children than adults since the overall objective of the cohort study was to focus on children, because they transition from malaria susceptibility to semiimmunity [15].

Blood smears and filter papers with dried blood spots were collected before the rain season (May 2006), at cross-sectional time points every 2 months during the malaria season (July,

October, and December 2006), and just prior to the start of the second malaria season (April 2007).

Clinical Episodes of Malaria

Participants were encouraged to report symptoms of malaria at the village health center, staffed 24 hours per day by a study physician during the study period (May 2006–April 2007). Individuals attending unscheduled so-called sick visits had a blood smear examined by microscopy for the presence of *P. falciparum*. Slide-positive patients were treated with a 3-day course of artesunate plus amodiaquine, following the national guidelines in Mali. Children with severe malaria, as defined by the World Health Organization [18], were referred to Kati District Hospital after receipt of an initial parenteral dose of quinine. The study definition of malaria was an axillary temperature of $>37.5^{\circ}\text{C}$, an asexual *P. falciparum* parasitemia level of ≥ 5000 parasitemia/ μL [19], and no signs indicative of any other disease.

Detection of *P. falciparum* Parasites by Microscopy

Thick blood smears were stained with Giemsa and analyzed by light microscopy. Parasites were counted against 300 leukocytes, and parasite densities were recorded as the number of asexual parasites per microliter of whole blood, based on an average leukocyte count of 7500 cells/ μL . Each smear was evaluated separately by 2 expert microscopists.

Genotyping *msp2*

DNA was extracted from filter paper blood spots (Whatman 3M), using the DNA MicroAmp Kit (Qiagen). Genotyping of *msp2* was performed by a nested polymerase chain reaction (PCR) assay [16]. In brief, an initial amplification of the outer *msp2* domain was followed by 2 separate nested reactions with fluorescently labeled primers targeting the 2 allelic types (families) of *msp2*: FC27 and IC-1 (also referred to as 3D7). PCR fragments were analyzed by capillary electrophoresis in a DNA sequencer (3730, Applied Biosystems) and Gene Mapper software (Applied Biosystems). Fragments were considered the same allele if their sizes were within the same 3 base pair (bp) bins within the 2 respective allelic types. The *msp2* alleles were named according to their allelic type (FC27 or IC) followed by the length of the amplified fragment (eg, 410-bp long FC27 fragments were named “FC410”).

Ethical Considerations

Ethical approval for this study was obtained from the Faculty of Medicine, Pharmacy, and Odonto-Stomatology Ethics Committee, University of Bamako; the Institutional Review Board at the National Institute of Allergy and Infectious Diseases, National Institutes of Health (Bethesda, Maryland); and the Central Ethical Review Board (Stockholm, Sweden). Written, informed consent was obtained from adult participants and from the parents or guardians of participating children.

Statistical Analysis

Analyses were performed with GraphPad PRISM, version 5, and Stata, version 13. The relationship between the number of clones and parasite densities was analyzed using Spearman's ρ . Parasite densities in different age categories were presented as geometric means. The time to first malaria episode (defined by study criteria) was assessed by Cox regression analyses, in which baseline was defined as either May 2006 or July 2006 and follow-up was until April 2007. The number of concurrent clones was categorized as 1 clone or ≥ 2 clones (ie, multiclonal infections). Age adjustments were made in the 4 predefined age groups (2–4, 5–7, 8–10, and 18–25 years). Treatment was included as a time-dependent covariate to adjust for the post-treatment protection mediated by amodiaquine. A 100% prophylactic effect against new infections was assumed the day after treatment, decaying exponentially with a half-life of 4 weeks. The Andersen-Gill regression (AG) model was used to model recurrent episodes of malaria. Since malaria incidence is overdispersed in this population, we included a γ -distributed frailty term in the AG model. Individuals were excluded from the analysis if information regarding the number of clones at baseline was missing (<1% for May 2006 and 3% in July 2006).

