Sickle Cell Trait Is Associated with a Delayed Onset of Malaria: Implications for Time-to-Event Analysis in Clinical Studies of Malaria

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Background. The World Health Organization (WHO) recently recommended that the time to first malaria episode serve as the primary end point in phase III malaria vaccine trials—the first of which will be held in Africa. Although common red blood cell (RBC) polymorphisms such as sickle hemoglobin (HbS) are known to protect against malaria in Africa, their impact on this end point has not been investigated.

Methods. A longitudinal study of 225 individuals aged 2–25 years was conducted in Mali. The association between common RBC polymorphisms and the time to first malaria episode was evaluated.

Results. Among children aged 2–10 years, sickle cell trait (HbAS) was associated with a 34-day delay in the median time to first malaria episode (P = 0.017). Cox regression analysis showed that greater age (hazard ratio [HR], 0.87 [95% CI, 0.80–0.94]; P = 0.001), HbAS (HR, 0.48 [95% CI, 0.26–0.91]; P = 0.024), and asymptomatic parasitemia at enrollment (HR, 0.35 [95% CI, 0.14–0.85]; P = 0.021) were associated with decreased malaria risk.

Conclusion. Given the delay in the time to first malaria episode associated with HbAS, it would be advisable for clinical trials and observational studies that use this end point to include Hb typing in the design of studies conducted in areas where HbAS is prevalent.
present article, we describe this observational cohort study designed to investigate the acquisition and maintenance of malaria immunity. We found that HbAS is associated with a 34-day delay in the median time to first malaria episode, an association that remained statistically significant in multivariate Cox regression analysis. HbAS was unique in this regard as we found no association between HbAC, α-thalassemia, G6PD deficiency, or blood group O and a delayed time to first malaria episode.

METHODS

Study site. The study was carried out in Kambila, a small (~1 km²), well-circumscribed, rural village with a population of 1500, situated 20 km north of Bamako, the capital of Mali (figure 1). The transmission of *P. falciparum* is seasonal and intense, peaking in September through November (figure 2). The entomological inoculation rate measured in a nearby village was near 0 during the dry season, and there were approximately 50–60 infective bites per person per month in October 2000 [13].

Sampling strategy, study participants, and malaria case definition. Approval for this study was obtained from the Faculty of Medicine, Pharmacy, and Odonto-Stomatology Ethics Committee; and the institutional review board at the National Institute of Allergy and Infectious Diseases, National Institutes of Health. Written, informed consent was obtained from adult participants, and from the parents or guardians of participating children. The study was externally monitored for protocol compliance, data integrity, and protection of human subjects.

This study is part of an ongoing observational study of the acquisition and maintenance of malaria immunity that began in May 2006. Individuals were invited to be screened for the study after being randomly selected from an age-stratified census of the entire village population. Enrollment exclusion criteria were hemoglobin level <7 g/dL, fever ≥37.5°C, acute systemic illness, use of antimalarial or immunosuppressive medications in the past 30 days, and pregnancy. Venous blood samples and blood smears were collected before the malaria season (May 2006), at cross-sectional time points every 2 months during the malaria season (July, October, and December 2006), prior to the second malaria season (May 2007), and 14 days after the first episode of malaria. Stool and urine samples were examined at enrollment for the presence of helminth and *Schistosoma haematobium* infection, respectively.

Participants were encouraged to report symptoms of malaria at the village health center, which was staffed 24 hours a day by a study physician. For subjects with signs or symptoms of malaria, blood smears were prepared and examined for the presence of *P. falciparum*. Patients with positive smear results were treated with a standard 3-day course of artesunate plus amodiaquine, following the guidelines of the Mali National Malaria Control Program. Children with severe malaria were referred to Kati District Hospital after an initial parenteral dose of quinine. The research
definition of malaria was an axillary temperature ≥37.5°C, *P. falciparum* asexual parasitemia ≥5000 parasites/μL, and a nonfocal physical exam by the study physician. Severe malaria, as defined by the WHO [14], was included in this definition. At the end of the malaria season, participants (or their parents or guardians, in the case of children) were asked whether or not they had used a bed net nightly during the rainy season.

