The Journal of Infectious Diseases

MAJOR ARTICLE

Multiplicity of Asymptomatic *Plasmodium falciparum* Infections and Risk of Clinical Malaria: A Systematic Review and Pooled Analysis of Individual Participant Data

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Background: The malaria parasite *Plasmodium falciparum* holds an extensive genetic polymorphism. In this pooled analysis, we investigate how the multiplicity in asymptomatic *P. falciparum* infections—that is, the number of coinfecting clones—affects the subsequent risk of clinical malaria in populations living under different levels of transmission.

Methods: A systematic search of the literature was performed to identify studies in which *P. falciparum* infections were genotyped in asymptomatic individuals who were followed up prospectively regarding the incidence of clinical malaria. Individual participant data were pooled from 15 studies (n = 3736 individuals).

Results: Multiclonal asymptomatic infections were associated with a somewhat increased subsequent risk of clinical malaria in the youngest children, followed by an initial declining risk with age irrespective of transmission intensity. At approximately 5 years of age, the risk continued the gradual decline with age in high-transmission settings. However, in older children in moderate-, low-, and seasonal-transmission settings, multiclonal infections were either not significantly associated with the risk of subsequent febrile malaria or were associated with an increased risk.

Conclusions: The number of clones in asymptomatic *P. falciparum* infections is associated with different risks of subsequent clinical malaria depending on age and transmission intensity.

Keywords. malaria; *Plasmodium falciparum*; immunity; clones; multiplicity of infection; risk analyses.

Malaria remains a major health problem in countries where the disease is endemic [1]. *Plasmodium falciparum* causes the majority of all malaria cases and deaths, especially in children in sub-Saharan Africa. Efforts to control and eliminate malaria have resulted in declining malaria incidence during the last decade. Nonetheless, the rate of decline has stalled and in some regions malaria incidence is increasing [1]. Malaria interventions aiming at eventually eliminating the disease need to reduce the reservoir of asymptomatic parasitemia that maintains transmission [1].

Individuals living in endemic areas gradually acquire immunity to *P. falciparum* after repeated infections. Protection is achieved against high parasite densities and symptoms of the infection, but the ability to clear infections is more limited, and apparently healthy children and adults often harbor low-density *P. falciparum* infections [2]. These infections are frequently composed of multiple genetically distinct clones [3]. Understanding the impact of asymptomatic multiclonal infections on the risk of clinical malaria and on the acquisition and maintenance of host immunity is important for guiding and optimizing interventions such as vaccines and preventive treatment strategies.

The number of coinfecting *P. falciparum* clones, also referred to as the multiplicity or complexity of infection, has been shown to vary by age and transmission intensity [3–5]. Several studies have assessed how the level of host immunity relates to

Received 19 June 2019; editorial decision 25 September 2019; accepted 1 October 2019; published online October 4, 2019.

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Abbreviations: msp1, Merozoite surface protein 1 gene; msp2, Merozoite surface protein 2 gene; PfPR, *Plasmodium falciparum* parasite rate; $PfPR_{2-10}$, *Plasmodium falciparum* parasite rate for the ages 2–10 years.

The Journal of Infectious Diseases® 2020;221:775–85

DOI: 10.1093/infdis/jiz510

the number of clones in asymptomatic *P. falciparum* infections. Some report a reduced risk [6–10], while others an increased risk, of subsequent febrile malaria attacks in asymptomatic individuals harboring multiclonal infections [5, 11–14]. Age and transmission intensity have been proposed to explain differences between studies [5, 9, 15]. However, these studies have been too small to conclusively differentiate the impact of these covariates on the associations between number of clones and risk of clinical malaria.

The aim of the current study was to investigate how the number of *P. falciparum* clones associates with the subsequent risk of malaria in relation to age in individuals living in areas of different transmission intensities. This analysis was performed through a systematic review and pooled analysis of individual participant data. Combining individual data from 3736 study participants from 15 studies revealed how the association between the number of coinfecting clones (ie, multiplicity of infection) and subsequent risk of malaria varies by age and transmission intensity.

METHODS

Search Strategy

A systematic review of the published literature was conducted according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses of Individual Participant Data (PRISMA-IPD) (Supplementary Table 1) [16]. The protocol was registered in PROSPERO (registration no. 2015:CRD42015025824). The search included PubMed, Cochrane Library, EMBASE, and Web of science through 9 November 2015, combining MeSH terms and free-text terms (full search presented in Supplementary Table 2). No restrictions were used regarding language, geography, or age.

Study Review

One investigator (M. E.) reviewed all abstracts and selected full-text articles using predetermined protocols (Supplementary Table 3). Consensus on the final study inclusion was achieved with a second investigator (A. F.). Population-based cohort studies were eligible for inclusion. Intervention studies were included only if they had a placebo group or if study participants were followed up for at least 3 months before the intervention.

Study Participants

The following were the criteria required for individual data to be included in the pooled analysis: (1) residence in a malaria-endemic area, (2) asymptomatic status at baseline (ie, when genotyping was performed), (3) prospective follow-up (with passive or active surveillance) of ≥90 days for clinical malaria, and (4) treatment with antimalarial drugs only if the participant presented with clinical malaria and not to clear asymptomatic infections.

