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MAJOR ARTICLE

Exposure to Diverse *Plasmodium falciparum* Genotypes Shapes the Risk of Symptomatic Malaria in Incident and Persistent Infections: A Longitudinal Molecular Epidemiologic Study in Kenya

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Background. Repeated exposure to malaria infections could protect against symptomatic progression as people develop adaptive immunity to infections acquired over time.

Methods. We investigated how new, recurrent, and persistent *Plasmodium falciparum* infections were associated with the odds of developing symptomatic compared with asymptomatic malaria. Using a 14-month longitudinal cohort in Western Kenya, we used amplicon deep sequencing of 2 polymorphic genes (*pfama1* and *pfcsp*) to assess overlap of parasite genotypes (represented by haplotypes) acquired within an individual's successive infections. We hypothesized infections with novel haplotypes would increase the odds of symptomatic malaria.

Results. After excluding initial infections, we observed 534 asymptomatic and 88 symptomatic infections across 186 people. We detected 109 *pfcsp* haplotypes, and each infection was classified as harboring novel, recurrent, or persistent haplotypes. Incident infections with only new haplotypes had higher odds of symptomatic malaria when compared with infections with only recurrent haplotypes [odds ratio (OR): 3.24; 95% confidence interval (CI), 1.20–8.78], but infections with both new and recurrent haplotypes (OR: 0.64; 95% CI: 0.15–2.65) did not. Assessing persistent infections, those with mixed (persistent with new or recurrent) haplotypes (OR: 0.77; 95% CI: 0.21–2.75) had no association with symptomatic malaria compared with infections with only persistent haplotypes. Results were similar for *pfama1*.

Conclusions. These results confirm that incident infections with only novel haplotypes were associated with increased odds of symptomatic malaria compared with infections with only recurrent haplotypes but this relationship was not seen when haplotypes persisted over time in consecutive infections.

Keywords. falciparum malaria; asymptomatic; adaptive immunity.

Plasmodium falciparum causes more than 200 million clinical malaria cases annually [1]. Many of these infections occur in young children, who are more likely to develop symptomatic malaria compared with adults [2–4]. This age-dependent risk of symptomatic disease is thought to be due to repeated exposure to *P falciparum* that produces adaptive, disease-controlling immune responses [5–9]. The targets and mechanisms of this naturally acquired, antidisease immunity remain largely obscure.

In the absence of measurable immune correlates, the contours of functional clinical immunity to disease have been inferred from patterns of disease risk and parasite genetics. Specifically, the dependence of antidisease immunity on the gradual accumulation of functional responses to genetically diverse parasites has been supported by studies reporting that symptomatic malaria is often associated with the presence of parasite genotypes that were unobserved in prior infections [10–15]; this suggests that symptomatic malaria results from new infections that exploit gaps in immunologic memory. These studies, though, have been limited in scope and follow-up [12–15], resolution of genotyping approach [10–15], and an inability to partition effects of parasite genotypes between newly acquired and persistent infections, which collectively limit generalizability of findings. Furthermore, most [10, 12–15] have interrogated neutral parasite genes that do not encode targets of functional immunity, which limits causal inference of immunologic mechanisms. A clearer understanding of the influence of parasite genetic diversity on disease risk would inform the development of polyvalent vaccines.

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To explore how specific *P falciparum* infections acquired over time influence the risk of symptomatic malaria, we investigated the association between *P falciparum* genotypes and an individual's risk of symptomatic infection using a 14-month longitudinal cohort in a high-transmission setting in Western Kenya. We classified each person's infections as harboring novel, recurrent, or persistent parasites on the basis of amplicon deep sequencing of 2 diverse parasite genes that encode targets of known functional immunity at the liver (circumsporozoite protein, *pfcsp*) and blood (apical membrane antigen-1, *pfama1*) stages, and analyzed associations between haplotype categories and odds of symptomatic malaria. We hypothesized that, compared with infections harboring parasite genotypes observed within a person's prior infections, those harboring hitherto-unobserved haplotypes would be associated with increased likelihood of symptomatic malaria.

MATERIALS AND METHODS

Study Population and Sample Collection

From June 2017 to July 2018, we followed a cohort in Webuye, Western Kenya, consisting of people aged 1 to 85 years residing in 38 households radially sampled in 3 villages [16] in an area of high malaria transmission primarily by *Anopheles gambiae*. Asymptomatic *P falciparum* infections were detected by active case detection using monthly dried blood spot (DBS) collection, in which parasites were detected by real-time polymerase chain reaction (qPCR). Symptomatic malaria infections were passively detected whereby participants experiencing malaria-like symptoms were tested for malaria using a rapid diagnostic test (RDT; Carestart Malaria HRP2 *Pf* from Accessbio) [17] and had a DBS collected. RDT-positive participants were treated with Artemether-Lumefantrine.