A summary measure of the number of clones for each individual was obtained by adding the number of clones from each survey. The association between HbAS genotype and this

summary measure was then modeled using Poisson regression. Individuals were excluded from the analysis if information from (at least) 1 survey was missing.

RESULTS

Clinical Episodes

The demarcated seasonal pattern of malaria transmission is illustrated by the number of malaria cases in the cohort recorded per day between May 2006 and January 2007 (Figure 1A [15]). Among the 495 sick visits at the clinic during the study period, 425 were *P. falciparum* positive by microscopy and received antimalarial treatment. Of the 425 treated malaria episodes, 298 met the more stringent study definition of a clinical malaria episode (ie, temperature of $>37.5^{\circ}\text{C}$ and parasite densities of >5000 asexual parasites/ μL of blood). Children had 0–5 episodes, whereas adults had 0–1 episodes during the 1-year follow-up period, with at least 1 malaria episode experienced in 79.0% of children and 8.2% of adults (Table 1). Severe malaria was diagnosed in 5 cases, all of which occurred in children <8 years of age.

Parasite Prevalence and Densities at Cross-sectional Surveys

Microscopy-determined parasite prevalence was 7.1% in May 2006 (just before the malaria season), 8.0% in July, 25.2% in

Figure 1. A, The number of clinical malaria episodes per day from May 2006 to January 2007, adopted from Crompton et al [15]. A malaria episode was defined as the presence of both an axillary temperature of $>37.5^{\circ}$ and >5000 asexual parasites/ μL blood as determined by microscopy. Arrows indicate time points for cross-sectional sampling. B, Parasite prevalence determined by microscopy at cross-sectional time points in different age groups. Error bars represent 95% exact binomial confidence intervals (CIs). C, Parasite prevalence measured by *msp2*-specific polymerase chain reaction (PCR) at cross-sectional time points in different age groups. Error bars represent 95% exact binomial CIs. D, Geometric mean parasite densities determined by microscopy at cross-sectional visits for the respective age groups, including only microscopy-positive individuals. None among the 2–4-year-old children had parasites detected by microscopy at the last survey in April 2007.

Table 1. Characteristics of the Study Cohort, by Age

Characteristic	2–4 y (n = 73)	5–7 y (n = 52)	8–10 y (n = 51)	18–25 y (n = 49)	Overall (n = 225)
Female sex	42 (57.5)	25 (48.1)	19 (37.3)	30 (61.2)	115 (51.6)
HbAS	8 (11.6)	4 (8.0)	5 (9.8)	5 (11.9)	22 (10.4)
Malaria episodes, no. ^a	1.99 ± 1.25	1.94 ± 1.21	0.98 ± 1.05	0.08 ± 0.28	1.33 ± 1.30
At least 1 malaria episode ^b	86.3 (63)	86.5 (45)	60.8 (31)	8.2 (4)	63.6 (143)
Sick visits with parasites detected, no. ^c	2.58 ± 1.19	2.74 ± 1.21	1.81 ± 1.19	0.33 ± 0.51	1.86 ± 0.95

Data are no. (%) of subjects or mean value ± SD.

Abbreviation: HbAS, sickle cell trait.

^a Defined as the number of malaria episodes over the 1-year follow-up. The study definition of a malaria episode was an axillary temperature of $\geq 37.5^{\circ}\text{C}$ and a parasite density of ≥ 5000 parasites/ μL .

^b During follow-up, defined as May 2006–April 2007.

^c Defined as detection of parasitemia at a sick visit, without regard to fever or parasite density.

October, 21.0% in December (at the end of the rainy season), and 9.3% in April 2007 (before the next rainy season; Figure 1B). Parasite prevalence determined by *msp2*-specific PCR was higher than that detected by microscopy, with values of 14.2%, 15.1%, 34.7%, 30.7%, and 16.4% in May, July, October, December, and April, respectively (Figure 1C). Children aged 2–4 years had the lowest prevalence both by microscopy and PCR (Figure 1B and 1C) and the highest mean parasite densities at cross-sectional surveys (Figure 1D). Fifty-eight individuals (25.8%) were parasite negative at all cross-sectional surveys.