Pregnant women and children <6 months old were excluded, as were study subjects with reported clinical malaria within 1 month before baseline or ongoing malaria or antipyretic treatment were excluded (these data were not accessible from all included studies). Study subjects with clinical malaria within 1 week after baseline were excluded from analysis (to ensure that all individuals were asymptomatic at baseline). In study sites with repeated sampling, 1 baseline per 5-year period was eligible for inclusion.

Parasite Genotyping

P. falciparum infections needed to be genotyped by polymerase chain reaction (PCR) targeting the genes coding for merozoite surface proteins 1 (*msp1* block 2) or 2 (*msp2*). The number of clones was defined as the total number of *msp1* or *msp2* alleles detected.

Clinical Outcome

The prespecified definition of a clinical episode of malaria was fever (temperature ≥37.5°C and/or reported fever), together with a positive blood slide by light microscopy.

Quality Criteria

The minimum quality criteria for inclusion were ethical approval granted, clinical malaria identified by active and/or passive case detection, and the presence of parasites at the time of fever confirmed by microscopy. Completeness and consistency of data were checked against original publications. Risk of bias in the selection of studies was assessed using National Institute of Health's Quality assessment tool (Supplementary Table 4) [17]. The risk of publication bias across studies was examined in a funnel-plot (Supplementary Figure 1).

Data Collection and Extraction

Authors were invited to share individual participant data from the published studies and any additional unpublished data relevant for the pooled analysis (deadline 23 May 2016, followed by 2 reminders). The requested parameters were sex, age, asymptomatic or symptomatic, number of clones, parasite density at baseline, follow-up time, time to a first clinical episode (parasite density and body-temperature at this event), number of episodes and or episodes (yes/no) during follow-up, and time since latest episode before baseline. Additional information was obtained from the publication or from the authors: malaria case definition, inclusion and exclusion criteria, genotyping method, time of baseline, and *P. falciparum* parasite rate (PfPR) by microscopy (including number and age range of examined subjects). The geographic coordinates of each study location were obtained from the publications or by using Google maps [18].

Ethical Approval and Consent to Participate

All 15 studies included in the meta-analysis had a granted ethical approval stated in the original publications or provided by authors of the original publication.

Data Analysis

Statistical analyses were conducted using Stata 13 (Stata: Release 13; StataCorp). Standardized PfPR for the age range 2–10 years ($PfPR_{2-10}$) were used to categorize studies conducted in perennial transmission areas based on transmission intensity, as low transmission ($PfPR_{2-10}$ <10%), moderate transmission ($PfPR_{2-10}$ 11%–50%), or high transmission ($PfPR_{2-10}$ >50%) [19]. For studies not covering the age range 2–10 years, age-standardized $PfPR_{2-10}$ values were generated from available data using a validated model (Supplementary Figure 2) [20]. If the parasite prevalence data needed for the validated model were not available for a given site, they were extracted from the corresponding time period and site through the Malaria Atlas Project database (Supplementary Table 5) [21]. Study locations without transmission for ≥3 months per year were defined as having strictly seasonal transmission [20].

A Cox proportional hazards model was used, with time to clinical event as a predefined outcome and number of clones and age as covariates, plus an interaction term between number of clones and age, with age as continuous variable. The follow-up period started 1 week after baseline, and subjects were censored at the time of an event, or 50 weeks after baseline or at the end of follow-up for each individual study (whichever occurred first).

The cubic spline model was first performed with predefined knots (10th, 50th and 90th percentiles). For age-stratified analyses, age groups were defined by using the results from this first risk analysis of the entire data, setting the boundaries for a middle age category by the limits of the age interval for which the entire 95% confidence interval (CI) of the hazard ratio (HR) was <1, resulting in 3 age categories (0.5–3.9, 4–7.9, and 8–16.9 years). In a second Cox regression analysis, with age treated as a continuous variable, the knots in the cubic spline were set to best fit the data for the respective transmission categories.

A threshold parasite density for clinical malaria [22] was not used, because data was available for only 6 studies, and it would affect the Cox regression analyses because all study subjects with fever and any parasitemia were treated with antimalarials. In all analyses, a fixed-effects partial likelihood approach was used to control for study site and for methodological heterogeneity, including genotyping method and laboratory [23–25].

RESULTS

Our systematic search found 1540 potential publications (Figure 1). Screening of all abstracts identified 79 studies eligible for full-text assessment, of which 23 met the study criteria and whose authors were invited to contribute data (Supplementary Table 5). Individual participant data was provided for 17 studies (16 published and 1 unpublished), of which 15 (13 conducted in Africa and 2 in Papua New Guinea) had the requested data and were included in the pooled analysis (Table 1). Quality control of the contributed data sets discovered no inaccuracies, except in 2 studies where the number of individuals was fewer than described in the original publication. From these 2 studies, a contribution of randomly selected subset of individual data from the original data set was accepted (Supplementary Table 5).