Sample Processing

Parasite genotyping has been previously described [18]. Briefly, genomic DNA from DBS was tested in duplicate for *P falciparum* parasites using a qPCR assay targeting the *P falciparum pfr364* motif [19, 20], *P falciparum*-positive samples were genotyped at *pfama1* and *pfcsp* using PCR amplification and sequencing on an Illumina MiSeq [21, 22]. Reads were quality-filtered and mapped to the 3D7 reference sequences for *pfama1* and *pfcsp* [22–24] before haplotype inference using DADA2 (version 1.8) in R (version 4.0.2) [25, 26], and haplotypes were filtered to mitigate false discovery risk using previously validated criteria [27]. The output was a catalog of all *pfcsp* and *pfama1* unique haplotypes in each qPCR-positive infection for each person. Sequences are available through GenBank (PRJNA646940).

Exposure and Outcome Assessment

For every person, we classified each parasite haplotype in each of their infections as: (1) new, a haplotype not previously observed in that person during the study period; (2) recurrent, one previously observed in that person but not in the most recent DBS; or (3) persistent, a haplotype previously observed in the most recent DBS (Figure 1). For the main exposure, we used these haplotype classifications to categorize each infection; categories were assigned independently for *pfama1* and *pfcsp*.

For the main outcome, each *P falciparum* infection was classified as asymptomatic or symptomatic. An asymptomatic infection was *P falciparum*-positive by qPCR in a person lacking symptoms during a monthly follow-up visit. A symptomatic infection was *P falciparum*-positive by RDT and qPCR in a person

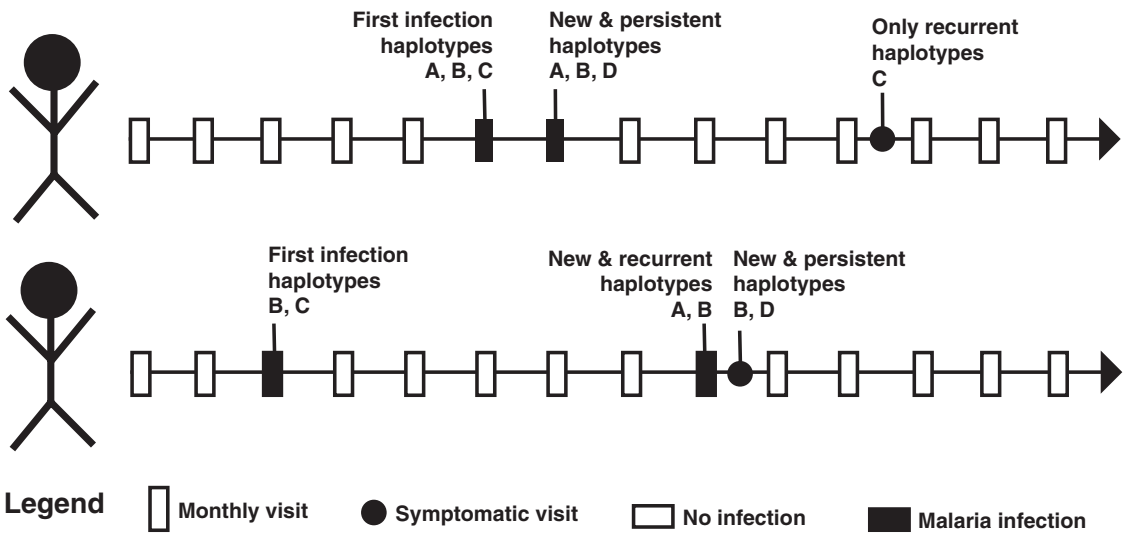

Figure 1. Haplotype categorization throughout participant follow-up. Two hypothetical scenarios illustrate how the malaria haplotypes that participants acquired over time were categorized as new, recurrent, or persistent. Visits in which participants did not have an actively detected asymptomatic malaria infection at a monthly visit are indicated by a white box.

with at least 1 malaria symptom (ie, fever, aches, vomiting, diarrhea, chills, cough, or congestion) during a sick visit. Infections were excluded from outcome ascertainment if they occurred within 14 days of taking Artemether-Lumefantrine or were the person's first infection during the study.

We assessed odds of symptomatic malaria as a function of haplotypes in 2 distinct types of malaria infections: (1) incident infections, where none of the haplotypes in the infection were previously observed in the participant's most recent DBS or (2) persistent infections, where at least 1 haplotype persisted between consecutive DBS collections, excluding infections where participants had a symptomatic infection, were prescribed antimalarials, and had another infection with persistent haplotypes within 30 days following the initial infection.