**Blood samples.** Blood samples were drawn by venipuncture into sodium citrate-containing cell preparation tubes (BD) and transported to the laboratory (20 km) for processing within 2 h. Following centrifugation (1800 relative centrifugal force; 20 min), plasma was collected and stored at −80°C for future studies. Peripheral blood mononuclear cells were collected, washed twice with sterile phosphate buffered saline (PBS; KD Medical), resuspended in 90% heat-inactivated fetal bovine serum (FBS; Gibco) and 7.5% dimethyl sulfoxide (DMSO; Sigma-Aldrich), kept at −80°C for 24 h, and transferred to liquid nitrogen for future studies. Two hundred microliters of whole blood was used to identify RBC polymorphisms.

**Identification of RBC polymorphisms.** Hemoglobin was typed by high-performance liquid chromatography (D-10 instrument; Bio-Rad). The 3.7-kb deletional determinant of α-thalassemia was detected by a nested polymerase chain reaction (PCR) protocol (Appendix) to identify heterozygous (−α/αα) and homozygous (−α/−α) individuals. The mutation responsible for G6PD deficiency in Mali (G6PD*A−) was identified by restriction-fragment length polymorphism analysis of PCR-amplified DNA samples, as described elsewhere [10]. ABO blood groups were determined by use of a monoclonal antibody–based kit (Linear Chemicals).

**Malaria slides.** Thick blood smears were stained with Giemsa and counted against 300 leukocytes. *P. falciparum* densities were recorded as the number of asexual parasites per microliter of whole blood, based on a mean leukocyte count of 7500 cells/μL. Each smear was evaluated separately by 2 expert microscopists blinded to the clinical status of study participants. Any discrepancies were resolved by a third expert microscopist.

**Stool and urine exam for helminth infection.** At enrollment, duplicate stool samples were examined for *S. mansoni* eggs and other intestinal helminths by use of the semiquantitative Kato-Katz method. To detect *S. haematobium* eggs, 10 mL of urine were poured over Whatman filter paper. One or two drops of ninhydrin were placed on the filter and left to air dry. After drying, the filter was dampened with tap water, and helminth eggs detected by microscopy.

**Geographic information system data collection.** Latitude and longitude coordinates and the altitude of study subjects’ households were measured by a handheld global positioning system receiver (GeoXM; Trimble).

**Data management and analysis.** Data were double entered and verified in a database (Microsoft Access 2003; Microsoft) and analyzed using Stata software (version 10.0; StataCorp). Fisher’s exact tests and Kruskall-Wallis tests were used to compare binary outcomes and continuous variables between groups, respectively. The probability of a subject remaining malaria-free over an 8-month period was estimated by the Kaplan-Meier method, and the time-to-event curves of different groups were compared by the log rank test. The Cox proportional hazards model was used to assess the effect of the following factors on risk of malaria: age, sex, weight, ethnicity, distance of resi-
RESULTS

Baseline characteristics of study cohort. Of the 237 individuals screened, 225 were enrolled during a 2-week period in May 2006, approximately 1 month before the malaria transmission season began (figure 2). The study cohort was divided into 4 predefined age groups: 2–4 years (n = 73), 5–7 years (n = 52), 8–10 years (n = 51), and 18–25 years (n = 49). Follow-up at scheduled cross-sectional visits was >99% for children and 82% for adults during the 8-month study. Table 1 shows baseline demographic and clinical characteristics according to age group. Overall, 115 (51.1%) of 225 subjects were female, and the predominant ethnic groups were Bambara (134 [59.6%]) and Sarakole (77 [34.2%]). The prevalence of asymptomatic parasitemia at enrollment was 7.1% (16 subjects) and did not vary significantly with age (P = .83). Of note, asymptomatic parasitemia during the dry season is commonly observed in Mali [15, 16]. Of 190 subjects with stool sample data available, 17 (9.0%) showed evidence of intestinal helminths; of 184 subjects with urine sample data available, 11 (6.0%) showed evidence of S. haematobium infection. These prevalences were lower than expected, likely due to communitywide albendazole treatment prior to this study. The mean distance between the study clinic and subjects’ residences was 382 meters (range, 127–881 meters), and 61 (27.1%) of 225 participants self-reported nightly bed net use during the rainy season.