In total, 3736 study subjects with individual participant data fulfilled the inclusion criteria (Table 1). The age range was 0.5–84 years (median, 7 years), with the majority of participants aged ≤10 years (62%; n = 2309) (Supplementary Figure 3). A total of 1146 study subjects (31%) had a febrile malaria episode within the 90-day to 50-week follow-up periods. Malaria episodes occurred mainly in children (Supplementary Figure 3), whereas adults (aged ≥17 years) had few episodes and were analyzed separately.

Transmission intensities in the respective study sites were categorized based on estimated $PfPR_{2-10}$ (Supplementary Figure 2). One study [5] included 2 sites with different transmission intensities, and the data were divided according to site. Overall, 7 studies (41% of individuals in the pooled analysis) were classified to be from high-transmission areas ($PfPR_{2-10}$ >50%), 5 (44% of individuals) from moderate-transmission ($PfPR_{2-10}$ 10%–50%), 1 (8%) from low-transmission ($PfPR_{2-10}$ <10%), and 2 (7%) from strictly seasonal-transmission areas.

A total of 1658 of 3629 study subjects (46%) were positive by *msp1* and/or *msp2* PCR at baseline (excluding 2 studies only reporting parasite positive individuals; n = 107); 62% in high-, 38% in moderate-, 18% in low-, and 14% in strictly seasonal-transmission settings (Figure 2). Infections with multiple clones (≥2 clones) were most prevalent in high-transmission areas (64%; n = 663), followed by moderate-transmission (61%; n = 381), strictly seasonal-transmission (32%; n = 18) and low-transmission areas (24%; n = 11) (Figure 2). When combining all data (n = 3736), the time to a subsequent malaria episode tended to be longer with increasing numbers of clones (Figure 3).

The risk of subsequent malaria for multiple versus a single clone was first assessed using a Cox proportional hazards model with age modeled as a cubic spline with predefined knots. This exploratory analysis showed that the HR changed with age, with multiple clones being associated with both increased and reduced risk (Figure 4). This significant association between age and the risk of clinical malaria in individuals with asymptomatic multiclonal infections was observed both when including all data ($P < .001$) (adults included) and when stratifying by transmission intensity (all $P \leq .03$); or stratifying for genotyping method, capillary or gel electrophoresis ($P \leq .002$) (data not shown).

We then analyzed the risk within each of the 4 transmission categories, using a second Cox regression model with the knots of the restricted cubic splines chosen to best fit the data for the respective transmission categories (Figure 5). In high-transmission settings (n = 1534), the risk of subsequent clinical malaria if

