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MECHANISMS OF RESISTANCE

Associations between Aminoquinoline Resistance Genotypes and Clinical Presentations of *Plasmodium falciparum* Infection in Uganda

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ABSTRACT Mutations that mediate resistance of *Plasmodium falciparum* to aminoquinoline antimalarials are selected by prior drug use and may alter parasite fitness, but associations with clinical presentations are uncertain. We evaluated genotypes in samples from a case-control study of determinants of severe malaria in Ugandan children 4 months to 10 years of age. We studied 274 cases with severe malaria matched by age and geography to 275 uncomplicated malaria controls and 179 asymptomatic parasitemic controls. The overall prevalence of mutations of interest (considering mixed results as mutant) was 67.0% for PfCRT K76T, 8.5% for PfMDR1 N86Y, 71.5% for PfMDR1 Y184F, and 14.7% for PfMDR1 D1246Y. Compared to asymptomatic controls, the odds of mutant PfCRT 76T were lower for uncomplicated (odds ratio, 0.42 [95% confidence interval, 0.24 to 0.72]; $P < 0.001$) or severe (0.56 [0.32 to 0.97]; $P = 0.031$) malaria; the odds of mutant PfMDR1 86Y were lower for uncomplicated (0.33 [0.16 to 0.65]; $P < 0.001$) or severe (0.21 [0.09 to 0.45]; $P < 0.001$) malaria; and the odds of mutant PfMDR1 1246Y were higher for uncomplicated (1.83 [0.90 to 3.98]; $P = 0.076$) or severe (2.06 [1.01 to 4.55]; $P = 0.033$) malaria. The odds of mutant PfMDR1 184F were lower in severe than asymptomatic (0.59 [0.37 to 0.92]; $P = 0.016$) or uncomplicated (0.61 [0.41 to 0.90]; $P = 0.009$) malaria. Overall, the PfCRT 76T and PfMDR1 86Y mutations were associated with decreased risk of symptomatic malaria, PfMDR1 1246Y was associated with increased risk of symptomatic malaria, and PfMDR1 184F was associated with decreased risk of severe malaria. These results offer insights into parasite genotypes in children with different presentations, although the basis for the identified associations is likely complex.

KEYWORDS malaria, *Plasmodium falciparum*, drug resistance, aminoquinoline, genotype, drug resistance mechanisms

Malaria, especially falciparum malaria, remains an enormous problem in Africa (1). The treatment of falciparum malaria is challenged by resistance to most available drugs (2). Of particular concern is delayed parasite clearance after treatment with artemisinins and resistance to some artemisinin-based combination therapy (ACT) partner drugs, both of which are now widespread in parts of Southeast Asia, leading to frequent treatment failures (3–6). However, the efficacy of ACTs, including the most widely used regimen, artemether-lumefantrine, appears to remain strong in Africa (7).

Genetic polymorphisms that mediate resistance to several antimalarials and that

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impact the response to key ACT partner drugs have been common in Africa, but prevalences have been changing (7). Mutations in two *Plasmodium falciparum* putative drug transporters, PfCRT and PfMDR1, mediate resistance to aminoquinoline antimalarials (8–10). In Africa, the PfCRT 76T mutation, which is the primary mediator of resistance to chloroquine, has been common (7), is selected by therapy with aminoquinolines (11–14), and is selected against by regimens including lumefantrine (12, 14–17). Mutations in PfMDR1, notably the 86Y mutation, are also associated with decreased sensitivity to aminoquinolines (8, 10), selected by therapy with aminoquinolines (12, 14, 18–20), and selected against by regimens including lumefantrine (12, 15–17, 19–21). Coincident with increased use of artemether-lumefantrine and decreased use of chloroquine to treat malaria, the prevalence of the PfCRT 76T, PfMDR1 86Y, and PfMDR1 1246Y mutations has decreased in many countries, including Uganda (7, 22–24). One additional PfMDR1 mutation, 184F, is also common in Africa. This mutation does not have a clear role in mediating drug sensitivity, and it may serve to improve the fitness of parasites with the resistance-mediating PfMDR1 86Y mutation; temporal trends in its prevalence in Uganda have not been straightforward (22–24). Overall, *P. falciparum* has changed remarkably in Africa in recent years, apparently due to countering selective pressures of different antimalarials and to fitness disadvantages of some mutations.

Resistance-mediating *P. falciparum* mutations may come with some fitness cost. Studies utilizing cultured parasites or animal models have demonstrated fitness advantages for certain PfCRT and PfMDR1 genotypes, although results have been complex (25). Direct demonstration of a fitness advantage for wild-type parasites has come from field studies of the consequences of changes in malaria treatment practices. Discontinuation of chloroquine as the standard antimalarial in Malawi in the 1990s was accompanied by reversion to wild-type sequence at the K76T locus (26) and a return to excellent treatment efficacy for chloroquine (27). Later, across much of Africa, with replacement of chloroquine as the standard antimalarial by artemether-lumefantrine, reversion to wild-type sequences at PfCRT K76T and PfMDR1 N86Y and D1246Y has been seen (7). Loss of mutant parasites after decreased chloroquine use offers evidence of negative fitness consequences of transporter mutations, but in many cases, results were confounded by coincident increased use of lumefantrine, which selects for wild-type parasites.