Comparing Odds of Symptomatic Malaria Among Incident Infections

Among incident infections, we conducted a multilevel logistic regression comparing the odds of having a symptomatic compared to an asymptomatic infection among people infected with (1) only new haplotypes; (2) new and recurrent haplotypes; or (3) only recurrent haplotypes (Equation).

$$\ln(\textit{Symptomatic malaria}_i) = \alpha_i + \beta_1 \textit{Age}_i + \beta_2 \textit{Number of prior infections}_i + \beta_3 \textit{Transmission season}_i + \beta_4 \textit{Multiplicity of infection}_i + \beta_5 \textit{Haplotype comparison}_i + \epsilon_i \quad (1)$$

The model included a participant-level random intercept and controlled for confounding covariates (Figure S1): participant age (≤15 or >15 years), number of prior malaria infections during the study (≤3 or >3 infections), transmission season (≤50 or >50 mosquitoes collected in the prior 14 days across study site), and multiplicity of infection (≤2 or >2 haplotypes). Categorization thresholds were determined by functional form assessment. Differences in model covariates stratified by symptomatic status were compared using the Pearson's χ^2 test.

We evaluated effect measure modification by age on the multiplicative scale by computing multilevel logistic regression models stratified by age category (≤15 or >15 years). Direction of effect and 95% confidence intervals were compared across age-stratified models. The log-likelihood ratio test compared output from an adjusted multilevel logistic regression with an interaction term between age and the haplotype categories to results from the model in the Equation.

Comparing Odds of Symptomatic Malaria Among Persistent Infections

We next focused on infections harboring persistent haplotypes, which were defined as haplotypes also observed in testing immediately before the episode. To do this, we restricted the data set to only infections with persistent haplotypes occurring within 30 days of a prior asymptomatic infection and classified the second infection within each of these pairs based on presence or absence of additional haplotypes: (1) only persistent; (2) new and persistent; (3) recurrent and persistent; or (4) new, recurrent, and persistent. We compared between categories the number of days since previous asymptomatic infection using the Kruskal-Wallis χ^2 test.

We then collapsed these persistent categories into infections with mixed types of haplotypes compared with only persistent haplotypes and computed a multilevel logistic regression similar to Equation 1. Model covariates and effect measure modification by age were evaluated as discussed previously.

To assess potential for presymptomatic infections, we analyzed pairs of symptomatic infections that had a preceding asymptomatic infection less than 30 days prior; among these asymptomatic-symptomatic pairings, we compared the time interval from asymptomatic to symptomatic infection between the symptomatic infections that did or did not harbor persistent haplotypes using a Kruskal-Wallis χ^2 test. Statistical analyses were performed using R (version 4.0.2) [26].

Ethical Review

The study was approved by the ethical review boards of Moi University (2017/36), Duke University (Pro00082000), and the University of North Carolina at Chapel Hill (19-1273). All study participants provided written informed consent or assent (for children).

RESULTS

Haplotype Classification and Infection Categorization

Over 14 months, we recorded 902 asymptomatic and 137 symptomatic *P falciparum* infections (Figure 2A). After parasite sequencing, we obtained genotypes for 861 *P falciparum* infections among 239 people, with a range from 1 to 14 infections during the study period (mean: 3.8). From these data, events meeting criteria for analysis as outcomes consisted of 622 infections (534 asymptomatic and 88 symptomatic) harboring 109 *pfcsp* haplotypes across 186 people; 435 infections (69.9%) harbored new haplotypes, 320 (51.4%) harbored recurrent haplotypes, and 213 (34.2%) had persistent haplotypes (Figure 2B). A plurality of infections (27.2%) harbored only new haplotypes (Table 1). Results for *pfama1* are recorded in the supplement (Table S1, Figure S2).

Analysis of Symptomaticity in Incident Infections

We first assessed if the presence of new haplotypes influenced odds of symptomatic malaria among 409 incident (358 asymptomatic and 51 symptomatic) infections consisting of: (1) only new (N = 169); (2) new and recurrent (N = 139); or (3) only recurrent (N = 101) *pfcsp* haplotypes. Among incident infections, symptomatic infections were more likely to consist of only new haplotypes, occur in children, arise during the high malaria transmission season, and have a lower multiplicity of infection (Tables 1, S1). Compared with infections composed of only recurrent *pfcsp* haplotypes, odds of symptomatic malaria were similar in those with both new and recurrent haplotypes (Odds