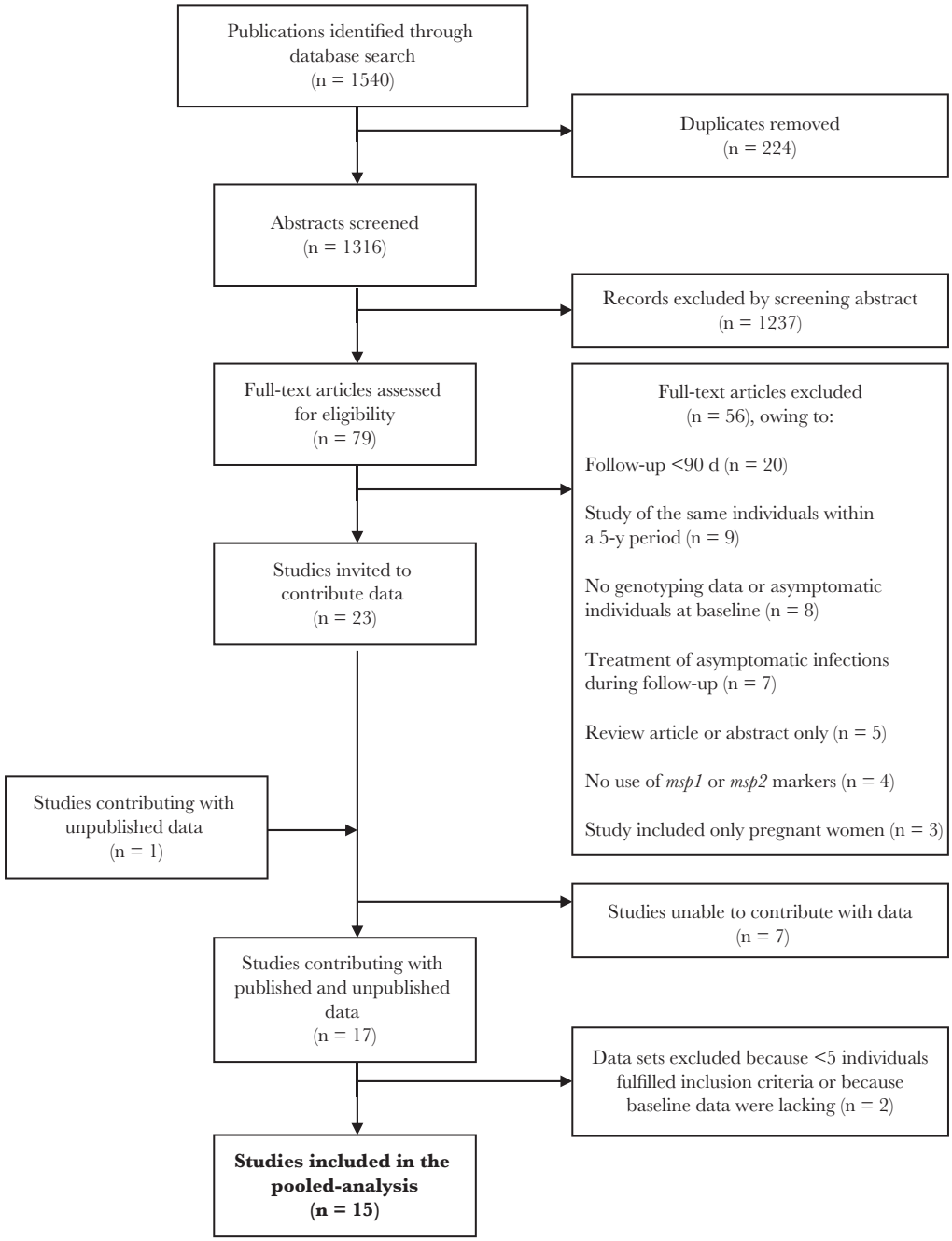


Figure 1. Flow chart of study identification. In total, 23 studies were considered for inclusion to the pooled analysis of which 17 provided data and 15 (14 published and 1 unpublished) had the requested data sets and were included in the pooled analysis.

infected with multiple clones (ie, ≥ 2 clones) compared with single clone, declined gradually from 0.5 years of age throughout childhood until 17 years, reaching a significantly decreased risk at approximately age 5 years (Figure 5*A*). In moderate-transmission sites (n = 1647), the association between clones and estimated risk of malaria was similar to the high-transmission settings during the first years of life. However, at about 5 years of age there was only a tendency of reduced risk in moderate-transmission sites, and the increased risk with age >5 years did not reach significance (Figure 5*B*). In low-transmission sites (n = 305), there was an increased risk for children 0.5–2 years of age infected with multiple ≥2 clones but also in older children (Figure 5*C*). In strictly seasonal-transmission settings (n = 250), again the youngest children tended to be at increased risk when having asymptomatic infections with ≥2 clones [1]; however, CIs were wide and did not reach significance in any age group (Figure 5*D*).

We also performed age-stratified Cox regression analyses for the respective transmission categories (Supplementary Table 6).

Table 1. Characteristics of the Populations From 15 Studies Included in Pooled Analysis[a]

Country	Study Authors, (Year) [Reference]	Year of Sampling	Follow-up Duration		Population			Malaria Outcome	
			Before Baseline, d	After Baseline, mo	Sample Size in Pooled Analysis[b] (in Original Study)	Age, y	Clone Analysis Genotyping Marker (Fragment Analysis[c]/Genotyping Method[d])	Source	Malaria Definition[e]
Kenya	Färnert et al (2009) Ngerenya [5]	2000	30	10	275 (380)	0.5–11	*msp2* (GE/1)	ACD, PCD	1
	Färnert et al (2009) Chonyi [5]	2000	30	10	273 (368)	0.5–11	*msp2* (GE/1)	ACD, PCD	1
	Liljander et al (2011) [13]	2005	28	3	280 (405)	1–7	*msp2* (CE/2)	ACD	2
Mali	Sondén et al (2015) [8]	2006	0	11	223 (225)	2–25	*msp2* (CE/2)	PCD	1
Papua New Guinea	al-Yaman et al (1997) [6]	1992	30	12	80 (236)[f]	1–19	*msp1, msp2* (GE/3)	ACD, PCD	1
	Mueller et al (2012) [26]	2006	30	12	91 (264)	1–3	*msp2* (CE/4)	ACD, PCD	1
Republic of Congo	Ibara-Okabande et al (2012) [27]	2010	14	12	305 (313)	1–9	*msp2* (GE/5)	PCD	1
São Tome	Müller et al (2001) [9]	1998	0	3	365 (365)	1–78	*msp2* (GE/6)	PCD	1
Senegal	Vafa et al (2008) [28	2002	0	7	151 (372)	2–10	*msp2* (GE/1)	ACD	3
	Zwetyenga et al (1999) [29]	1994	2	12	120 (143)	0.5–69	*msp1, msp2* (GE/7)	ACD	2
Sudan	Roper et al (1998) [30]	1994–1996	30	12	27 (106)[f]	6–34	*Msp1, msp2* (GE/1)	ACD, PCD	1
Tanzania	Bereczky et al (2007) [10]	1999	30	11	562 (792)	1–79	*msp2* (CE[g]/2)	PCD	1
	Felger et al (1999) [14]	1993	30	8	24 (99)	0.5–1	*msp2* (GE/6)	ACD, PCD	2
	Fraser-Hurt et al (1999) [31]	1996	0	6	103 (122)	0.5–2	*msp2* (GE/6)	ACD, PCD	1
	Henning et al (2004) [15]	1998	0	9	202 (610)	0.5–6	*msp2* (GE/6)	PCD	2
	Färnert et al (unpublished)	1994	30	9	655 (890)	1–84	*msp2* (CE/2)	PCD	1

Abbreviations: ACD, active case detection; CE, capillary electrophoresis; GE, gel electrophoresis; *msp1*, merozoite surface protein 1 gene; *msp2*, merozoite surface protein 2 gene; PCD, passive case detection.