It is of interest to determine the impacts of drug resistance-mediating *P. falciparum* polymorphisms on the clinical consequences of malaria. Available studies searching for associations between *P. falciparum* transporter mutations and severe malaria have been inconsistent, with studies from India (28) and Mali (29), but not Sudan (30) or Gabon (31), showing association between the PfCRT 76T mutation and risk of severe malaria. Considering risk of asymptomatic versus symptomatic infection, in Uganda the prevalence of mutant PfCRT 76T, PfMDR1 86Y, and PfMDR1 1246Y genotypes was higher in children with asymptomatic parasitemia than in those with parasitemia and fever (32). In another Ugandan study considering paired samples from the same subject, the odds of symptomatic malaria were lower for infections with mutant than with wild-type sequence at PfMDR1 N86Y but not PfCRT K76T (33). In these studies, it is difficult to assign causality for parasite polymorphisms in mediating malaria outcomes, as other determinants of outcomes, including parasite density, host genetic polymorphisms, and prior antimalarial drug use, may have varied among study subjects. To better characterize associations between resistance-mediating parasite polymorphisms and malaria outcomes, we evaluated key polymorphisms in samples from a case-control study that included subjects with severe malaria, subjects with uncomplicated malaria, and asymptomatic community controls.

RESULTS

Study populations. Ages of the three study groups were similar, all with median age of ~2 years (Table 1). Each group had nearly equal distributions of boys and girls. Mean parasite density was slightly higher in uncomplicated than severe malaria cases,

TABLE 1 Study populations

Variable	Asymptomatic controls ($n = 179$)	Uncomplicated malaria controls ($n = 275$)	Severe malaria cases ($n = 274$)
Age [yr, median (IQR)]	2.08 (1.22–3.15)	2.12 (1.30–3.14)	1.97 (1.14–2.98)
Gender [*n* (%) female]	91 (50.8)	132 (48.0)	135 (49.2)
Parasite density [geometric mean (95% CI)]	1,852 (1,080–3,174)	29,240 (24,730–34,560)	25,900 (20,770–32,290)
Gametocytemia, *n* (%)	21 (11.7)	43 (15.6)	81 (29.5)
Hyperparasitemia (>200,000/μl), *n* (%)	0	13 (4.7)	31 (11.3)

but hyperparasitemia (>200,000 parasites/μl of blood) was more common with severe malaria. Of the 275 asymptomatic controls in the parent study, 179 (65%) had parasitemia detected by blood smear; parasite density in this group was much lower than that in children with symptomatic malaria. Use of at least one medication with antimalarial activity prior to presentation was reported in 44.0% of uncomplicated and 48.9% of severe malaria subjects (Table 2). In both groups the most commonly used antimalarial was artemether-lumefantrine, and 77.2% of those who received antimalarials prior to presentation received an artemisinin-containing regimen. Data on prior drug use were not collected for asymptomatic children.

Prevalence of *P. falciparum* genetic polymorphisms associated with aminoquinoline resistance. We studied the prevalence of four polymorphisms, PfCRT K76T and PfMDR1 N86Y, Y184F, and D1246Y, in all available samples from children with uncomplicated or severe malaria and in samples from asymptomatic children with positive blood smears for *P. falciparum*. Mutations at each of these loci were previously very common in Uganda, but the prevalence of PfCRT 76T and PfMDR1 86Y and 1246Y has decreased, probably due to decreased use of chloroquine and widespread use of artemether-lumefantrine to treat malaria (22–24). Overall, the prevalence of mutations of interest (considering mixed results as mutant) was 67.0% for PfCRT K76T, 8.5% for PfMDR1 N86Y, 71.5% for PfMDR1 Y184F, and 14.7% for PfMDR1 D1246Y.

Comparative odds of genetic polymorphisms with different presentations of malaria. The prevalence of mutations of interest varied among children with different clinical presentations (see Table S1 in the supplemental material). Our primary analysis was unadjusted and considered mixed genotypes as mutant. Compared to asymptomatic controls, the odds of mutant PfCRT 76T were lower for uncomplicated (odds ratio, 0.42 [95% confidence interval, or CI, 0.24–0.72]; $P < 0.001$) or severe (0.56 [0.32 to 0.97]; $P = 0.031$) malaria, the odds of mutant PfMDR1 86Y were lower for uncomplicated (0.33 [0.16 to 0.65]; $P < 0.001$) or severe (0.21 [0.09 to 0.45]; $P < 0.001$) malaria, and the odds of mutant PfMDR1 1246Y were higher for uncomplicated (1.83 [0.90 to 3.98], $P = 0.076$) or severe (2.06 [1.01 to 4.55], $P = 0.033$) malaria (Table 3). Compared to uncomplicated malaria controls, the odds of mutant PfCRT 76T, PfMDR1 86Y, or PfMDR1 1246Y did not differ from those for severe malaria cases. The odds of mutant PfMDR1 184F were lower

TABLE 2 Antimalarial drug use prior to presentation[a]

Antimalarial	No. (%) for: Uncomplicated malaria controls ($n = 275$)	Severe malaria cases ($n = 274$)	*P* value
Took at least one type of antimalarial	121 (44.0)	134 (48.9)	0.274
Antimalarial used			
Chloroquine	1 (0.3)	1 (0.3)	1.0
Artemether-lumefantrine	87 (31.6)	86 (31.3)	0.39
Oral artemether	2 (0.7)	0	0.21
Intravenous artesunate	4 (1.4)	23 (8.3)	0.0018
Oral quinine	5 (1.8)	9 (3.2)	0.59
Intravenous quinine	2 (0.7)	16 (5.8)	0.0031
Sulfadoxine-pyrimethamine	3 (1.0)	5 (1.8)	0.73
Trimethoprim-sulfamethoxazole	27 (9.8)	14 (5.1)	0.019

[a]Some children received more than one antimalarial. *P* values are based on Fisher's exact test.