Parasite Diversity at Cross-sectional Surveys

A total of 1064 DNA samples (mean, 4.72 samples/individual; range, 2–5 samples/individual) were analyzed by *msp2*-specific PCR. Between 1 and 7 clones were detected in PCR-positive individuals at single time points. Among the 249 PCR-positive samples, single *msp2* alleles (ie, clones) were detected in 137 samples (55.0%), and ≥ 2 clones were detected in 112 (45.0%). The correlation between parasite density and the number of clones was 0.13 (Spearman ρ ; 95% confidence interval [CI], .01–.25).

The number of clones and proportion of multiclonal infections increased in October and December 2006 in all age groups (Figure 2). The cumulative number of clones from all 5 surveys was highest in 8–10-year-old children, among whom there was an average of 5.3 clones (95% CI, 3.7–6.8 clones, range, 1–19 clones), whereas the average was 2.0 clones (95% CI, 1.4–2.8 clones; range, 1–8 clones) in the 2–4-year age group, 3.2 clones (95% CI, 1.8–4.5 clones; range, 1–20 clones) in the 5–7-year age group, and 3.6 clones (95% CI, 2.6–4.6 clones; range, 1–12 clones) among adults. The highest total number of *msp2* alleles detected within an individual over time (summarizing numbers from 5 time points) was 20, which was found in a 5-year-old child. HbAS was associated with a lower total number of clones when summarizing results from all 5 cross-sectional surveys in 167 individuals with complete data (incidence rate ratio, 0.63;

95% CI, .43–.92). The mean cumulative number of clones was 1.52 in HbAS individuals, compared with 2.48 in the rest of the population. More details of individual infection patterns are described in the [Supplementary Materials](#).

msp2 Diversity

In total, 104 unique *msp2* alleles were detected among the 249 positive samples; 23 were of the FC allelic family, and 81 were of the IC allelic family. Seven alleles were identified as common (ie, they were detected in >10 individuals): FC292, FC329, FC375, IC471, IC498, IC509, and IC515. FC292, the most common allele, was detected in 11.3% of samples ([Supplementary Figure 1](#)). Among the PCR-positive samples, 24.5% consisted of only FC type alleles, 46.5% of only IC types, and 29.0% contained both types.

Genotyping Profiles at Cross-sectional Surveys and Risk of Subsequent Clinical Malaria

The risk of febrile malaria was assessed using May or July 2006 as baseline. Follow-up was until April 2007, and no episodes were recorded after 206 days from May 2006. Having a multiclonal infection at baseline in May 2006 (at the end of the dry season), compared with being parasite negative, was associated with a reduced risk of subsequent febrile malaria (hazard ratio [HR], 0.40; 95% CI, .17–.99; Table 2, Figure 3A). In contrast, there was no significant association between the risk of febrile malaria and having 1 clone at baseline (HR, 0.71; 95% CI, .36–1.40). As expected, the risk of malaria during follow-up decreased with age (Table 2, Figure 3B). However, the association between multiple clones and malaria risk became more pronounced after adjustment for age and treatment (HR, 0.28; 95% CI, .11–.69). Similar results were observed when the July survey was used as the baseline, as well as when multiple events of malaria were taken into account (Table 2). Adjustment for HbAS did not affect the association with reduced risk of clinical malaria among individuals with multiclonal infections (data not shown).

Figure 2. Proportion of individuals with different number of clones at in the respective age groups at the 5 cross-sectional time points.

DISCUSSION

In this setting of seasonal malaria, multiclonal asymptomatic *P. falciparum* infections at the end of the dry season predicted a reduced risk of febrile malaria during the following high-transmission season.