[a]Two studies were excluded after the data collection was completed since they did not include the requested data or did not meet the inclusion criteria [7, 32]. All data refer to the publication or in contact with authors of the studies, unless otherwise specified.

[b]Study subjects with complete data.

[c]Method of electrophoresis (GE or CE) used for fragment sizing in the genotyping method.

[d]Sources for genotyping methods are as follows: method 1, Snounou et al [33]; 2, Liljander et al al [34]; 3, Reeder and Marshall [35]; 4, Falk et al [36]; 5, Ntoumi et al al [37]; 6, Felger et al al [38]; and 7, Zwetyenga et al [39].

[e]Malaria definitions were as follows: definition 1, temperature ≥37.5°C or history of fever plus parasitemia (by microscopy); definition 2, temperature ≥37.5°C plus parasitemia (by microscopy); and definition 3, temperature ≥37.5°C or history of fever plus parasitemia with cutoff value (by microscopy).

[f]Study contributed with partial data.

[g]Fragment analysis was performed by means of GE in the original publication, however, data from a repeated analysis with the higher-resolution CE method are included for the purpose of the current study.

Asymptomatic parasitemia, (irrespective of number of clones) detected by means of microscopy or PCR, was associated with an increased risk of clinical malaria during follow-up in the youngest age groups in moderate- and high-transmission sites. Considering the large volume of pooled data set, we attempted to assess the risk for the actual number of clones. The lowest risk was found in high-transmission settings, (≥5 clones) (HR, 0.20; 95% CI, .05–.85; $P = .03$). However, results for specific numbers of clones should be interpreted with caution, because sample sizes in these subgroups are small and multiple analyses may generate by-chance significant findings. Hence, we grouped multiclonal infections in ≥2 or ≥3 versus 1 clone, and in both analyses, the lowest risk was again found in children aged 8–16.9 years in the high-transmission settings (HR, 0.50 [95% CI .29–.87; $P = .02$] and 0.44 [.21–.91; $P = .03$], respectively). Differences with age and transmission were also apparent in stratified Kaplan-Meier curves (Supplementary Figure 4).

Analyses for adults were restricted by the low number of malaria episodes in adults in moderate- and seasonal-transmission settings, and the lack of data from adults in low-transmission sites (Supplementary Table 6). Adults in high-transmission areas infected with ≥2 clones, however, were at increased risk of febrile malaria during follow-up (HR, 2.61; 95% CI, 1.03–6.57; $P = .04$).

DISCUSSION

Combining individual participant data from 15 studies resulted in a total of 3736 prospectively followed up study subjects, allowing us to investigate the impact of age and transmission intensity on

