BRIEF REPORT

Prolonged Selection of *pfmdr1* Polymorphisms After Treatment of Falciparum Malaria With Artemether-Lumefantrine in Uganda

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We compared the prevalence of key *pfmdr1* alleles between pretreatment *Plasmodium falciparum* parasite isolates and parasites that emerged after treatment of uncomplicated malaria in a longitudinal cohort of Ugandan children. The *pfmdr1* 86N, 184F, and 1246D alleles were selected after treatment with artemether-lumefantrine, but not after artesunate-amodiaquine or amodiaquine-sulfadoxine-pyrimethamine. Remarkably, selection persisted in infections presenting up to about 60 days after treatment with artemether-lumefantrine. Thus, parasites selected for decreased drug sensitivity can appear long after predicted exposure to antimalarial drugs. Continued surveillance of the clinical efficacy and in vitro activity of new combination therapies is warranted.

Artemisinin-based combination therapies (ACTs) are recommended for treating uncomplicated falciparum malaria because of widespread resistance to older drugs. ACTs contain a fast-acting artemisinin and a longer-acting partner drug. Artemisinins rapidly reduce parasite biomass and control malaria symptoms, but treatment with artemisinin monotherapies is often followed by recrudescence. Longer-acting partner drugs increase ACT efficacy by eliminating parasites not cleared during the initial days of treatment. The partner drugs may also protect against selection for artemisinin resistance. However, infections that occur after artemisinins have been cleared, but while long-acting partner drugs continue to circulate, may select for resistance to the partner drugs.

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Artemether-lumefantrine (AL) is the most widely used ACT in Africa. Artemether has a plasma half-life of approximately 1 hour. The half-life of lumefantrine has been measured at approximately 3–4 days [1], but it was shorter (33 hours) in children with uncomplicated malaria [2]. These half-lives are considerably shorter than those of the other principal ACT partners amodiaquine, mefloquine, and piperaquine, which have half-lives of approximately 2–4 weeks [1]. AL selects for parasites with genetic polymorphisms that reduce sensitivity to artemether, lumefantrine, and other antimalarial drugs. At various study sites in Africa, new infections that emerged soon after AL therapy had increased prevalence of single-nucleotide polymorphisms (SNPs) in *pfcrt* and/or *pfmdr1*, 2 putative drug transporters [3–6]. Of note, AL exerted the opposite effect of chloroquine and amodiaquine on these loci. Specifically, chloroquine and amodiaquine selected for the *pfcrt* 76T and *pfmdr1* 86Y, 184Y, and 1246Y alleles, but AL selected for the *pfcrt* 76K and *pfmdr1* 86N, 184F, and 1246D alleles [3–6].

The polymorphisms in *pfcrt* and *pfmdr1* selected by prior therapy with antimalarials have been associated with alterations in the in vitro sensitivity of *Plasmodium falciparum* to various drugs. Considering key ACT components, the *pfcrt* 76K, *pfmdr1* 86N, and *pfmdr1* 1246D alleles (generally considered wild-type based on the reference 3D7 strain [6]) have been linked to increased sensitivity to amodiaquine, but decreased sensitivity to lumefantrine (and its analogue halofantrine), mefloquine, and artemisinins [7–10]. Three polymorphisms primarily seen outside Africa, pfmdr1 1034C, pfmdr1 1042D, and increased copy number of pfmdr1, have been associated with decreased sensitivity of P. falciparum to mefloquine, halofantrine, and artemisinins [4, 9]. Most relevant to this report, polymorphisms 86N and 1246D selected in Africa by prior therapy with AL were associated with decreased sensitivity to both components of this drug [7–10]. Correlations between individual polymorphisms in *pfcrt* or *pfmdr1* and treatment efficacy have not been seen, as AL remains highly efficacious for treating uncomplicated falciparum malaria in Africa [11].

The selective pressure of AL for parasites with diminished drug sensitivity would be predicted to be short-lived, considering the approximately 1 hour and 3–4 day half-lives of its components. To determine the duration of selection by AL and other drugs, we compared the prevalence of key alleles between pretreatment *P. falciparum* isolates and isolates that emerged over extended periods after treatment with combination regimens in a cohort of children in Kampala, Uganda.