Asymptomatic *P. falciparum* infections detected at the first 2 surveys, in May and July (ie, at the end of the dry season and before any malaria episodes were reported), likely represent persistent infections acquired during the high-transmission season the previous year. Repeated parasite positivity through the dry season is a phenomenon previously observed by microscopy in Mali [20, 21] and other settings of seasonal transmission, such as Sudan, where the same genotypes were repeatedly detected by PCR throughout the dry season [22]. Here, some individuals had infections composed of up to 7 clones, despite negligible

transmission during the 6-month dry season, and the PCR assay detected more individuals who carried parasites through the dry season, compared with microscopy, although a majority of individuals who were parasite positive at the end of the malaria transmission season became PCR negative during the following dry season, even without antimalarial treatment (in agreement with previous reports [23]). These persistent infections are likely to contribute to transmission by maintaining a parasite reservoir while the vector is absent [24].

The number of clones was highest in 8–10-year-old children and increased in all age groups during the peak of the malaria season. When including the 5 time points over 1 year, up to 20 different *msp2* alleles were detected within a single individual over time (in one 5-year-old child). Although the number of clones might reflect the level of individual exposure, the association between the number of clones and the risk of malaria was found

Figure 3. Time to first malaria episode with regard to the number of *Plasmodium falciparum* clones at the end of the dry season in children 2–10 years of age. *A*, At baseline in May 2006, 152 children were parasite negative, 12 were positive for 1 parasite clone, and 12 were positive for ≥ 2 parasite clones ($P=.011$, by the log-rank test). *B*, In July 2006, 142 children were parasite negative, 17 were positive for 1 parasite clone, and 12 were positive for ≥ 2 parasite clones ($P=.033$, by the log-rank test). Abbreviation: PCR, polymerase chain reaction.

when the surveys before the transmission season were used as baselines, thus decreasing the likelihood of recent exposure.

Protection from febrile malaria in children with multiclonal infections has previously been reported from areas with high and moderate transmission [8–10], while in young children the presence of multiclonal infections has been associated with an increased risk [11, 12]. The lower level of immunity in young children might suggest that the diversity of infections represents exposure and recent inoculations, whereas in older children persistent multiclonal infections might reflect tolerance to antigenically diverse infections. Moreover, within a trial of intermittent preventive treatment in Ghana, in which clearance of infections was achieved by repeated courses of antimalarials during a 6-month period, multiclonal infections were associated with a reduced risk of febrile malaria only in the placebo group. This suggests that it is not a certain number of clones at a single time point that predicts protection (as in the intervention groups),

but rather infections persisting over time that influence malaria immunity [25]. This is also supported by the present study, in which infections carried over several months (the dry season) predicted a reduced risk of subsequent malaria.

The mechanisms by which multiclonal infections contribute to protective immunity need to be elucidated. Antibodies are a key component of antimalarial immunity, and the breadth of antibody responses to merozoite antigens has been associated with protection against clinical malaria [26, 27]. Nonetheless, antibodies specific for merozoite antigens are rather short lived, and antibody levels wane within a few months without reexposure, particularly in children [28, 29]. Production of *P. falciparum*-specific antibodies might be enhanced in individuals who harbor parasites through the dry season. Alternatively, children with broader antibody responses are more likely to have multiclonal infections from the previous season and also enjoy a higher level of protection. Observation of the reduced risk of malaria only in individuals with multiclonal infections, rather than in those with single-clone infections, suggests that the diversity per se might be important to maintain broad antibody responses against several variants of different antigens. This is supported by the recent findings that children with multiclonal infections have broader antibody responses and that, in combination, they predicted a higher level of protection than they did individually [26].

An alternative explanation for the observed association between multiple parasite clones and protection from clinical malaria is that children who already have a higher degree of clinical immunity receive antimalarial treatment less often and as a consequence tend to accumulate more clones during the transmission season that then persist through the dry season. These individuals have an immunity that allows for low-grade parasite densities to persist without symptoms and multiclonal infections can also be seen as a marker of exposure in a semi-immune population.