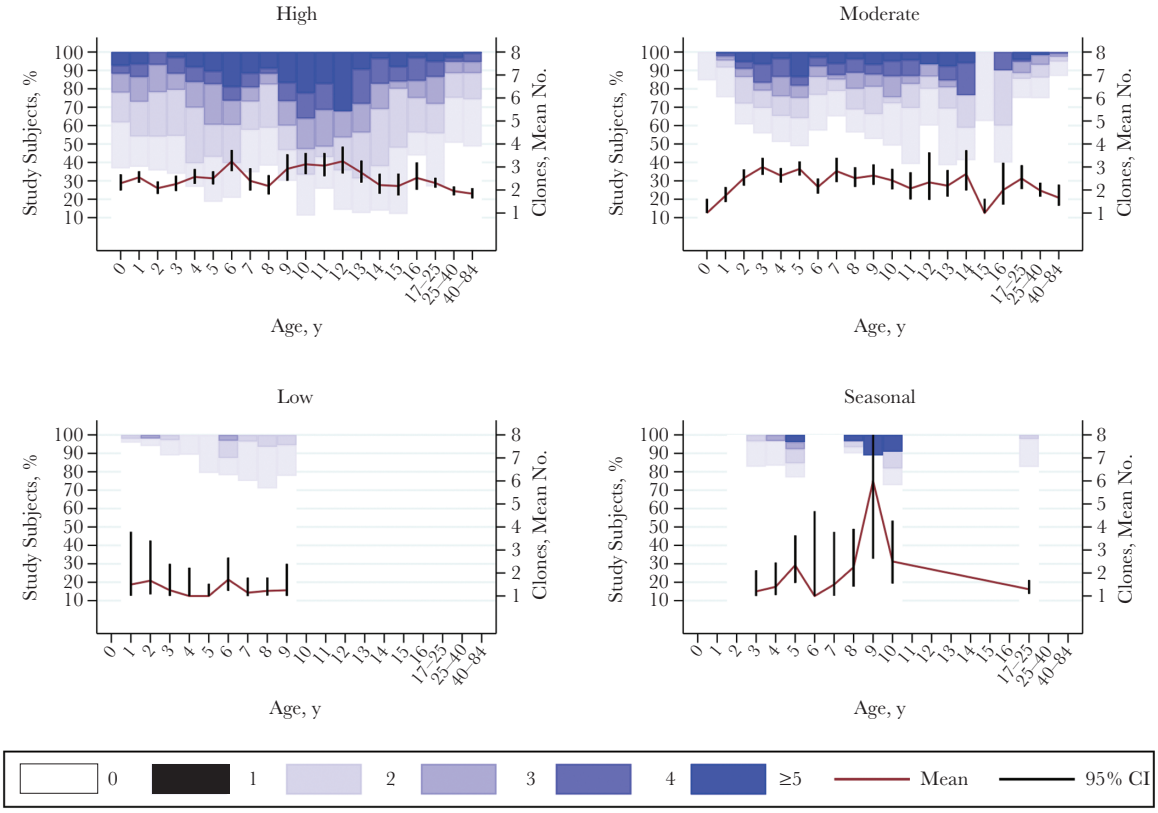
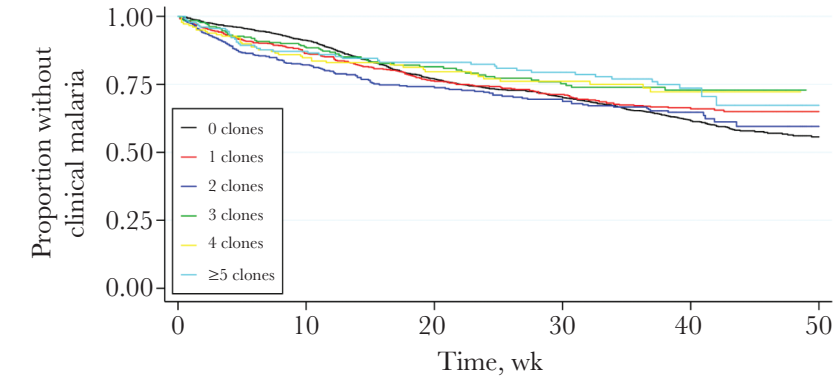


Figure 2. Distribution of number of clones by age and transmission intensity, displayed as percentage of asymptomatic study subjects aged 0.5–84 years infected with different numbers of merozoite surface protein 2 (*msp2*) (and/or *msp1*) genotypes (ie, clones) at baseline, grouped by transmission intensity. Red graphed lines represent the mean number of *msp2* (and/or *msp1*) genotypes in polymerase chain reaction–positive individuals (excluding those who were negative) by subject age, with 95% confidence intervals displayed as vertical black lines. The shades of blue reflects the individual participant data of the number of infected clones, are pooled from 13 studies (n = 3629), not including 2 studies (n = 107) that reported only subjects who were parasite positive at baseline.



Clones, No.	No. at Risk					
0	1972	1737	1287	1105	507	128
1	681	566	394	327	159	51
2	459	364	219	176	80	25
3	265	227	136	110	42	0
4	179	146	94	76	28	0
≥5	180	153	116	102	47	1

Figure 3. *Top,* Kaplan-Meier estimates of time to first subsequent clinical episode of malaria in relation to the number of *Plasmodium falciparum* clones in asymptomatic individuals at baseline. Pooled analysis (unadjusted) is shown for all 15 studies combined (n = 3736). *Bottom,* Number at risk over time.

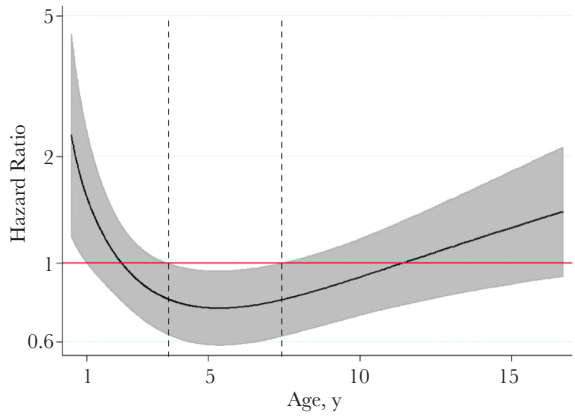


Figure 4. Association between multiple clones of *Plasmodium falciparum* at baseline and time to first episode of clinical malaria for all included individuals <17 years of age (pooled analysis of 15 studies; n = 3736). The hazard ratio (HR) (*black line*), with 95% confidence interval (CI) (*gray area*), corresponds to the risk of a clinical episode of *P. falciparum* for multiple clones (≥2 clones) versus 1 clone (reference HR, 1). An HRs <1 indicates that multiple clones are associated with reduced risk, and an HRs >1 indicates increased risk of clinical malaria during follow-up of ≥3 months. The figure was created using a restricted cubic spline model with prespecified knots (10th, 50th, and 90th percentiles). Dotted lines demarcate the 3 age categories, 0.5–3.9,4–7.9, and 8–16.9 years, used in the age stratified analysis.

the association between the number of clones in asymptomatic *P. falciparum* infections and the risk of subsequent malaria.