METHODS

We analyzed samples collected between 2004 and 2008 from a previously described clinical trial involving 690 children aged 1–10 years at enrollment [11, 12]. After informed consent, children were assigned to artemether-lumefantrine (AL), artesunate-amodiaquine (AS+AQ), or amodiaquine-sulfadoxine-pyrimethamine (AQ+SP) upon their first episode of malaria. Each child received the assigned treatment regimen for subsequent malaria episodes. They were asked to return for examination on days 1, 2, 3, 7, 14, and 28 after malaria diagnosis and whenever they felt ill. Blood for microscopy was obtained by finger prick, and samples for molecular analyses were stored on filter paper before treatment and on all follow-up days, except day 1. Treatment outcomes were classified following standard criteria, with genotyping based on 6 loci to distinguish recrudescence and new infection after therapy, as previously reported [11, 12].

Molecular analyses were done on 198 randomly selected pretreatment samples and on all 498 parasites that emerged within 120 days after a prior antimalarial treatment. For analysis of polymorphisms of interest and *pfmdr1* copy number, DNA was extracted from filter paper with Chelex resin, alleles were identified by nested polymerase chain reaction (PCR) followed by restriction fragment length polymorphism analysis, and copy number was determined by quantitative PCR, all as previously reported [3, 13]. The presence of the *pfmdr1* 86N/184F/1246D combination was assessed by considering only samples that amplified at all 3 loci. Allele prevalence at various time points was compared by Fisher 2-tailed exact test, considering $P < .05$ as significant, using GraphPad software version 5.01.

RESULTS AND DISCUSSION

A total of 696 samples were analyzed, but 24 were excluded for failing to amplify after repeated PCR, leaving 672 *P. falciparum* isolates—195 obtained before the first treatment of study subjects, and 154, 143, and 180 obtained within 120 days after treatment with AL, AS+AQ, and AQ+SP, respectively. Notably, recrudescences were uncommon in the ACT arms of the study, and seen in only 4 of 521 (0.8%) treatments with AL between 2005 and 2008 [12]. Thus, for the AL treatment arm, nearly all of the isolates that caused recurrent disease were from new infections.

A total of 316 isolates (93 randomly selected from the pretreatment samples plus 52, 70, and 101 obtained within 40 days after prior treatment with AL, AS+AQ, and AQ+SP, respectively) were screened for mutations at *pfcrt* 76, *pfmdr1* 1034, and *pfmdr1* 1042. Consistent with recent reports from Uganda, all 305 evaluable isolates (11 did not amplify) had the *pfmdr1* 1034S and *pfmdr1* 1042N sequences. For 296 of the 316 samples studied at *pfcrt* 76 (20 did not amplify), 287 had the *pfcrt* 76T sequence, 5 76K, and 4 a mixed genotype. Samples with 76K included 1 collected before any treatment, 4 collected after prior treatment with AL, and 4 collected after prior treatment with AQ+SP. Because these loci showed little variability in parasites presenting soon after therapy, when they would be expected to be under the strongest drug pressure, they were not studied further. We also tested for amplification of the *pfmdr1* gene in the 52 samples collected within 40 days of prior AL therapy. Consistent with other reports from Africa [3–5], amplification of *pfmdr1* was not seen in any of 43 successfully tested samples.

We were particularly interested in the impact of therapy on *pfmdr1* N86Y, Y184F, and D1246Y, alleles that are polymorphic in Africa and affected by various antimalarial drugs. For samples collected before the first treatment in our trial, the prevalence of the *pfmdr1* 86N, 184F, and 1246D alleles was low, with these pure alleles occurring in 8%–15% of samples and mixed genotypes in an additional 13%–25% of samples (Figure 1). In infections that emerged after treatment with AS+AQ or AQ+SP, the prevalences of the *pfmdr1* 86N, 184F, and 1246D polymorphisms