Prevalence of RBC polymorphisms. HbAS and HbAC phenotypes were present in 22 (10.4%) and 29 (13.7%) of 212 individuals, respectively (table 2). The prevalence of the G6PD*– genotype was 17.0% (19 of 112) among females (heterozygous and homozygous individuals) and 14.1% (14 of 99) among males (hemizygous individuals). −α/−α and −α/–α genotypes were found in 84 (39.8%) and 2 (1.0%) of 211 individuals, respectively. Because of the low frequency of the −α/–α genotype, individuals with this genotype were excluded from further analyses. Blood groups O, B, A, and AB were identified in 63 (33.2%), 58 (30.5%), 50 (26.3%), and 19 (10.0%) of 190 individuals, respectively.
Malaria outcomes by age group. A record of the number of malaria cases per day from May 2006 to January 2007 (figure 2) illustrates the intense, seasonal malaria transmission at this site. During 495 clinic visits that occurred during the study period, 298 episodes of malaria were diagnosed. Table 3 summarizes malaria outcomes according to age group. As expected, malaria incidence decreased with age (P < .001), and the 5 cases of severe malaria were confined to children <5 years old. Among those who presented with malaria, the median time to first malaria episode (as measured in days from study enrollment) increased with age (101 days for subjects 2–4 years old vs. 153 days for subjects 18–25 years old; P < .016). The geometric mean parasite density per microliter at time of the first malaria episode decreased with age (P = .036).

Impact of RBC polymorphisms on malaria outcomes among children aged 2–10 years. Because adult subjects had few episodes of malaria (table 3), the analysis of RBC polymorphisms and malaria outcomes focused on children aged 2–10 years. Time-to-event analysis (figure 3A) showed that HbAS was associated with a significant delay in the time to first malaria episode (P = .038 by log rank test). Among children who presented with malaria, HbAS was associated with a median 34-day delay to the first episode, compared with the non-HbAS group (median, 145 days for the HbAS group vs. 111 days for the non-HbAS group; P = .017). HbAS was also associated with a 53% reduction in malaria incidence (mean malaria incidence, 0.82 episodes for the HbAS group [95% confidence interval {CI}, 0.48–1.17] vs. 1.76 episodes for the non-HbAS group [95% CI, 1.56–1.96]; P < .003). Although the geometric mean parasite densities at the time of the first malaria episode were lower in HbAS children, this difference was not statistically significant (9033 parasites/L in the HbAS group [95% CI, 1364–59,825] vs. 21,257 parasites/L in the non-HbAS group [95% CI, 16,312–27,701]; P = .83). The 5 cases of severe malaria occurred in non-HbAS children (3 HbAA children and 2 HbAC children). HbAC phenotype, G6PD*A— genotype (hemizygosity, heterozygosity, and homozygosity), α-thalassemia, and blood group O were not associated with a delayed time to first malaria episode (figure 3B–3D) or a decreased incidence of malaria (not shown).

Baseline characteristics of children aged 2–10 years, stratified by hemoglobin type. To assess for potential confounding of the protective effect of HbAS, factors that might influence malaria risk were stratified by Hb type (table 4). While there was a higher proportion of females in the HbAS group (P = .022), HbAS children did not differ significantly from non-HbAS chil-

Table 2. Frequency of red blood cell polymorphisms, according to age group.

<table>
<thead>
<tr>
<th>Polymorphism</th>
<th>2–4 years</th>
<th>5–7 years</th>
<th>8–10 years</th>
<th>18–25 years</th>
<th>All</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemoglobin type</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>50/69 (72.5)</td>
<td>43/50 (86.0)</td>
<td>35/51 (68.6)</td>
<td>32/42 (76.2)</td>
<td>160/212 (75.5)</td>
</tr>
<tr>
<td>AS</td>
<td>8/69 (11.6)</td>
<td>4/50 (8.0)</td>
<td>5/51 (9.8)</td>
<td>5/42 (11.9)</td>
<td>22/212 (10.4)</td>
</tr>
<tr>
<td>AC</td>
<td>10/69 (14.5)</td>
<td>3/50 (6.0)</td>
<td>11/51 (21.6)</td>
<td>5/42 (11.9)</td>
<td>29/212 (13.7)</td>
</tr>
<tr>
<td>CC</td>
<td>1/69 (1.5)</td>
<td>0/50 (0.0)</td>
<td>0/51 (0.0)</td>
<td>0/42 (0.0)</td>
<td>1/212 (0.5)</td>
</tr>
</tbody>
</table>