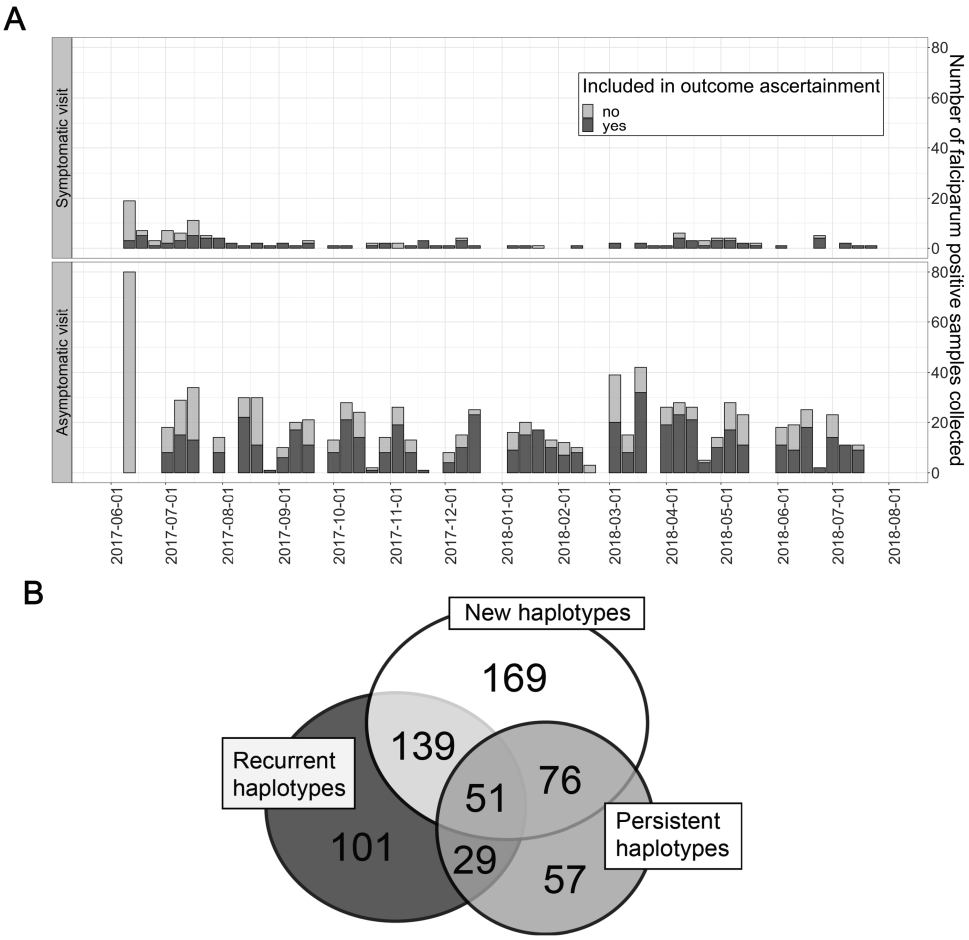

Figure 2. Total number of *Plasmodium falciparum* infection types and categorization of *pfcsp* haplotypes within these infections. *A,* Asymptomatic and symptomatic *P falciparum*-positive samples were captured during 14 months of sampling. Symptomatic infections were captured during as-needed sick visits and asymptomatic infections during monthly visits. A person's initial infection is light gray. Subsequent infections for that person were used for outcome ascertainment (dark gray). *B,* Overlap of *pfcsp* haplotype categories across all symptomatic and asymptomatic *P falciparum* infections (N = 622). Numbers indicate the number of infections that had haplotypes within each category: new, recurrent, or persistent.

Ratio [OR]: 0.64; 95% confidence interval [CI]: 0.15–2.65) but significantly higher for those harboring only new haplotypes (OR: 3.24; 95% CI: 1.20–8.78) (Figure 3A). Results were similar but not statistically significant for *pfama1* (Figure S3). In age-stratified models, the association was similar in children ≤15 years (OR: 3.01; 95% CI: 1.02–8.84) and adults >15 years (OR: 4.00; 95% CI: 0.44–36.08), indicating, along with similarity in model fit between models with and without an interaction term for age (P = .996 by log-likelihood ratio test), that age did not modify the association between haplotype classification and symptoms (Figure 3B).

Analysis of Symptomaticity in Persistent Infections

Restricting the data set to consecutive infections occurring within 30 days, persistent *pfcsp* haplotypes were identified in 139 infections (109 asymptomatic and 30 symptomatic) categorized into those containing: (1) only persistent (N = 40); (2) new and persistent (N = 49); (3) recurrent and persistent (N = 17); or (4) new, recurrent, and persistent (N = 33) *pfcsp* haplotypes. The number of days since previous asymptomatic infection differed between haplotype categories (P = .023 by Kruskal-Wallis χ^2 test), owing to a cluster of symptomatic infections consisting solely of persistent haplotypes with very small intervals (Figure 4A). A total of 66.7% of symptomatic infections with persistent haplotypes occurred within 14 days (N = 20/30). Results were similar for *pfama1* (Figure S4).