The association between protection from clinical malaria and HbAS is well established and was also observed in this study population [15]. Individuals with the HbAS phenotype had a lower number of clones at the 5 cross-sectional surveys. Previous studies have either reported a higher number of clones within HbAS individuals [30] or have not found such differences [31, 32]. Here, the lower number of clones was found despite HbAS individuals being treated less frequently, owing to their lower incidence of clinical malaria, possibly suggesting that these individuals either have fewer successful inoculations from mosquito bites or clear infecting parasite clones more rapidly. The results indicate that HbAS individuals, either have different mechanisms of protection that are independent of the number of infecting parasite clones, or they maintain chronic parasitemia at lower level which may affect the detectability of clones. We did not find an association between HbAS and clones in relation to risk.

Overall, 104 unique *msp2* alleles were detected, which is perhaps surprising given that there is little-to-no malaria

Table 2. Risk of Malaria During Follow-up in Relation to the Number of Clones in Asymptomatic *Plasmodium falciparum* Infections at Cross-sectional Surveys in May 2006 and July 2006

Variable	Baseline Hazard Ratio (95% Confidence Interval)							
	May 2006				July 2006			
	Unadjusted ^a	Adjusted ^b	Adjusted ^c	Adjusted ^d	Unadjusted ^a	Adjusted ^b	Adjusted ^c	Adjusted ^d
Genotyping result, clones, no.								
0	1 (reference)	1 (reference)	1 (reference)	1 (reference)	1 (reference)	1 (reference)	1 (reference)	1 (reference)
1	0.58 (.30–1.15)	0.70 (.35–1.39)	0.71 (.36–1.40)	0.69 (.38–1.29)	0.90 (.49–1.67)	0.75 (.40–1.40)	0.76 (.41–1.43)	0.82 (.45–1.50)
≥2	0.40 (.17–.99)	0.34 (.14–0.84)	0.28 (.11–.69)	0.34 (.16–.74)	0.53 (.25–1.13)	0.50 (.23–1.09)	0.40 (.18–.87)	0.31 (.14–.71)
Age, y								
2–4	...	1 (reference)	1 (reference)	1 (reference)	...	1 (reference)	1 (reference)	1 (reference)
5–7	...	0.98 (.66–1.44)	1.03 (.70–1.52)	1.11 (.71–1.44)	...	1.00 (.68–1.48)	1.04 (.70–1.53)	1.06 (.73–1.52)
8–10	...	0.47 (.30–.72)	0.45 (.29–.70)	0.37 (.25–.56)	...	0.48 (.31–.76)	0.45 (.29–.70)	0.34 (.22–.53)
18–25	...	0.04 (.02–0.12)	0.03 (.01–.09)	0.02 (.00–.05)	...	0.03 (.01–.10)	0.03 (.01–.08)	0.01 (.00–.04)
Previous treatment ^e								
No	...	...	1 (reference)	1 (reference)	...	...	1 (reference)	1 (reference)
Yes	...	...	0.18 (.08–.43)	0.10 (.06–.16)	...	...	0.15 (.06–.38)	0.08 (.05–.13)

Follow-up time was from the respective surveys until April 2007.

^a By Cox regression analysis, assessing time to the first episode of malaria.

^b Adjusted for age, using Cox regression analysis to assess time to the first episode of malaria.

^c Adjusted for age and posttreatment prophylactic, using Cox regression analysis to assess time to the first episode of malaria.

^d Adjusted for recurrent events (frailty), age, and posttreatment prophylactic effect, using the Anderson-Gill model.