G6PD*A—

<table>
<thead>
<tr>
<th>Gender</th>
<th>Normal</th>
<th>Heterozygous</th>
<th>Homozygous</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td>32/42 (76.2)</td>
<td>22/52 (89.5)</td>
<td>20/42 (95.2)</td>
</tr>
<tr>
<td>Male</td>
<td>26/29 (89.7)</td>
<td>15/24 (62.5)</td>
<td>15/29 (51.7)</td>
</tr>
</tbody>
</table>

α-thalassemia

<table>
<thead>
<tr>
<th>Gender</th>
<th>Normal</th>
<th>Heterozygous</th>
<th>Homozygous</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td>35/67 (52.2)</td>
<td>26/30 (86.7)</td>
<td>26/30 (86.7)</td>
</tr>
<tr>
<td>Male</td>
<td>32/67 (47.8)</td>
<td>25/31 (80.6)</td>
<td>25/31 (80.6)</td>
</tr>
</tbody>
</table>

ABO blood group

<table>
<thead>
<tr>
<th>Gender</th>
<th>O</th>
<th>A</th>
<th>B</th>
<th>AB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td>20/49 (41.0)</td>
<td>12/49 (25.0)</td>
<td>8/49 (16.3)</td>
<td>5/49 (10.2)</td>
</tr>
<tr>
<td>Male</td>
<td>16/49 (33.7)</td>
<td>10/49 (20.4)</td>
<td>6/49 (12.2)</td>
<td>1/49 (0.2)</td>
</tr>
</tbody>
</table>

NOTE. Data are no. of subjects with a given trait/no. of subjects for whom data was available (%). There were 225 subjects enrolled: 73 in the 2–4 years group, 52 in the 5–7 years group, 51 in the 8–10 years group, and 49 in the 18-25 group.
Table 3. Outcomes for the 298 episodes of malaria diagnosed during the study period, according to age group.

<table>
<thead>
<tr>
<th>Outcome</th>
<th>2–4 years</th>
<th>5–7 years</th>
<th>8–10 years</th>
<th>18–25 years</th>
<th>All</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n = 73)</td>
<td>(n = 52)</td>
<td>(n = 51)</td>
<td>(n = 49)</td>
<td>(N = 225)</td>
<td></td>
</tr>
<tr>
<td>Malaria incidence, mean ± SD, no. of episodes</td>
<td>1.99 ± 1.25</td>
<td>1.94 ± 1.21</td>
<td>0.98 ± 1.05</td>
<td>0.08 ± 0.28</td>
<td>1.33 ± 1.30</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Severe malaria, no. of episodes</td>
<td>4</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>&gt;1 malaria episode, no. (%) of subjects</td>
<td>63 (86.3)</td>
<td>45 (86.5)</td>
<td>31 (60.8)</td>
<td>4 (8.2)</td>
<td>143 (63.6)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Time to first malaria episode, median, days</td>
<td>101</td>
<td>114</td>
<td>130</td>
<td>153</td>
<td>115</td>
<td>.016</td>
</tr>
<tr>
<td>Parasitemia at first malaria episode, geometric mean no. of parasites/μL (95% CI)</td>
<td>34,374 (24,965–47,348)</td>
<td>15,687 (9623–25,574)</td>
<td>10,433 (5079–21,427)</td>
<td>8816 (4082–19,037)</td>
<td>19,625 (15,004–25,668)</td>
<td>.036</td>
</tr>
</tbody>
</table>

*a A malaria episode was defined as axillary temperature ≥37.5°C, asexual parasitemia ≥5000 parasites/μL, and a nonfocal physical examination by the study physician.