To test whether the acquisition of new or recurrent haplotypes affected the odds of symptomatic compared with asymptomatic malaria among people with a background of persistent parasite haplotypes, we collapsed these categories into having only persistent *pfcsp* haplotypes or having mixed types of haplotypes (persistent haplotypes + at least 1 new or recurrent haplotype). Compared with infections with only persistent *pfcsp* haplotypes, the acquisition of additional haplotypes (either new or recurrent) was not associated with symptoms (OR: 0.77; 95% CI: 0.21–2.75) (Figure 4B). Results

Table 1. Distribution of Covariates Across Infections With Haplotype Results for pfcsp

		Infection Type[a]				
		Incident Infections (N = 409)			Persistent Infections Occurring With 30 Days (N = 139)	
	All (N = 622)	Asymptomatic Infections (N = 358)	Symptomatic Infections (N = 51)	*P* Value	Asymptomatic Infections (N = 109)	Symptomatic Infections (N = 30)	*P* Value
Haplotype category, N (%)				<.001[b]			.002[b]
Only new	169 (27.2)	133 (37.2)	36 (70.6)		…	…	
New and recurrent	139 (22.3)	134 (37.4)	5 (9.8)		…	…	
Only recurrent	101 (16.2)	91 (25.4)	10 (19.6)		…	…	
Persistent + ≥1 new or recurrent	156 (25.1)	…	…		86 (78.9)	13 (43.3)	
Only persistent	57 (9.2)	…	…		23 (21.1)	17 (56.7)	
Age, N (%)				.016[b]			1.000[b]
≤15 years	408 (65.6)	213 (59.5)	42 (82.4)		76 (69.7)	23 (76.7)	
>15 years	214 (34.4)	145 (40.5)	9 (17.6)		33 (30.3)	7 (23.3)	
Number of prior malaria infections[c], N (%)				1.000[b]			.801[b]
≤3	425 (68.3)	252 (70.4)	40 (78.4)		65 (59.6)	23 (76.7)	
>3	197 (31.7)	106 (29.6)	11 (21.6)		44 (40.4)	7 (23.3)	
Transmission season[d], N (%)				.004[b]			.755[b]
Low	387 (62.2)	245 (68.4)	22 (43.1)		70 (64.2)	14 (46.7)	
High	235 (37.8)	113 (31.6)	29 (56.9)		39 (35.8)	16 (53.3)	
Multiplicity of infection, N (%)				.022[b]			<.001[b]
1–2 *pfcsp* haplotypes	317 (51.0)	200 (55.9)	40 (78.4)		29 (26.6)	21 (70.0)	
>2 *pfcsp* haplotypes	305 (49.0)	158 (44.1)	11 (21.6)		80 (73.4)	9 (30.0)	

Abbreviations: IQR, interquartile range; NE, not evaluated

[a]Incident infections were defined as *Plasmodium falciparum* infections in which none of the haplotypes in the infection were previously observed in the participant's most recent dried blood spot. Persistent infections were defined as those in which at least 1 haplotype persisted between consecutive dried blood spot collections, excluding infections where participants had a symptomatic infection, were prescribed antimalarials, and had another infection with persistent haplotypes within 30 days following the initial infection. Some persistent infections occurred greater than 30 days apart; these were excluded from the persistent infection analysis.

[b]Pearson's χ^2 test with Bonferroni correction for repeated measures for 6 infections.

[c]During their participation in the cohort before the event.

[d]Low: ≤50 mosquitoes collected in the two weeks prior; high: > 50 mosquitoes.

were similar using *pfama1* (Figure S5). Owing to small sample sizes, we could not assess effect measure modification of these associations by age.

The shorter time interval between infections with persistent *pfcsp* haplotypes (median: 8; range, 2–28) compared with that for those without persistent haplotypes (median: 21.5; range, 4–30) (*p* value 0.022 by Kruskal-Wallis χ^2 test) suggested that some of these persistent infections could have been presymptomatic (Figures S6, S7). Sensitivity analyses removing potential presymptomatic infections could not be conducted due to data sparsity.

DISCUSSION

In a high-transmission setting in Western Kenya, incident *P falciparum* infections composed of parasite haplotypes that were hitherto unobserved within an individual increased that person's odds of symptomatic malaria. In contrast, the appearance of new haplotypes in a person who was already infected with persistent haplotypes did not increase the odds of symptoms. Collectively, our results are consistent with a model of antidisease immunity in which genetically distinct parasites can overcome immunity and cause disease in incident infections, but this ability is attenuated by the presence of persistent, tolerated parasites.