^e The posttreatment prophylactic effect was incorporated in the final models with a gradual decrease from a value of 1 the first day after treatment and then exponentially decreasing with a half-life of 4 weeks.

transmission during the 6-month dry season each year. The method used for genotyping *P. falciparum* infection is based on fragment analysis by capillary electrophoresis in a DNA sequencer, which generates reproducible high resolution sizing and enables more-detailed assessments than the gel-based methods used for *msp2* genotyping [16]. Here, most alleles were found at low frequencies; however, certain alleles were repeatedly detected in the cohort, in both the dry and rainy seasons. Some of these alleles have also been reported in other studies using the same genotyping technique, in both asymptomatic and symptomatic (mild and severe) infections in Kenya and Uganda [33, 34]. Identical fragment lengths might represent identical sequences, but sequencing would be required to confirm this. The sample size did not allow for analysis of specific alleles and risk of malaria, but the finding of specific *msp2* alleles that appear frequently in both asymptomatic infections and disease-causing infections warrants investigation and could possibly have importance for the development of new vaccine candidates.

Although the association between risk and number of clones was found in an age-adjusted model, a limitation of the study is that detailed risk assessments in relation to increasing number of clones and different ages was restricted by the sample size. Moreover, the diversity of infections is generally underestimated because of within-host dynamics of human *P. falciparum* populations [35, 36]. Models used in this study took into account the effect of the posttreatment prophylactic effect of artesunate and amodiaquine, rather than excluding individuals who were recently treated, which increased the statistical power of the study in this setting, where >80% of children aged 2–7 years received at least 1 treatment during follow-up. Moreover, the strength of the current study is that the findings regarding multiclonal infections and reduced risk of malaria are comparable with those of previous studies using the same genotyping and statistical methods in other transmission settings [25, 33] and thus confirm the associations in a setting with highly seasonal transmission.

With more regions focusing on malaria elimination, there is greater interest in strategies targeting asymptomatic parasite carriage as a reservoir of ongoing transmission. Clinical trials targeting asymptomatic parasitemia during the dry season have, however, failed to interrupt malaria epidemics following the annual rains [37]. In this area of seasonal malaria, genetically diverse parasites carried through the dry season may contribute to the maintenance of clinical immunity; therefore, it may be prudent in the preelimination phase to expect and prepare for a higher incidence of clinical malaria following interventions that decrease asymptomatic carriage during the dry season. Furthermore, interventions aiming at interrupting the asymptomatic carriage during the dry season might enhance the selection of resistant parasites. For example, in Sudan it has been shown that the proportion of wild-type parasites increases when transmission ceases and the environment remains drug free [38]. A clearer understanding of the biology of asymptomatic

P. falciparum infections will likely inform the optimal interventions that aim to eliminate and ultimately eradicate malaria.

In summary, the study shows that, in an area of seasonal malaria, multiclonal infections carried through the dry season predict a reduced risk of febrile malaria during the ensuing malaria season. It remains to be determined whether higher multiplicity of clones per se enhances blood-stage immunity and thus clinical protection or whether it is an epiphenomenon in clinically immune individuals that arises from less frequent antimalarial treatment, although it seems likely that these effects may be self-reinforcing. Further studies are needed to elucidate the mechanisms underlying the association between multiclonal *P. falciparum* infections and a reduced risk of clinical malaria.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online (<http://jid.oxfordjournals.org>). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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Potential conflicts of interest. All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