*b In accordance with the World Health Organization definition of severe malaria [14].

*c Days since study enrollment.
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Figure 3. Kaplan-Meier estimates of the time from study enrollment to the first episode of malaria in children aged 2–10 years. Subjects were compared in the following ways: sickle cell trait (HbAS) vs. non-HbAS (A), α-thalassemia heterozygosity vs. normal α hemoglobin (B), G6PD*A− hemizygous versus G6PD*A− heterozygous versus G6PD*A− homozygous versus normal G6PD (C), blood group O vs. blood groups A, B, and AB (D), and Plasmodium falciparum parasitemia status at enrollment (E). *P values were obtained using the log rank test.

Children with regard to age, weight, ethnicity, distance of residence from the clinic, or frequency of G6PD*A− and −α/αα genotypes. The prevalence of asymptomatic parasitemia at enrollment was 9.4% (12 of 128) among HbAA children and 0% (0 of 41) among HbAC and HbAS children. However, asymptomatic parasitemia at enrollment was associated with a delayed time to first malaria episode (figure 3E; P = .026, by log rank test) and a trend toward decreased malaria incidence. Thus, HbAS children were not delayed in their time to first malaria episode as a result of the effect of asymptomatic parasitemia. There were too few HbAS children to make meaningful comparisons with respect to the remaining variables.
Predictors of malaria outcomes, multivariate analysis of children aged 2–10 years. Cox regression analysis revealed that greater age (HR, 0.87 [95% CI, 0.80–0.94]; P = .001), HbAS phenotype (HR, 0.48 [95% CI, 0.26–0.91]; P = .024), and asymptomatic parasitemia at enrollment (HR, 0.35 [95% CI, 0.14–0.85]; P = .021) were associated with decreased malaria risk. Poisson regression analysis showed that greater age (incidence rate ratio [IRR], 0.90 [95% CI, 0.85–0.95]; P < .001) and HbAS phenotype (IRR, 0.46 [95% CI, 0.27–0.79]; P = .005) were significant predictors of decreased malaria incidence, whereas asymptomatic parasitemia at enrollment was not (P > .100). Removal of the −α/α genotype from the analysis decreased the hazard ratio (HR, 0.44; P = .020) and incidence rate ratio (IRR, 0.44; P = .004) for HbAS, indicating negative epistasis between these polymorphisms. Factors that did not independently predict either measure of malaria risk (hazard ratio or incidence rate ratio) were sex, weight, distance of residence from clinic, bed net use, helminth infection, HbAC phenotype, G6PD*A− genotype (hemizygous, heterozygous, or homozygous), −α/α genotype, and blood group O.

**DISCUSSION**

In the present study, we found that HbAS was associated with a delayed time to first malaria episode, an association that remained statistically significant in multivariate Cox regression analysis. Numerous case-control studies [7, 17–20] and longitudinal studies [12, 21] have established that HbAS decreases the risk of malaria, as measured by odds and incidence rate ratios, respectively. However, the effect of HbAS on the time to first malaria episode had not been established using time-to-event analysis. Aidoo et al. showed by time-to-event analysis that HbAS is associated with a reduction in all-cause mortality, but malaria-specific outcomes, including severe malarial anemia and high-density parasitemia, were reported as incidence rates [21]. In Gabon, investigators found no association between HbAS and time to malaria episodes [19], but the results of this study are difficult to interpret for the following reasons: (1) individuals were initially enrolled in a hospital-based, case-control study of severe malaria; (2) age and other covariates were not taken into account when HbAS and non-HbAS individuals were compared longitudinally; and (3) statistical methods were chosen that were inappropriate for the analysis of correlated sojourn times between consecutive malaria episodes for the same subject. This approach is further complicated by the problem of defining when a malaria episode ends and when an individual becomes susceptible to subsequent episodes after treatment.