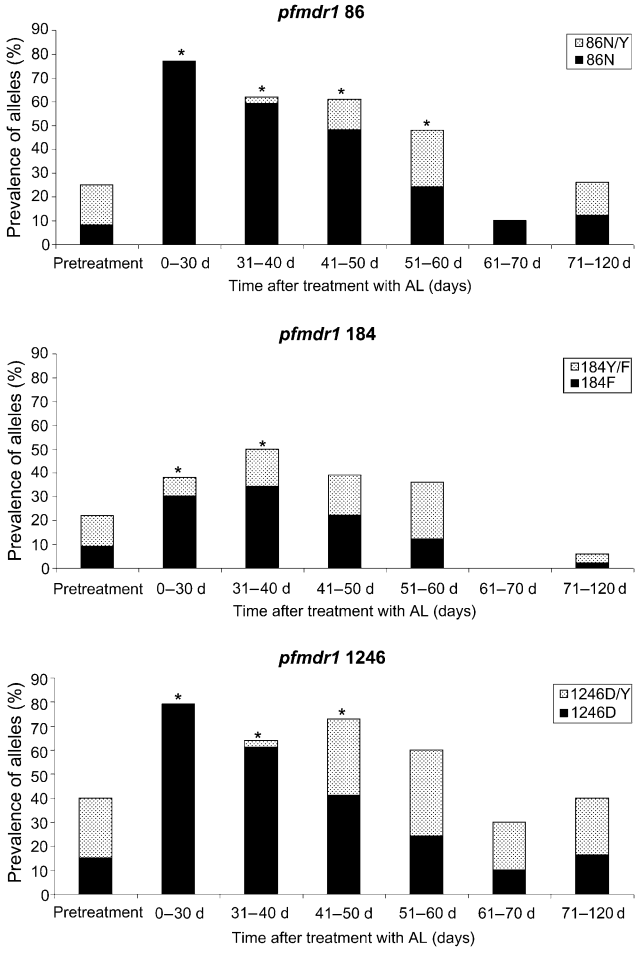

Figure 1. Prevalence of *pfmdr1* alleles in pretreatment samples and in samples collected after treatment with artemether-lumefantrine in a cohort of children in Kampala, Uganda. For clarity, prevalences of the pure 86Y, 184Y, and 1246Y alleles are not shown. *Significantly higher than pretreatment prevalence by Fisher exact test (2-tailed; $P < .05$).

Table 1. *Pfmdr1* Polymorphism Seen in *Plasmodium falciparum* Isolates From Kampala, Uganda, Following Treatment With Different Drugs

	pfmdr1 86N			*pfmdr1* 184F			*pfmdr1* 1246D			*pfmdr1* 86N/184F/1246D		
Time since treatment (d)	AL	AS+AQ	AQ+SP	AL	AS+AQ	AQ+SP	AL	AS+AQ	AQ+SP	AL	AS+AQ	AQ+SP
Pretreatment	16/194 (8%)			18/194 (9%)			30/194 (15%)			15/193 (8%)		
0–30	10/13 (77%)[a]	2/46 (4%)	4/73 (5%)	4/13 (30%)[a]	3/46 (7%)	8/73 (11%)	11/14 (79%)[a]	5/46 (11%)	6/68 (9%)	4/13 (31%)[a]	1/46 (2%)	3/68 (4%)
31–40	19/32 (59%)[a]	1/17 (6%)	1/28 (4%)	11/32 (34%)[a]	1/17 (6%)	2/28 (7%)	19/31 (61%)[a]	3/19 (16%)	7/26 (27%)	10/31 (32%)[a]	0/17 (0%)	3/26 (12%)
41–50	11/23 (48%)[a]	0/10 (0%)	0/17 (0%)	5/23 (22%)	0/10 (0%)	1/17 (6%)	9/22 (41%)[a]	1/11 (9%)	3/17 (18%)	6/23 (26%)[a]	0/10 (0%)	1/17 (6%)
51–60	6/25 (24%)[a]	0/13 (0%)	1/13 (8%)	3/25 (12%)	0/10 (0%)	1/13 (8%)	6/25 (24%)	2/13 (15%)	3/13 (23%)	6/25 (24%)[a]	0/13 (0%)	0/13 (0%)
61–70	1/10 (10%)	0/10 (0%)	0/10 (0%)	0/10 (0%)	1/10 (10%)	1/10 (10%)	1/10 (10%)	3/11 (27%)	2/10 (20%)	0/10 (0%)	1/10 (10%)	0/10 (0%)
71–120	6/49 (12%)	4/40 (10%)	5/37 (14%)	1/48 (2%)	4/39 (10%)	6/37 (16%)	8/50 (16%)	8/41 (20%)	9/38 (24%)	0/48 (0%)	2/39 (5%)	1/36 (3%)