Compared with infections with haplotypes a person experienced previously during the 14-month study, we found incident infections with only new haplotypes increased odds of symptomatic malaria more than 3-fold. These results are consistent with the phenomena that partial variant-specific immunity is acquired over time to provide antidisease protection, and extend the findings of prior studies that report an increased risk of symptomatic malaria when infected with novel haplotypes [10–15]. Notably, our findings resulted from approaches that overcame limitations in these studies, including small sample sizes with brief follow-up [12–14], infrequent sampling [14, 15], genotyping approaches with high failure rates [10], and an inability to capture multiclonal genotypes [10–15]. Specifically, genotyping approaches that use PCR-restricted fragment length polymorphism to detect size variants [10–15] capture only 30% of the unique clones present compared with amplicon deep sequencing [28]. Under these approaches, complex infections common in high-transmission areas [29] are incompletely captured

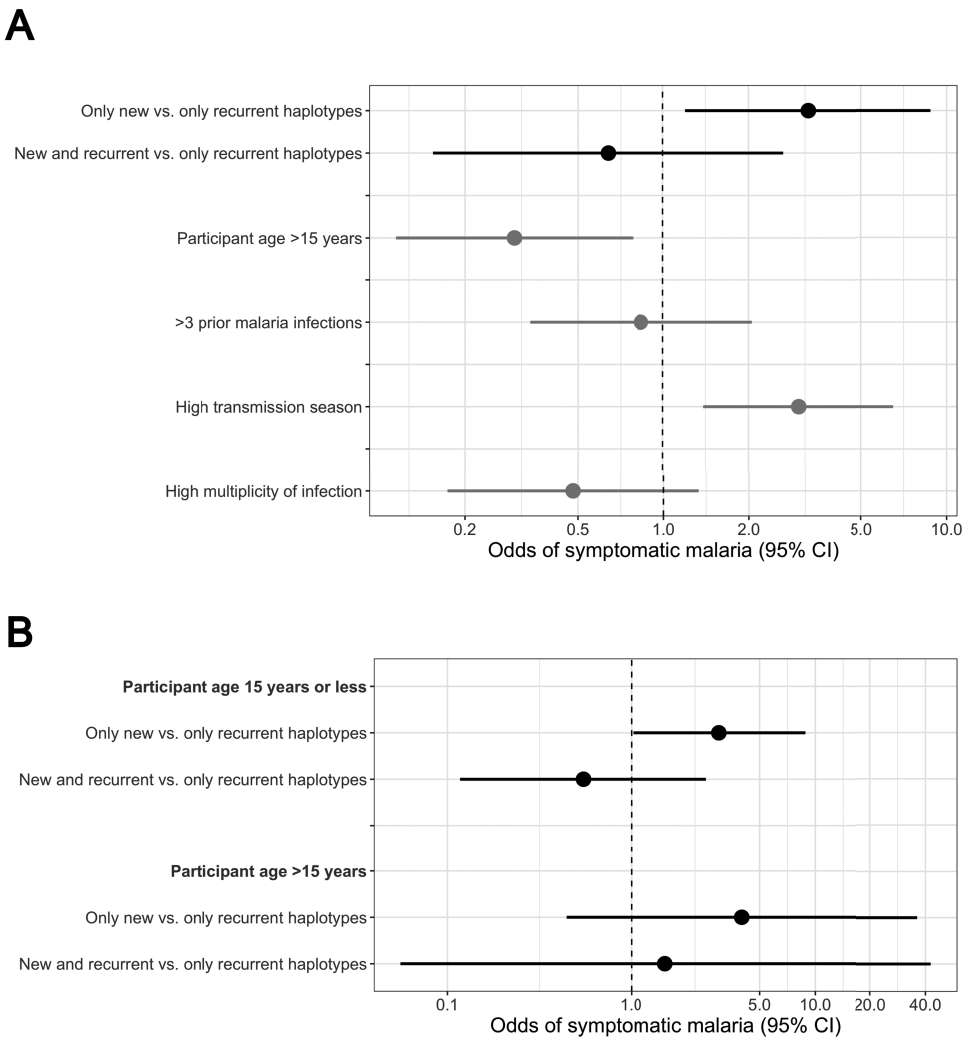

Figure 3. Incident infections: comparison of odds of symptomatic malaria between infections harboring new versus recurrent *pfcsp* haplotypes. *A,* Multilevel logistic regression results for the odds of symptomatic malaria comparing (1) only new versus only recurrent (black) and (2) new and recurrent versus only recurrent (black) *pfcsp* haplotypes. Dots indicate the odds ratios and lines the 95% confidence intervals. *B,* Assessment of effect measure modification on symptomatic disease by age. Adjusted multilevel logistic regression models comparing the odds of developing symptomatic malaria between (1) only new versus only recurrent and (2) new and recurrent versus only recurrent haplotypes were computed conditioned on age category. Dots indicate the odds ratios and lines the corresponding 95% confidence intervals.

and can be incorrectly classified as new or recurrent. Using fine-scale genotypes created by the more sensitive amplicon deep sequencing method [28], we more definitively partitioned the distinct effects of new or recurrent haplotypes within incident infections. Our results suggest that symptomatic malaria among frequently infected residents of a high-transmission setting is associated with the acquisition of blood-stage parasites to which a person has been hitherto unexposed.