Several features of our study made it well-suited to assess the impact of RBC polymorphisms on the time to first malaria episode. The study population was an age-stratified, random sample that represented 15% of all individuals living in a rural, well-circumscribed, nonmigratory community where antimalarial drugs were provided exclusively by the study investigators. En-
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rollment occurred over a short period of time, approximately 1 month prior to the abrupt onset of malaria transmission (figure 2). Ninety-three percent of individuals had blood smears negative for *P. falciparum* at the time of enrollment, obviating the need for a treatment–reinfection study design [22], an approach that may alter the subsequent risk of malaria [23]. Moreover, the following aspects of our study favored an unbiased detection of malaria episodes: (1) follow-up at regular cross-sectional visits was >99% among children aged 2–10 years, indicating a high degree of study awareness and participation; (2) the average distance from individuals’ homes to the study clinic was 382 meters, minimizing geographic and logistic barriers to study participation; and (3) a study physician was available at all times at the only easily accessible health care facility in the area.

The use of time to first malaria episode as a study end point is increasingly common in clinical trials of malaria vaccines [24] and antimalarial drugs [2] and in observational studies of malaria immunity [3–5]. Indeed, the WHO recently recommended that the time to first malaria episode serve as the primary end point in phase III clinical trials of candidate malaria vaccines [1]. An imbalance in the distribution of HbAS between comparator groups, whether in randomized trials or observational studies, may occur by chance and lead to invalid conclusions. The clear impact of HbAS on this end point and the possibility of a biological interaction between HbAS and certain interventions (e.g., candidate blood stage vaccines) provide a rationale for routine Hb typing in such studies, which are often conducted in areas where the prevalence of HbAS exceeds 25% [6]. To date, Hb typing has not been consistently reported in such studies [5, 24, 25]. Hb typing not only determines whether HbAS was successfully randomized in clinical trials, but incorporating the information into data analyses can increase the statistical power for detecting the effect of an intervention and hence reduce the sample size required. This is particularly important in malaria vaccine trials in which the current vaccine candidates are anticipated to have relatively low efficacy (e.g., ≤50%) [26].

Several mechanisms have been proposed to explain how HbAS confers protection against malaria, including decreased RBC invasion or poor growth under low-oxygen tension [27, 28], enhanced removal of parasitized RBCs [29, 30], accelerated acquisition of antibodies specific for *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP-1) and other variant surface antigens [31, 32], and more recently, reduced cytoadherence of parasitized RBCs [33]. Anti–PfEMP-1 antibodies could thus work cooperatively with HbS to further reduce cytoadherence [33], which may explain the apparent effects of age-associated acquired immunity on malaria protection by sickle cell trait [34]. It is conceivable that any one of these processes, or a combination of them, underlies the association between HbAS and the delayed time to first malaria episode observed in this study, either by prolonging the time it takes to achieve symptomatic parasite densities or increasing the probability that asymptomatic infections will be aborted. Because we did not actively survey for asymptomatic parasitemia, we could not distinguish between these 2 possibilities. The former idea is supported by the results of a study in Senegal in which weekly blood smears performed after quinine treatment showed a delay in the reappearance of asymptomatic parasitemia in HbAS individuals [22]. However, the relationship between asymptomatic parasitemia and the subsequent risk of malaria is complex and not fully understood [35, 36].

It is unlikely that differential mosquito exposure confounded the association between HbAS and the time to first malaria episode because HbAS individuals appeared to be randomly distributed in this small, well-circumscribed village, which lacks a dominant body of water (figure 1). Moreover, the frequency of bed net use was evenly distributed between HbAS and non-HbAS individuals. It is also unlikely that access to the study clinic played a significant role because the distance to the clinic from the individuals’ residences did not differ by hemoglobin type.

*G6PD*<sup>A−</sup> and −α/αα genotypes, as well as blood group O, have been associated with protection from severe malaria [9–11], but their role in protection against uncomplicated malaria is less clear. Consistent with this, we did not observe an association between these polymorphisms and the time to first malaria episode or malaria incidence, although more subtle effects may have been detectable with a larger sample size. Notably, we did not detect an increase in the incidence of uncomplicated malaria among females heterozygous for *G6PD*<sup>A−</sup>, which has been observed by others [19, 37, 38]. Consistent with a recent study [20], we observed negative epistasis between HbAS and the −α/αα genotype. However, the frequency of the −α/−α genotype in this cohort was too low to confirm the negative epistatic interaction with HbAS reported elsewhere [39].