TABLE 3 Association of polymorphisms and different malaria outcomes[a]

Genotype	Prevalence [no. positive/total no. (%)]			OR (95% CI); *P* value		
	Asymptomatic controls	Uncomplicated malaria controls	Severe malaria cases	Uncomplicated malaria cases vs asymptomatic controls	Severe malaria cases vs uncomplicated malaria controls	Severe malaria cases vs asymptomatic controls
PfMDR1 N86Y						
Wild type	121/148 (81.7)	255/274 (93.0)	254/266 (95.5)	1	1	1
Mutant (univariate)	27/148 (18.2)	19/274 (6.9)	12/267 (4.5)	0.33 (0.16–0.65); <0.001	0.63 (0.27–1.40); 0.226	0.21 (0.09–0.45); <0.001
Mutant (adjusted)				0.65 (0.25–1.70); 0.389	0.62 (0.29–1.36); 0.239	0.23 (0.06–0.760; 0.017
PfMDR1 Y184F						
Wild type	42/173 (24.3)	68/274 (24.8)	95/271 (35.0)	1	1	1
Mutant (univariate)	132/173 (75.7)	206/274 (75.2)	176/271 (65.0)	0.97 (0.60–1.54); 0.897	0.61 (0.41–0.90); 0.009	0.59 (0.37–0.92); 0.016
Mutant (adjusted)				0.95 (0.48–1.86); 0.882	0.61 (0.41–0.91); 0.016	0.68 (0.36–1.28); 0.233
PfMDR1 D1246Y						
Wild type	122/134 (91.0)	227/268 (84.7)	212/256 (83.1)	1	1	1
Mutant (univariate)	12/134 (8.9)	41/268 (15.3)	43/255 (16.8)	1.83 (0.90–3.98); 0.076	1.12 (0.68–1.84); 0.626	2.06 (1.01–4.55); 0.033
Mutant (adjusted)				1.06 (0.45–2.53); 0.880	1.02 (0.62–1.67); 0.936	1.91 (0.86–4.23); 0.108
PfCRT K76T						
Wild type	24/114 (21.0)	101/261 (38.7)	85/266 (31.9)	1	1	1
Mutant (univariate)	90/114 (78.9)	160/261 (61.3)	181/266 (68.0)	0.42 (0.24–0.72); <0.001	1.34 (0.92–1.95); 0.105	0.56 (0.32–0.97); 0.031
Mutant (adjusted)				0.50 (0.26–0.95); 0.036	1.31 (0.90–1.92); 0.150	0.62 (0.33–1.18); 0.151

[a]OR, odds ratio. Mutants included mixed genotypes. Results are presented based on univariate analyses and after adjustments for each of the other polymorphisms studied, prior use of artemether-lumefantrine (prior use information was not available for asymptomatic controls), and parasite density.

in severe compared to asymptomatic (0.59 [0.37 to 0.92]; $P = 0.016$) or uncomplicated (0.61 [0.41 to 0.90]; $P = 0.009$) malaria. The odds of mutant PfMDR1 184F did not differ between uncomplicated malaria and asymptomatic controls.

For each genotype, we considered adjustment for other polymorphisms, prior use of artemether-lumefantrine, and parasite density (Table 3). After these adjustments, the identified associations were similar to unadjusted associations, but odds were generally less pronounced, with loss of some significant associations. These results are consistent with complex associations between genotypes, other factors, and clinical outcomes.

DISCUSSION

We assessed associations between *P. falciparum* genetic polymorphisms linked to aminoquinoline resistance and clinical presentations in Ugandan children enrolled in a case-control study of severe malaria. The two mutations most clearly associated with aminoquinoline resistance, PfCRT 76T and PfMDR1 86Y, were less common in parasites causing clinical illness than in isolates from children with asymptomatic infections; thus, these mutations were associated with decreased risk of symptomatic malaria. The PfMDR1 1246Y mutation had the opposite association; it was more common in isolates from symptomatic children and, thus, was associated with increased risk of symptomatic malaria. The PfMDR1 184F mutation, which has unclear impacts on drug sensitivity but may play a role in maintaining parasite fitness, was less common in severe compared to uncomplicated or asymptomatic infections and, thus, was associated with decreased risk specifically of severe malaria. Adjustment for other studied polymorphisms, prior use of artemether-lumefantrine, and parasite density decreased some associations, highlighting complex interactions between individual genetic polymorphisms and relevant variables. Our results identify significant associations between transporter mutations and clinical outcomes, but multiple factors account for clinical presentations of African children with malaria, and we cannot conclude that specific genotypes had causal relationships with clinical presentations. Nonetheless, our results offer insights into the likelihood of sensitivity to different antimalarials in parasites associated with different presentations of malaria.