References

1. Crompton PD, Moebius J, Portugal S, et al. Malaria immunity in man and mosquito: insights into unsolved mysteries of a deadly infectious disease. *Annu Rev Immunol* 2014; 32:157–87.
2. Marsh K, Kinyanjui S. Immune effector mechanisms in malaria. *Parasite Immunol* 2006; 28:51–60.
3. Richards JS, Beeson JG. The future for blood-stage vaccines against malaria. *Immunol Cell Biol* 2009; 87:377–90.
4. Polley SD, Tetteh KK, Cavanagh DR, et al. Repeat sequences in block 2 of *Plasmodium falciparum* merozoite surface protein 1 are targets of antibodies associated with protection from malaria. *Infect Immun* 2003; 71:1833–42.
5. Felger I, Smith T, Edoh D, et al. Multiple *Plasmodium falciparum* infections in Tanzanian infants. *Trans R Soc Trop Med Hyg* 1999; 1:29–34.
6. Ntoumi F, Contamin H, Rogier C, et al. Age-dependent carriage of multiple *Plasmodium falciparum* merozoite surface antigen-2 alleles in asymptomatic malaria infections. *Am J Trop Med Hyg* 1995; 52:81–8.
7. Smith T, Beck HP, Kitua A, et al. Age dependence of the multiplicity of *Plasmodium falciparum* infections and of other malariological indices in an area of high endemicity. *Trans R Soc Trop Med Hyg* 1999; 93:15–20.
8. al-Yaman F, Genton B, Reeder JC, et al. Reduced risk of clinical malaria in children infected with multiple clones of *Plasmodium falciparum* in a highly endemic area: a prospective community study. *Trans R Soc Trop Med Hyg* 1997; 91:602–5.