An overall survival analysis, including all individuals from all transmission sites, indicated a tendency for longer time to clinical malaria during follow-up with increasing number of clones. In multivariable analyses, the risk was found to vary with age and transmission setting. For the youngest children with asymptomatic multiclonal infections, the age-spline model predicted an increased subsequent risk of clinical malaria followed by an initial decline irrespective of transmission intensity. However, above approximately 5 years of age, the risk patterns varied by transmission. A gradually declining relative risk until age >17 years was observed in high-transmission settings, and an increasing relative risk (not always to significant levels) in children >5 years old in moderate-, low-, and seasonal-transmission settings.

Previous studies (several included here) investigating the association between number of clones and subsequent risk of malaria have presented somewhat divergent results but have been underpowered to demonstrate the effect of age in different transmission settings [5–14]. Moreover, heterogeneity between these studies restricts meta-analyses of published data. The

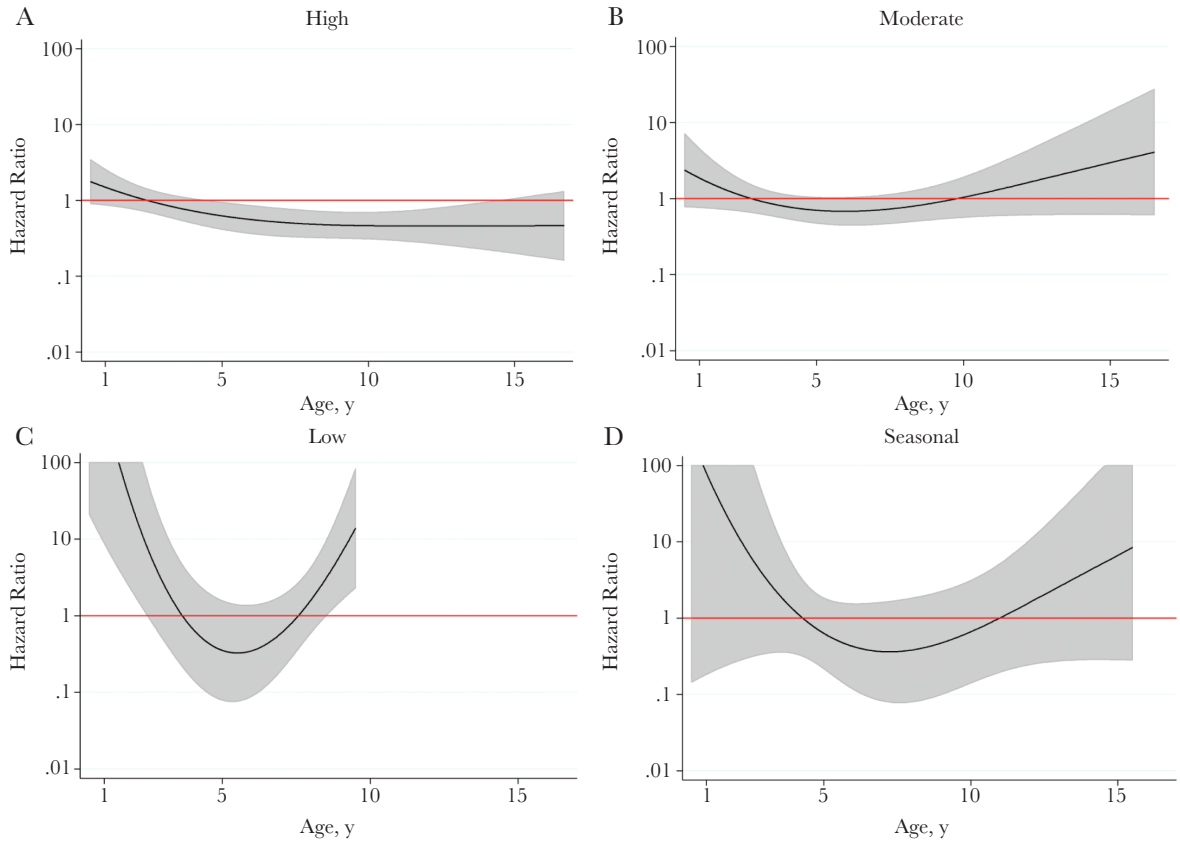


Figure 5. Association between multiple clones of *Plasmodium falciparum* at baseline and time to first episode of clinical malaria in children (aged <17 years) presented by transmission intensity. *A,* High transmission. *B,* Moderate transmission. *C,* Low transmission. *D,* Strictly seasonal transmission. Hazard ratio (HR) (black line) with 95% confidence interval (light gray area) corresponds to risk of clinical malaria episode for multiple clones (≥2 clones) versus 1 clone (reference HR, 1). HRs <1 indicate that multiple clones are associated with reduced risk, and HRs >1 indicate an increased risk of clinical malaria during follow-up of ≥90 days. The figures are created using restricted cubic spline models, with knots chosen to fit the data as well as possible.

access to individual data enabled us to standardize the malaria outcome definition and perform a uniform risk assessment.