Interestingly, asymptomatic parasitemia at enrollment (at the end of the dry season) was associated with a decreased risk of subsequent malaria in multivariate analysis. This finding is consistent with those of a recent longitudinal study in Senegal, where malaria is also highly seasonal [36]. In Uganda, however, where malaria is mesoendemic, asymptomatic parasitemia was reported as a risk factor for malaria [35], suggesting that the clinical outcome of asymptomatic parasitemia might vary with the epidemiological setting. In neither of these studies, however, were HbAS and other malaria-protective polymorphisms investigated as potential contributing factors. In-depth studies of both host and parasite factors may reveal important mechanisms by which host immune responses and parasite immune evasion are balanced.

The delayed presentation of malaria associated with HbAS in this study adds to the measures by which the protective effect of this remarkable polymorphism has been documented. Clinical studies of malaria that use the time to first malaria episode as an end point should incorporate routine Hb typing in the study design to account for its effect. It appears that other common
RBC polymorphisms do not significantly impact this end point, though this may vary in different epidemiological settings. Although this study was designed to investigate the acquisition and maintenance of malaria immunity, the observed effects of HbAS validate this study’s ability to detect factors that influence malaria morbidity.

Acknowledgments

We sincerely thank the residents of Kambila, Mali, for participating in this study. We also thank Cheick Tidiane Dabo for clinical laboratory support, Boubacar Guindo and Dansine Diarra for collecting geographic information system data, Seydou Dia and Tonkoro Diarra for assisting at the study clinic, Bakary Coblilay and Daouda Kane for helping to prepare the study site, and Richard Sakai and Julie Kim for logistic support.

APPENDIX

POLYMERASE CHAIN REACTION (PCR) PROTOCOL FOR DETECTION OF THE 3.7-kb DELETIONAL DETERMINANT OF α-THALASSEMA

The 3.7-kb deletional determinant of α-thalassemia (α-3.7) was identified by a nested PCR protocol. Approximately 5 ng of extracted genomic DNA (Qiagen) was amplified in a 25-μL reaction volume consisting of 20 mmol/L Tris-HCl, pH 8.5, 50 mmol/L KCl, 1.5 mmol/L MgCl2, 1 mol/L betaine (Sigma), 0.3 μmoles/L of each primer, 0.2 mmol/L each dNTP and 1.25 units Platinum Taq polymerase (Invitrogen). In the first round (multiplexed), forward 5’-CCCCTGCAAAGTCACC C-3’ [40] and reverse 5’- AAAGCACTCTAGGGTCCAG CG-3’ [40] primers were used to generate a product that would only amplify if α-3.7 was present. A different reverse primer 5’- AGACACGGAAAGGCCTGGTG-3’ [40] was used in the same reaction mixture to generate a product that would only amplify if α-3.7 was present. Denaturation at 95°C for 5 min was followed by 35 cycles of denaturation at 97°C for 45 s, annealing at 60°C for 75 s, and extension at 72°C for 2.5 min with a final extension at 72°C for 5 min. Separate nested amplifications of the first round products (1 μL of 1:20 dilution) were performed in the same reaction buffer with forward primer 5’- CTTTCCTACCCGAGCGAGTT -3’ [41] and reverse primer 5’- AGAGG GGGCGTTGGGAAGGC-3’ (to generate a 1.8-kb product that amplifies in the absence of α-3.7) or forward primer 5’- CTTTCCTACCCGAGCGAGTT -3’ [41] and reverse primer 5’- CCACTTTCCTCCTCCATCCC-3’ (to generate a 2.0-kb product that amplifies in the presence of α-3.7). Thermal cycling steps were the same as for the first round. Amplified products were separated and visualized on 1.2% agarose gels (Lonza). The sole presence of the 1.8-kb band indicated no deletion, the sole presence of the 2-kb band indicated α-3.7 homozygosity (−α/−α), and the presence of both the 1.8-kb and 2-kb bands indicated α-3.7 heterozygosity (−α/α).

References