9. Berezcky S, Liljander A, Rooth I, et al. Multiclonal asymptomatic *Plasmodium falciparum* infections predict a reduced risk of malaria disease in a Tanzanian population. *Microbes Infect* **2007**; 9:103–10.
10. Farnert A, Rooth I, Svensson G, et al. Complexity of *Plasmodium falciparum* infections is consistent over time and protects against clinical disease in Tanzanian children. *The J Infect Dis* **1999**; 179:989–95.
11. Branch OH, Takala S, Kariuki S, et al. *Plasmodium falciparum* genotypes, low complexity of infection, and resistance to subsequent malaria in participants in the Asembo Bay Cohort Project. *Infect Immun* **2001**; 69:7783–92.
12. Henning L, Schellenberg D, Smith T, et al. A prospective study of *Plasmodium falciparum* multiplicity of infection and morbidity in Tanzanian children. *Trans R Soc Trop Med Hyg* **2004**; 98:687–94.
13. Farnert A, Williams TN, Mwangi TW, et al. Transmission-dependent tolerance to multiclonal *Plasmodium falciparum* infection. *J Infect Dis* **2009**; 200:1166–75.
14. Sergent E, Parrot L. L'immunité, la prémunition et la résistance innée. *Archives de l'Institut Pasteur d'Algérie* **1935**; 13:279–319.
15. Crompton PD, Traore B, Kayentao K, et al. Sick cell trait is associated with a delayed onset of malaria: implications for time-to-event analysis in clinical studies of malaria. *J Infect Dis* **2008**; 198:1265–75.
16. Liljander A, Wiklund L, Falk N, et al. Optimization and validation of multi-coloured capillary electrophoresis for genotyping of *Plasmodium falciparum* merozoite surface proteins (msp1 and 2). *Malar J* **2009**; 8:78.
17. Snounou G, Zhu X, Siripoon N, et al. Biased distribution of msp1 and msp2 allelic variants in *Plasmodium falciparum* populations in Thailand. *Trans R Soc Trop Med Hyg* **1999**; 93:369–74.
18. WHO. Severe falciparum malaria. World Health Organization, Communicable Diseases Cluster. *Trans R Soc Trop Med Hyg* **2000**; 94:S1–90.
19. Rogier C, Commenges D, Trape JF. Evidence for an age-dependent pyrogenic threshold of *Plasmodium falciparum* parasitemia in highly endemic populations. *Am J Trop Med Hyg* **1996**; 54:613–9.
20. Delley V, Bouvier P, Breslow N, et al. What does a single determination of malaria parasite density mean? A longitudinal survey in Mali. *Trop Med Int Health* **2000**; 5:404–12.
21. Dicko A, Mantel C, Kouriba B, et al. Season, fever prevalence and pyrogenic threshold for malaria disease definition in an endemic area of Mali. *Trop Med Int Health* **2005**; 10:550–6.
22. Roper C, Richardson W, Elhassan IM, et al. Seasonal changes in the *Plasmodium falciparum* population in individuals and their relationship to clinical malaria: a longitudinal study in a Sudanese village. *Parasitology* **1998**; 116:501–10.
23. Babiker HA, Abdel-Muhsin AM, Ranford-Cartwright LC, et al. Characteristics of *Plasmodium falciparum* parasites that survive the lengthy dry season in eastern Sudan where malaria transmission is markedly seasonal. *Am J Trop Med Hyg* **1998**; 59:582–90.
24. Lindblade KA, Steinhardt L, Samuels A, et al. The silent threat: asymptomatic parasitemia and malaria transmission. *Expert Rev Anti Infect Ther* **2013**; 11:623–39.
25. Liljander A, Chandramohan D, Kweku M, et al. Influences of intermittent preventive treatment and persistent multiclonal *Plasmodium falciparum* infections on clinical malaria risk. *PloS One* **2010**; 5:e13649.
26. Rono J, Osier FH, Olsson D, et al. Breadth of anti-merozoite antibody responses is associated with the genetic diversity of asymptomatic *Plasmodium falciparum* infections and protection against clinical malaria. *Clin Infect Dis* **2013**; 57:1409–16.
27. Crompton PD, Pierce SK, Miller LH. Advances and challenges in malaria vaccine development. *J Clin Invest* **2010**; 120:4168–78.
28. Kinyanjui SM, Conway DJ, Lanar DE, Marsh K. IgG antibody responses to *Plasmodium falciparum* merozoite antigens in Kenyan children have a short half-life. *Malaria J* **2007**; 6:82.
29. Portugal S, Pierce SK, Crompton PD. Young lives lost as B cells falter: what we are learning about antibody responses in malaria. *J Immunol* **2013**; 190:3039–46.
30. Kiwanuka GN, Joshi H, Isharaza WK, Eschrich K. Dynamics of *Plasmodium falciparum* alleles in children with normal haemoglobin and with sickle cell trait in western Uganda. *Trans R Soc Trop Med Hyg* **2009**; 103:87–94.
31. Vafa M, Troye-Blomberg M, Anchang J, et al. Multiplicity of *Plasmodium falciparum* infection in asymptomatic children in Senegal: relation to transmission, age and erythrocyte variants. *Malaria J* **2008**; 7:17.
32. Ntoumi F, Rogier C, Dieye A, et al. Imbalanced distribution of *Plasmodium falciparum* MSP-1 genotypes related to sickle-cell trait. *Mol Med* **1997**; 3:581–92.
33. Lundblom K, Murungi L, Nyaga V, et al. *Plasmodium falciparum* infection patterns since birth and risk of severe malaria: a nested case-control study in children on the coast of Kenya. *PloS One* **2013**; 8:e56032.
34. Kiwuwa MS, Ribacke U, Moll K, et al. Genetic diversity of *Plasmodium falciparum* infections in mild and severe malaria of children from Kampala, Uganda. *Parasitol res* **2013**; 112:1691–700.
35. Farnert A. *Plasmodium falciparum* population dynamics: only snapshots in time? *Trends Parasitol* **2008**; 24:340–4.
36. Farnert A, Snounou G, Rooth I, Bjorkman A. Daily dynamics of *Plasmodium falciparum* subpopulations in asymptomatic children in a holoendemic area. *Am J Trop Med Hyg* **1997**; 56:538–47.
37. von Seidlein L, Walraven G, Milligan PJ, et al. The effect of mass administration of sulfadoxine-pyrimethamine combined with artesunate on malaria incidence: a double-blind, community-randomized, placebo-controlled trial in The Gambia. *Trans R Soc Trop Med Hyg* **2003**; 97:217–25.
38. Babiker HA, Gadalla AA, Ranford-Cartwright LC. The role of asymptomatic *P. falciparum* parasitaemia in the evolution of antimalarial drug resistance in areas of seasonal transmission. *Drug Resist Updat* **2013**; 16:1–9.