Surprisingly, this increased risk of symptomatic disease with new parasite haplotypes was attenuated when new haplotypes were mixed with recurrent ones. Because this analysis was restricted to incident infections, this could not be attributed to the "persistence" of recurrent haplotypes, suggesting the acquisition of recurrent parasite strains may mediate the disease-causing effects of new haplotypes. Attenuation could result from cross-reacting immune recognition of recurrent parasites that enhances parasite clearance or diminishes immune activation [9, 30], with competition between haplotypes that reduces pathogenesis [31] or alternate mechanisms. Also surprising, and in contrast to a prior report [10], we observed an increased risk of symptomatic malaria when new haplotypes were present both in adults and in children. The ability to register this effect is likely from use of a sensitive genotyping method that could capture diverse clones in polygenomic infections, which are more common in adults. Additionally, the enhanced though partial control of malaria transmission in Kenya over decades may have mitigated the durability of disease-controlling responses. The presence of this risk in adults supports an age-independent mechanism for this phenomenon,

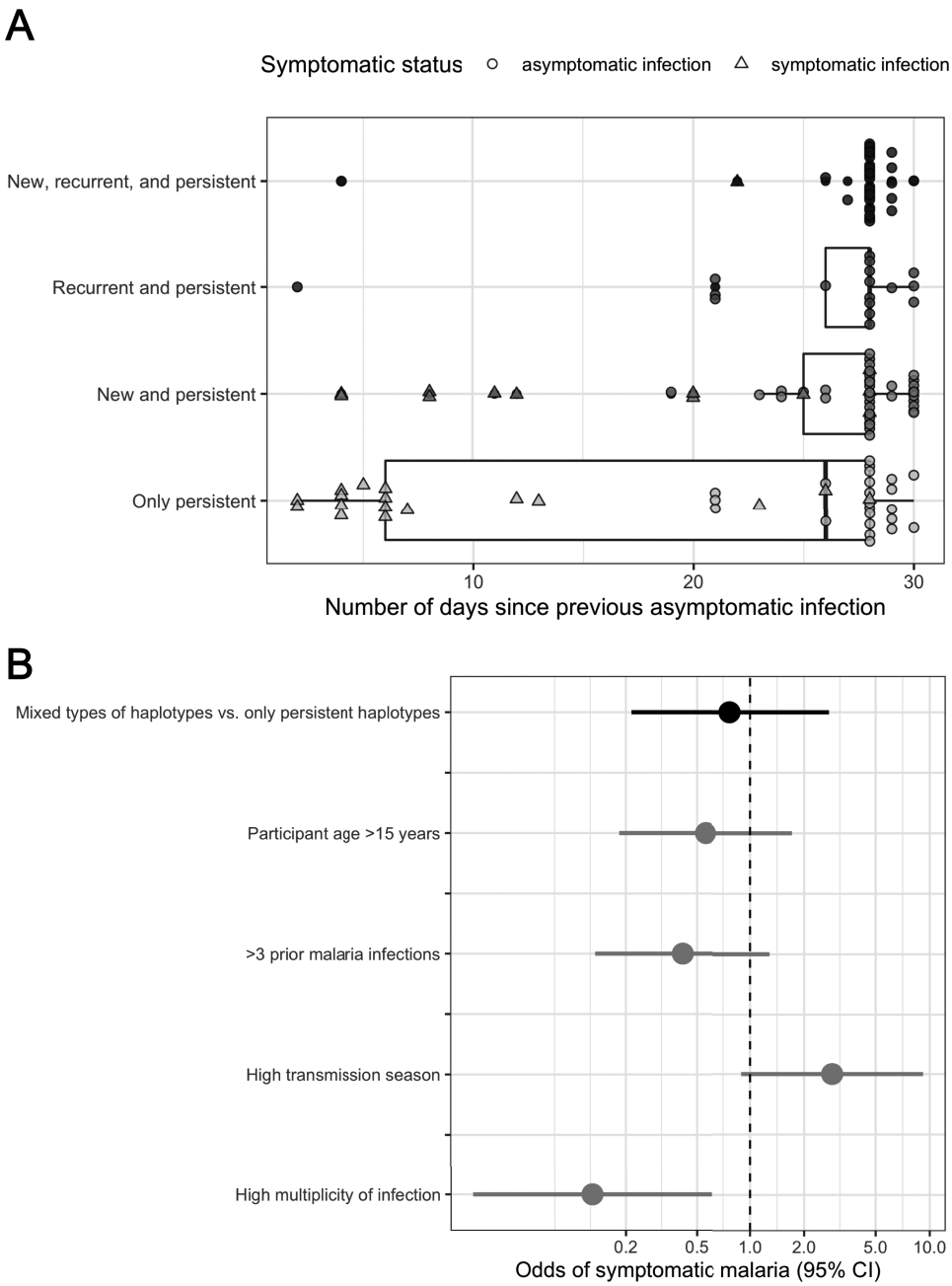

Figure 4. Persistent infections: comparison of odds of symptomatic malaria between infections harboring mixed (persistent mixed with new or recurrent) versus only persistent *pfcsp* haplotypes that occurred within 30 days. *A*, Distribution of the number of days since previous asymptomatic infection for malaria infections with persistent *pfcsp* haplotypes occurring within 30 days. Current infections were categorized into: (1) only persistent; (2) new and persistent; (3) recurrent and persistent; and (4) new, recurrent, and persistent. Current asymptomatic infections were represented by circles and symptomatic ones by triangles. *B*, Adjusted multilevel logistic regression results for the odds of symptomatic malaria comparing consecutive infections occurring within 30 days with mixed (persistent mixed with new or recurrent) types of haplotypes versus only persistent haplotypes (black). Dots represent odds ratios and lines the corresponding 95% confidence intervals.

despite the common assumption that by reaching adulthood one has acquired durable immunity to diverse parasites.

In contrast to incident infections, persistent infections were not likely to be symptomatic when supplemented by new or recurrent parasite haplotypes. Asymptomatic infections with persistent haplotypes could be presymptomatic and, thus, not greatly impacted by the acquisition of additional haplotypes; this has been observed in previous work in which many asymptomatic infections later became symptomatic [32, 33] and is supported by the short time intervals we observed between asymptomatic-symptomatic infection pairs with persistent haplotypes. Alternatively, the presence of persistent haplotypes may limit immune responses [30] or the efficiency of establishing superinfections