Prior studies have shown varied associations between *P. falciparum* drug resistance-mediating polymorphisms and clinical presentations. Considering associations with asymptomatic versus symptomatic disease in Uganda, the prevalence of mutant PfCRT 76T, PfMDR1 86Y, and PfMDR1 1246Y genotypes was higher in children with asymp-

tomatic parasitemia than in those with parasitemia and fever, suggesting that mutant parasites were less likely than the wild type to be associated with symptomatic disease (32). However, in a subsequent study considering paired samples from different episodes in the same subject, to control for potential confounders, differences in prevalences of mutant genotypes between asymptomatic and symptomatic disease were less pronounced and significant only for the PfMDR1 86Y mutation (33). These results highlight the challenge of defining causal associations between specific genotypes and clinical presentations. Potential confounders include the following. First, children presenting with symptomatic disease presumably were more likely to have had recent prior episodes of malaria and, thus, were more likely to have recently received antimalarial therapy with artemether-lumefantrine, which selects for wild-type genotypes. Second, those with symptomatic disease had higher parasite densities and, thus, higher multiplicities of infection than those who were asymptomatic, increasing the likelihood of identifying multiple genotypes, including wild-type isolates, in symptomatic children. Our new study again showed associations between the PfCRT 76T and PfMDR1 86Y mutations and decreased risk of symptomatic infection. Interestingly, the PfMDR1 1246Y mutation had the opposite result; it was associated with increased risk of symptomatic infection, although associations for this polymorphism were modest and not significant after adjustments for other genotypes and prior drug use. This result was unexpected and is not easily explained. As in prior studies, our case-control design did not allow us to control for all potential confounders, but our results show clear differences in parasites seen in symptomatic compared to asymptomatic infections.

Considering genotypes of parasites causing uncomplicated versus severe malaria, potential increased virulence of wild-type parasites might be balanced by risks of progression to severe malaria in those with mutant parasites unresponsive to initial therapy, in particular in older studies in which the standard therapy was chloroquine (28–31). We gathered information on prior drug therapy at the time of study enrollment. Prior use of chloroquine or other aminoquinolines was rare. Prior use of artemether-lumefantrine was common and of similar prevalence in isolates from children presenting with uncomplicated or severe malaria. Unfortunately, we did not collect data on prior drug use from asymptomatic controls, but it is anticipated that recent drug use was uncommon in those who were clinically well. Thus, the selective pressure of recent use of artemether-lumefantrine may explain, at least in part, differences in genotypes between those with symptomatic and asymptomatic parasitemia. Only one studied polymorphism, PfMDR1 Y184F, showed an association with severe malaria: 184F mutant parasites were associated with decreased risk of severe compared to uncomplicated or asymptomatic infections. Interestingly, the PfMDR1 184F mutation, which is common across Africa, may play a role in maintaining the fitness of resistant parasites. In another new study characterizing relative *in vitro* fitness of parasites with different genotypes, the 184F mutation appeared to stabilize parasites with the 86Y mutation, but in parasites with the wild-type N86 sequence, the wild-type Y184 genotype conferred improved fitness (unpublished data). Although fitness and virulence are distinct properties, these *in vitro* results are consistent with the conclusion that parasites with the wild-type PfMDR1 Y184 genotype are more likely than mutant parasites to be associated with severe malaria.

Multiple factors influence the clinical outcomes of *P. falciparum* infections, including inherent parasite fitness, host immunity, multiplicity of infection, drug pressure over the course of infection, and host genetics, and it is difficult to determine whether impacts of genotypes on parasite fitness or virulence directly influenced clinical outcomes in our study children. Nonetheless, our results offer insight into the genotypes of parasites in children with different clinical presentations. Notably, children presenting with uncomplicated or severe malaria were more likely to harbor PfCRT K76 and PfMDR1 N86 wild-type parasites that are typically highly sensitive to aminoquinolines but also less sensitive than mutant parasites to artemether-lumefantrine, the first-line therapy for uncomplicated malaria in Uganda and most African countries. Our current understanding of drug resistance in Africa suggests that, even in the setting of PfCRT/PfMDR1

wild-type parasites, artemether-lumefantrine is highly efficacious for the treatment of uncomplicated malaria, but, as has been the case with other artemisinin-based combinations, some loss of activity of lumefantrine might foretell decreasing efficacy of artemether-lumefantrine to treat symptomatic malaria in Africa.

Our study had important limitations. First, any case-control study is necessarily limited by imperfect matching, although in our case efforts were made to match all three groups by age and place of residence, limiting impacts of varied immunity on results. Second, consideration of multiple comparisons limited available sample size and, thus, statistical power for some comparisons. Third, our decision to classify mixed isolates as mutant in all comparisons may have introduced bias for some analyses, but we chose this approach due to its clarity and simplicity. Fourth, information on prior drug use was not obtained for asymptomatic individuals. We assume that asymptomatic children were much less likely than those with symptomatic infections to have used antimalarials shortly before presentation and study enrollment, and decreased selective pressure of AL may well explain the increased prevalence of PfMDR1 86Y and PfCRT 76T mutant genotypes in this group. Fifth, two important polymorphisms, PfMDR1 N86Y and PfMDR1 D1246Y, were quite uncommon, after a remarkable decrease in prevalence in recent years (23, 24), limiting sample size for some comparisons. Finally, we studied only a small set of parasite polymorphisms; full impacts of diverse parasite sequences on clinical outcomes of *P. falciparum* infection were beyond the scope of our study.