Malaria transmission intensity and individual exposure affect the risk of being infected and the number of concurrent clones (multiplicity of infection), as well as the rate at which antimalarial immunity is acquired [40]. We therefore stratified the analyses according to transmission intensity and used age-standardized estimates of PfPR from available data or from a validated model [20]. Dividing the areas into only 4 types of transmission intensity may be crude, but at the expense of resolution, the models gained stability. The age-specific effect of multiclonal infections on the risk of clinical malaria differed with transmission intensity. Pooling data without taking transmission into account would have missed these associations.

As previously proposed for both mild [15, 41] and severe malaria [42, 43], the youngest children seem to be at increased risk of subsequent malaria when infected with multiclonal *P. falciparum* infections. The large study population enabled analyses in relation to age and transmission. Despite harboring the same number of clones, older children clearly had different risk of disease depending on transmission environment. These results emphasize the importance of considering transmission intensity when relating the number of *P. falciparum* clones to host factors.

In contrast to children, adults in high-endemic settings with multiclonal infections were at an increased risk of clinical malaria. Adults in high-endemic areas have generally acquired a considerable degree of protective immunity and symptomatic infections are relatively uncommon, but most individuals still harbor low-level parasitemia [2, 41]. Multiclonal infections might thus be found in individuals with high exposure and possibly a lower degree of immunity. Moreover, adults who are sick in malaria tend to have low parasite densities [44], and the ability to control malaria infections may be affected by comorbid conditions [45, 46].

The number of concurrent *P. falciparum* clones was defined by genotyping MSP1 and MSP2, among the most polymorphic merozoite surface antigens [25]. The true genetic diversity and number of clones is likely to be underestimated (especially in areas of high and moderate transmission), owing both to competition in the PCR assay [47] and to within host-dynamics with some clones being entirely below the detection limit or temporarily below this limit in circulating blood because of sequestration [48, 49]. Other limitations of the study were the uneven distribution of data sets included from different transmission intensities, with a majority of study subjects living in high- and moderate-transmission areas.

The results are therefore most reliable regarding children in sites with high and moderate transmission. Moreover, the patterns might differ before and after rainy seasons, and although precise data on time of sampling were not available, the majority of samples were collected at the beginning or just before the peak transmission season. Having this large pooled data, we attempted to study the effect of specific number of clones. However, that analysis was restricted by sample size especially when stratifying according to age and transmission categories. In addition, age-stratified analyses are highly affected by how the age groups are chosen. We therefore believe that the spline models with age treated as a continuous variable and with the best fit to data for the respective transmission category provide the most stable and reliable risk estimates.

Acquired immunity to malaria is believed to depend on cumulative exposure and a broad repertoire of antibodies against many highly polymorphic antigens. Hence, the antigenic diversity of chronic multiclonal infections might stimulate and strengthen broad protective antibody responses. Conversely, antibodies generated from previous infections might permit harboring multiclonal infections without developing symptoms. To be infected with multiple clones might have a direct effect; that is, detecting multiple clones means that one of them is likely to soon develop into a symptomatic infection, or multiple clones may simply be a marker of exposure, meaning that someone is more likely to get a new infection. In small children who have not yet acquired immunity that can control multiple antigenically diverse infections, multiclonal infections are likely to be found in the children with the highest exposure, irrespective of transmission setting.

The risk of clinical malaria associated with multiclonal infections then decreases with increasing age, at least to a certain degree, in all 4 settings, as shown in Figure 5, suggesting that multiclonal infections found in the older children are a sign of acquired immunity capable of controlling, but not clearing, their infections. Similar patterns were noted for all transmission levels, but the decreased risk of malaria observed in older children in high-transmission sites might reflect the need for a high level of exposure to acquire and maintain protection to multiclonal infections. Understanding the exact biological mechanism by which the presence of parasites in the blood contributes to the maintenance of immunity is vital to achieve a better understanding of human immunity to malaria. The present study, however, was not designed to investigate exact biological mechanisms, and further research is needed to understand whether multiclonal infection is only a marker of previous exposure and immunity, or whether these asymptomatic *P. falciparum* infections per se may contribute to maintenance of immunity in the most highly exposed.

In conclusion, this pooled analysis of individual data has provided comprehensive data on how the number of clones in asymptomatic *P. falciparum* infections predicts the subsequent risk of febrile malaria across different transmission intensities. The results have implications for our understanding of how multiclonal infections predict clinical malaria and why previous studies in this field have presented contradicting results. The findings should be considered in future design of malaria control interventions, such

as vaccines and seasonal malaria chemoprevention, because these interventions may have varying effects in different age groups, depending on transmission intensity.

Supplementary Data

Supplementary materials are available at *The Journal of 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

Acknowledgments. We are grateful to all authors replying to our invitation and wish to acknowledge all study participants and researchers contributing to the original studies. We also thank Linnea Widman for her much appreciated input on the statistical analysis.

Author contributions. M. E. and A. F. had full access to all data and had the final responsibility for the decision to submit for publication.

Disclaimer. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Financial support. This work was supported by the Swedish Research Council (grants 2015-09277 and 2018-02688 to A. F.).

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.

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