[34]; this would be consistent with the original meaning of "premunition," wherein contemporaneous infection confers resistance to superinfection [35]. Regardless of mechanism, these results illustrate the importance of distinguishing between recurrent and persistent haplotypes in incident and persistent infections, which has previously not been done [10–15, 33]. Moreover, future work could assess variability in haplotype within-host competition [31], virulence of specific haplotypes [36], and host immune responses to more directly measure how new, recurrent, and persistent haplotypes affect symptomatic malaria risk.

Broadly, our results highlight not only the role that recurrent and persistent haplotypes have in reducing odds of symptomatic disease, but also the critical influence of parasite genetic diversity on this relationship. In population-based studies, reduced transmission can increase and shift the severity of disease [37], possibly by reduced acquisition of antidisease immunity in childhood. In our study, such antidisease immunity manifested in incident infections such that symptomaticity was prevented by the presence of recurrent haplotypes. Because these recurrent haplotypes require exposure to prior diverse infections, reduced exposure would increase the likelihood that incident infections are composed of new haplotypes and likely to manifest symptoms. However, if reduced transmission is accompanied by reductions in parasite genetic diversity, as has been reported in several settings [38, 39], even with fewer prior infections, the per-infection likelihood that a parasite will harbor recurrent haplotypes would remain high and thereby attenuate symptoms. Future studies could explore whether specific haplotypes at disease-mediating loci differentially modify the risk of malaria and furnish targets for surveillance.

The study had limitations. Although amplicon deep sequencing was a sensitive method for identifying different malaria infections [28], it might not have captured all genetically distinct infections that occurred during the study. To account for this, we compared results across 2 unlinked parasite gene targets, *pfama1* and *pfcsp*. We did not observe malaria infections that participants acquired before the study; misclassifying a haplotype as new when it might have been present in an individual before the study would bias results toward the null. Additionally, persistent infections were possibly presymptomatic. Future studies could have more frequent longitudinal sampling to distinguish between asymptomatic and presymptomatic infections.

In conclusion, infections harboring novel haplotypes increased the likelihood of symptomatic malaria in incident infections, but not when acquired in the presence of persistent infections. Future research could explore the immunological mechanisms by which new haplotypes change the risk of symptomatic malaria when compared to recurrent or persistent haplotypes.

Supplementary Data

Supplementary materials are available at *Clinical Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

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