In summary, in a case-control study in Uganda, the PfCRT 76T and PfMDR1 86Y mutations were associated with decreased risk of symptomatic *P. falciparum* infections, and the PfMDR1 184F mutation was specifically associated with decreased risk of severe malaria. It is not clear if the studied mutations had causal relationships with clinical presentations, but our results offer insight into genotypes of parasites in children presenting with symptomatic and severe malaria in Africa, including the possibility that parasites from those with symptomatic disease are less responsive to standard therapy with artemether-lumefantrine than are parasites from those with asymptomatic infection.

MATERIALS AND METHODS

Study design. A matched case-control study was conducted in Jinja District, Uganda, in 2015 to 2016 to identify determinants of severe malaria in Ugandan children (34, 35). Cases, recruited from the Children's Ward of Jinja Regional Referral Hospital, were children aged 4 months to 10 years with severe malaria, defined as a positive malaria blood smear (>2,500 parasites/μl) and severe anemia (hemoglobin, <5g/dl), impaired consciousness (Blantyre coma score, <4), or respiratory distress (intercostal or subcostal recession without crackles or other evidence of pneumonia upon auscultation). Two types of controls were selected for each case, both recruited from the Busoga Subregion, the catchment area of the Jinja Regional Referral Hospital. First, uncomplicated malaria controls, recruited from level III or IV health centers, were defined as children with fever and a blood smear positive for *P. falciparum* parasites (>2,500 parasites/μl) but without clinical evidence of severe malaria, danger signs (36), or other known causes of febrile illness. Second, asymptomatic controls were defined as children, recruited from the community, without illness or a history of fever in the 2 weeks prior to enrollment. Both control groups were matched to village and date (within 1 month) of a case. Target age groupings for matching were 6 to <12 months, 1 to <3 years, 3 to <5 years, and ≥5 years. Caregivers of children with severe or uncomplicated malaria were asked to detail all medicines received during the acute illness, with specific drugs verified using physical descriptions, observations of medicines, and/or prescriptions.

Ethics. Institutional approval was obtained from the Uganda National Council of Science and Technology and the Institutional Review Boards of the College of Health Sciences, Makerere University, and the University of California, San Francisco. Written informed consent was obtained from the parent or guardian of each study subject.

Malaria blood smears. Thick blood smears were stained with 10% Giemsa for 10 min and initially read by health facility staff as part of routine care. The eligibility of severe malaria cases and uncomplicated malaria controls was confirmed upon ascertainment of a positive malaria smear and adequacy of parasite density by a study laboratory technician. Thick blood smears reported as positive were sent to a central laboratory to be read by two expert microscopists for confirmation and determination of parasite densities. If reads were discordant (>25% in parasite density), a third read was performed.

Characterization of *P. falciparum* genetic polymorphisms. Parasite DNA was extracted from dried blood spots from samples positive by microscopy using Chelex (37). Sequences of *pfcrt* and *pfmdr1* alleles of interest were determined using a ligase detection reaction-fluorescent microsphere assay, as described previously (38), with minor modifications, including nested PCR amplification of templates, also as described previously (39).

Analysis. Data were entered using Microsoft Access and analyzed using STATA (version 14). Descriptive statistics were reported as proportions and medians with interquartile ranges. Baseline characteristics in each study group were compared using the chi-square test and the Wilcoxon signed rank test for categorical and continuous data, respectively. Associations between drug resistance-mediating *P. falciparum* polymorphisms and malaria outcomes were initially assessed using cross-tabulations with chi-square tests. To account for confounding and interaction between different polymorphisms, logistic regression was used to measure independent associations between each polymorphism (with adjustment for other polymorphisms, prior use of artemether-lumefantrine, and parasite density) and the clinical outcome groups.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.02 MB.

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REFERENCES

1. World Health Organization. 2019. World Malaria Report, 2019. World Health Organization, Geneva, Switzerland.
2. Blasco B, Leroy D, Fidock DA. 2017. Antimalarial drug resistance: linking *Plasmodium falciparum* parasite biology to the clinic. Nat Med 23:917–928. https://doi.org/10.1038/nm.4381.
3. Ashley EA, Dhorda M, Fairhurst RM, Amaratunga C, Lim P, Suon S, Sreng S, Anderson JM, Mao S, Sam B, Sopha C, Chuor CM, Nguon C, Sovannaroth S, Pukrittayakamee S, Jittamala P, Chotivanich K, Chutasmit K, Suchatsoonthorn C, Runcharoen R, Hien TT, Thuy-Nhien NT, Thanh NV, Phu NH, Htut Y, Han K-T, Aye KH, Mokuolu OA, Olaosebikan RR, Folaranmi OO, Mayxay M, Khanthavong M, Hongvanthong B, Newton PN, Onyamboko MA, Fanello CI, Tshefu AK, Mishra N, Valecha N, Phyo AP, Nosten F, Yi P, Tripura R, Borrmann S, Bashraheil M, Peshu J, Faiz MA, Ghose A, Hossain MA, Samad R, Rahman MR, Hasan MM, Islam A, Miotto O, Amato R, MacInnis B, Stalker J, Kwiatkowski DP, Bozdech Z, Jeeyapant A, Cheah PY, Sakulthaew T, Chalk J, Intharabut B, Silamut K, Lee SJ, Vihokhern B, Kunasol C, Imwong M, Tarning J, Taylor WJ, Yeung S, Woodrow CJ, Flegg JA, Das D, Smith J, Venkatesan M, Plowe CV, Stepniewska K, Guerin PJ, Dondorp AM, Day NP, White NJ, Tracking Resistance to Artemisinin Collaboration (TRAC). 2014. Spread of artemisinin resistance in *Plasmodium falciparum* malaria. N Engl J Med 371:411–423. https://doi.org/10.1056/NEJMoa1314981.
4. Amaratunga C, Lim P, Suon S, Sreng S, Mao S, Sopha C, Sam B, Dek D, Try V, Amato R, Blessborn D, Song L, Tullo GS, Fay MP, Anderson JM, Tarning J, Fairhurst RM. 2016. Dihydroartemisinin-piperaquine resistance in *Plasmodium falciparum* malaria in Cambodia: a multisite prospective cohort study. Lancet Infect Dis 16:357–365. https://doi.org/10.1016/S1473-3099(15)00487-9.
5. Phyo AP, Ashley EA, Anderson TJC, Bozdech Z, Carrara VI, Sriprawat K, Nair S, White MM, Dziekan J, Ling C, Proux S, Konghahong K, Jeeyapant A, Woodrow CJ, Imwong M, McGready R, Lwin KM, Day NPJ, White NJ, Nosten F. 2016. Declining efficacy of artemisinin combination therapy against P falciparum malaria on the Thai-Myanmar border (2003–2013): the role of parasite genetic factors. Clin Infect Dis 63:784–791. https://doi.org/10.1093/cid/ciw388.
6. van der Pluijm RW, Imwong M, Chau NH, Hoa NT, Thuy-Nhien NT, Thanh NV, Jittamala P, Hanboonkunupakarn B, Chutasmit K, Saelow C, Runjarern R, Kaewmok W, Tripura R, Peto TJ, Yok S, Suon S, Sreng S, Mao S, Oun S, Yen S, Amaratunga C, Lek D, Huy R, Dhorda M, Chotivanich K, Ashley EA, Mukaka M, Waithira N, Cheah PY, Maude RJ, Amato R, Pearson RD, Goncalves S, Jacob CG, Hamilton WL, Fairhurst RM, Tarning J, Winterberg M, Kwiatkowski DP, Pukrittayakamee S, Hien TT, Day NP, Miotto O, White NJ, Dondorp AM. 2019. Determinants of dihydroartemisinin-piperaquine treatment failure in Plasmodium falciparum malaria in Cambodia, Thailand, and Vietnam: a prospective clinical, pharmacological, and genetic study. Lancet Infect Dis 19:952–961. https://doi.org/10.1016/S1473-3099(19)30391-3.
7. Conrad MD, Rosenthal PJ. 2019. Antimalarial drug resistance in Africa: the calm before the storm? Lancet Infect Dis 19:e338–e351. https://doi.org/10.1016/S1473-3099(19)30261-0.
8. Valderramos SG, Fidock DA. 2006. Transporters involved in resistance to antimalarial drugs. Trends Pharmacol Sci 27:594–601. https://doi.org/10.1016/j.tips.2006.09.005.
9. Eyase FL, Akala HM, Ingasia L, Cheruiyot A, Omondi A, Okudo C, Juma D, Yeda R, Andagalu B, Wanja E, Kamau E, Schnabel D, Bulimo W, Waters NC, Walsh DS, Johnson JD. 2013. The role of Pfmdr1 and Pfcrt in changing chloroquine, amodiaquine, mefloquine and lumefantrine susceptibility in Western-Kenya *P falciparum* samples during 2008–2011. PLoS One 8:e64299. https://doi.org/10.1371/journal.pone.0064299.
10. Rasmussen SA, Ceja FG, Conrad MD, Tumwebaze PK, Byaruhanga O, Katairo T, Nsobya SL, Rosenthal PJ, Cooper RA. 2017. Changing antimalarial drug sensitivities in Uganda. Antimicrob Agents Chemother 61:e01516-17. https://doi.org/10.1128/AAC.01516-17.
11. Djimde A, Doumbo OK, Cortese JF, Kayentao K, Doumbo S, Diourte Y, Dicko A, Su XZ, Nomura T, Fidock DA, Wellems TE, Plowe CV, Coulibaly D. 2001. A molecular marker for chloroquine-resistant falciparum malaria. N Engl J Med 344:257–263. https://doi.org/10.1056/NEJM200101253440403.
12. Zongo I, Dorsey G, Rouamba N, Tinto H, Dokomajilar C, Guiguemde RT, Rosenthal PJ, Ouedraogo JB. 2007. Artemether-lumefantrine versus amodiaquine plus sulfadoxine-pyrimethamine for uncomplicated falciparum malaria in Burkina Faso: a randomised non-inferiority trial. Lancet 369:491–498. https://doi.org/10.1016/S0140-6736(07)60236-0.
13. Yeka A, Kigozi R, Conrad MD, Lugemwa M, Okui P, Katureebe C, Belay K, Kapella BK, Chang MA, Kamya MR, Staedke SG, Dorsey G, Rosenthal PJ. 2016. Artesunate/amodiaquine versus artemether/lumefantrine for the treatment of uncomplicated malaria in Uganda: a randomized trial. J Infect Dis 213:1134–1142. https://doi.org/10.1093/infdis/jiv551.
14. Sondo P, Derra K, Diallo Nakanabo S, Tarnagda Z, Kazienga A, Zampa O, Valéa I, Sorgho H, Owusu-Dabo E, Ouédraogo J-B, Guiguemdé TR, Tinto H. 2016. Artesunate-amodiaquine and artemether-lumefantrine therapies and selection of Pfcrt and Pfmdr1 alleles in Nanoro, Burkina Faso. PLoS One 11:e0151565. https://doi.org/10.1371/journal.pone.0151565.
15. Some AF, Sere YY, Dokomajilar C, Zongo I, Rouamba N, Greenhouse B, Ouedraogo JB, Rosenthal PJ. 2010. Selection of known *Plasmodium falciparum* resistance-mediating polymorphisms by artemether-lumefantrine and amodiaquine-sulfadoxine-pyrimethamine but not dihydroartemisinin-piperaquine in Burkina Faso. Antimicrob Agents Chemother 54:1949–1954. https://doi.org/10.1128/AAC.01413-09.

16. Baraka V, Tinto H, Valea I, Fitzhenry R, Delgado-Ratto C, Mbonye MK, Van Overmeir C, Rosanas-Urgell A, Van Geertruyden JP, D'Alessandro U, Erhart A. 2015. In vivo selection of *Plasmodium falciparum* Pfcrt and Pfmdr1 variants by artemether-lumefantrine and dihydroartemisinin-piperaquine in Burkina Faso. Antimicrob Agents Chemother 59:734–737. https://doi.org/10.1128/AAC.03647-14.
17. Otienoburu SD, Maïga-Ascofaré O, Schramm B, Jullien V, Jones JJ, Zolia YM, Houzé P, Ashley EA, Kiechel J-R, Guérin PJ, Le Bras J, Houzé S. 2016. Selection of *Plasmodium falciparum* pfcrt and pfmdr1 polymorphisms after treatment with artesunate-amodiaquine fixed dose combination or artemether-lumefantrine in Liberia. Malar J 15:452. https://doi.org/10.1186/s12936-016-1503-3.
18. Nsobya SL, Dokomajilar C, Joloba M, Dorsey G, Rosenthal PJ. 2007. Resistance-mediating *Plasmodium falciparum* pfcrt and pfmdr1 alleles after treatment with artesunate-amodiaquine in Uganda. Antimicrob Agents Chemother 51:3023–3025. https://doi.org/10.1128/AAC.00012-07.
19. Humphreys GS, Merinopoulos I, Ahmed J, Whitty CJ, Mutabingwa TK, Sutherland CJ, Hallett RL. 2007. Amodiaquine and artemether-lumefantrine select distinct alleles of the *Plasmodium falciparum* mdr1 gene in Tanzanian children treated for uncomplicated malaria. Antimicrob Agents Chemother 51:991–997. https://doi.org/10.1128/AAC.00875-06.
20. Dahlström S, Aubouy A, Maïga-Ascofaré O, Faucher J-F, Wakpo A, Ezinmègnon S, Massougbodji A, Houzé P, Kendjo E, Deloron P, Le Bras J, Houzé S. 2014. *Plasmodium falciparum* polymorphisms associated with ex vivo drug susceptibility and clinical effectiveness of artemisinin-based combination therapies in Benin. Antimicrob Agents Chemother 58:1–10. https://doi.org/10.1128/AAC.01790-12.
21. Sisowath C, Stromberg J, Martensson A, Msellem M, Obondo C, Bjorkman A, Gil JP. 2005. In vivo selection of *Plasmodium falciparum* pfmdr1 86N coding alleles by artemether-lumefantrine (Coartem). J Infect Dis 191:1014–1017. https://doi.org/10.1086/427997.
22. Mbogo GW, Nankoberanyi S, Tukwasibwe S, Baliraine FN, Nsobya SL, Conrad MD, Arinaitwe E, Kamya M, Tappero J, Staedke SG, Dorsey G, Greenhouse B, Rosenthal PJ. 2014. Temporal changes in prevalence of molecular markers mediating antimalarial drug resistance in a high malaria transmission setting in Uganda. Am J Trop Med Hyg 91:54–61. https://doi.org/10.4269/ajtmh.13-0647.
23. Tumwebaze P, Tukwasibwe S, Taylor A, Conrad M, Ruhamyankaka E, Asua V, Walakira A, Nankabirwa J, Yeka A, Staedke SG, Greenhouse B, Nsobya SL, Kamya MR, Dorsey G, Rosenthal PJ. 2017. Changing antimalarial drug resistance patterns identified by surveillance at three sites in Uganda. J Infect Dis 215:631–635. https://doi.org/10.1093/infdis/jiw614.
24. Asua V, Vinden J, Conrad MD, Legac J, Kigozi SP, Kamya MR, Dorsey G, Nsobya SL, Rosenthal PJ. 2018. Changing molecular markers of antimalarial drug sensitivity across Uganda. Antimicrob Agents Chemother 63:e01818-18. https://doi.org/10.1128/AAC.01818-18.
25. Rosenthal PJ. 2013. The interplay between drug resistance and fitness in malaria parasites. Mol Microbiol 89:1025–1038. https://doi.org/10.1111/mmi.12349.
26. Kublin JG, Cortese JF, Njunju EM, Mukadam RA, Wirima JJ, Kazembe PN, Djimde AA, Kouriba B, Taylor TE, Plowe CV. 2003. Reemergence of chloroquine-sensitive *Plasmodium falciparum* malaria after cessation of chloroquine use in Malawi. J Infect Dis 187:1870–1875. https://doi.org/10.1086/375419.
27. Laufer MK, Thesing PC, Eddington ND, Masonga R, Dzinjalamala FK, Takala SL, Taylor TE, Plowe CV. 2006. Return of chloroquine antimalarial efficacy in Malawi. N Engl J Med 355:1959–1966. https://doi.org/10.1056/NEJMoa062032.
28. Ranjit MR, Das A, Chhotray GP, Das BP, Das BN, Acharya AS. 2004. The PfCRT (K76T) point mutation favours clone multiplicity and disease severity in *Plasmodium falciparum* infection. Trop Med Int Health 9:857–861. https://doi.org/10.1111/j.1365-3156.2004.01286.x.
29. Wele M, Djimde AA, Guindo A, Beavogui AH, Traore IZ, Sadou A, Blaise D, Diallo DA, Wellems TE, Doumbo OK. 2011. High frequency of PfCRT 76T in two Malian villages and its prevalence in severe relative to non-severe malaria. Acta Trop 119:11–13. https://doi.org/10.1016/j.actatropica.2011.01.002.
30. Giha HA, Elbashir MI, A-Elbasit IE, A-Elgadir TM, ElGhazali GE, Mackinnon MJ, Babiker HA. 2006. Drug resistance-virulence relationship in *Plasmodium falciparum* causing severe malaria in an area of seasonal and unstable transmission. Acta Trop 97:181–187. https://doi.org/10.1016/j.actatropica.2005.10.004.
31. Mayengue PI, Kalmbach Y, Issifou S, Kremsner PG, Ntoumi F. 2007. No variation in the prevalence of point mutations in the Pfcrt and Pfmdr1 genes in isolates from Gabonese patients with uncomplicated or severe *Plasmodium falciparum* malaria. Parasitol Res 100:487–493. https://doi.org/10.1007/s00436-006-0287-8.
32. Tukwasibwe S, Mugenyi L, Mbogo GW, Nankoberanyi S, Maiteki-Sebuguzi C, Joloba ML, Nsobya SL, Staedke SG, Rosenthal PJ. 2014. Differential prevalence of transporter polymorphisms in symptomatic and asymptomatic falciparum malaria infections in Uganda. J Infect Dis 210:154–157. https://doi.org/10.1093/infdis/jiu044.
33. Tukwasibwe S, Tumwebaze P, Conrad M, Arinaitwe E, Kamya MR, Dorsey G, Nsobya SL, Greenhouse B, Rosenthal PJ. 2017. Drug resistance mediating *Plasmodium falciparum* polymorphisms and clinical presentations of parasitaemic children in Uganda. Malar J 16:125. https://doi.org/10.1186/s12936-017-1777-0.
34. Mpimbaza A, Ndeezi G, Katahoire A, Rosenthal PJ, Karamagi C. 2017. Demographic, socioeconomic, and geographic factors leading to severe malaria and delayed care seeking in Ugandan children: a case-control study. Am J Trop Med Hyg 97:1513–1523. https://doi.org/10.4269/ajtmh.17-0056.
35. Mpimbaza A, Walakira A, Ndeezi G, Katahoire A, Karamagi C, Nsobya SL, Tukwasibwe S, Asua V, Rosenthal PJ. 2018. Associations between erythrocyte polymorphisms and risks of uncomplicated and severe malaria in Ugandan children: a case control study. PLoS One 13:e0203229. https://doi.org/10.1371/journal.pone.0203229.
36. World Health Organization. 2014. Severe malaria. Trop Med Int Health 19:7–131. https://doi.org/10.1111/tmi.12313_2.
37. Plowe CV, Djimde A, Bouare M, Doumbo O, Wellems TE. 1995. Pyrimethamine and proguanil resistance-conferring mutations in *Plasmodium falciparum* dihydrofolate reductase: polymerase chain reaction methods for surveillance in Africa. Am J Trop Med Hyg 52:565–568. https://doi.org/10.4269/ajtmh.1995.52.565.
38. Leclair NP, Conrad MD, Baliraine FN, Nsanzabana C, Nsobya SL, Rosenthal PJ. 2013. Optimization of a ligase detection reaction fluorescent microsphere assay for the characterization of resistance-mediating polymorphisms in African samples of *Plasmodium falciparum*. J Clin Microbiol 51:2564–2570. https://doi.org/10.1128/JCM.00904-13.
39. Conrad MD, LeClair N, Arinaitwe E, Wanzira H, Kakuru A, Bigira V, Muhindo M, Kamya MR, Tappero JW, Greenhouse B, Dorsey G, Rosenthal PJ. 2014. Comparative impacts over 5 years of artemisinin-based combination therapies on *Plasmodium falciparum* polymorphisms that modulate drug sensitivity in Ugandan children. J Infect Dis 210:344–353. https://doi.org/10.1093/infdis/jiu141.