For pretreatment samples, 3 of 198 did not amplify for the *pfmdr1* gene and 2 additional samples did not amplify for at least 1 allele, leading to the denominators shown. For 86N, *P* values were $< .0001$ up to 50 days and .0253 for the 51–60-days time point. For 184F, *P* values were .0362 for 0–30 days and .0005 for 31–40 days. For 1246D, *P* values were $< .0001$ up to 40 days and .007 for 41–50 days. For the combined alleles, *P* values were .0219 for 0–30 days, .0005 for 31–40 days, .0139 for 41–50 days, and .0204 for 51–60 days. For the individual polymorphisms, only data for the pure alleles are shown. For the combined polymorphisms, data include mixed sequences.

[a] Significantly higher than pretreatment prevalence by Fisher exact test (2-tailed) at $P < .05$.

remained similar to those seen in baseline samples, and there was no significant association between time after treatment and prevalence of a particular polymorphism (Table 1). In contrast, as seen previously in a number of African studies [3–5], the prevalences of the 86N, 184F, and 1246D polymorphisms were much higher soon after treatment with AL (Table 1; Figure 1).

The longitudinal design of our trial offered a unique opportunity to determine the duration of selection of alleles of interest after treatment with AL. Remarkably, strong selection of *pfmdr1* 86N, 184F, and 1246D persisted for about 2 months after treatment with AL (Table 1; Figure 1). Selection was significant for the 86N allele at all studied intervals up to 60 days, for 1246D up to 50 days, and for 184F up to 40 days when considering selection of either only pure or pure and mixed genotypes (except for 184F at 0–30 days, which was significant only for pure genotypes) (Table 1; Figure 1). Moreover, considering all isolates studied, samples containing the 3 polymorphisms (86N, 184F, and 1246D) were more prevalent in parasites from the AL treatment arm (26 of 150, 17%) than in the pretreatment samples (15 of 193, 8%; $P = .0075$) or in parasites from the AS+AQ (4 of 135, 3%; $P < .0001$) and AQ+SP (8 of 170, 5%; $P = .0004$) arms of the study. In vivo, these 3 polymorphisms have been associated with recrudescence after AL treatment in some studies [4, 5]. In vitro, polymorphisms 86N [8] and 1246D [9] have been associated with decreased sensitivity to lumefantrine and artemisinin, respectively. Thus, treatment with AL selected strongly for polymorphisms associated with decreased sensitivity to both components of the combination therapy, and the selective pressure of AL was evident far beyond the half-lives of both components.

Clinically, prolonged selection by AL for particular alleles in *pfmdr1* suggests the possibility that resistance to AL may develop as it is increasingly used in Africa. Evidence for this possibility is that the 86N allele has been associated with diminished in vitro sensitivity to artemether, dihydroartemisinin (the active metabolite of artemether) and lumefantrine [7, 8, 10]. Furthermore, the 1246D allele has been associated with diminished sensitivity to halofantrine (an analogue of lumefantrine) [9]. In our study, 17% of samples from children treated at least once with AL had the 86N/184F/1246D SNP combination, which has been associated with AL treatment failure in some studies [4, 5]. Moreover, assessment of children from our cohort with uncomplicated malaria indicated that the current weight-based dosing regimen for AL resulted in a shorter half-life (33 hours) and lower exposure of lumefantrine than in adults [2], potentially also contributing to treatment failures if parasite sensitivity to the drug diminishes over time.

The only other available information on the duration of selection for resistance by antimalarial drugs comes from studies of murine malaria. In mice infected with *Plasmodium chabaudi* and treated with pyrimethamine, the strength of selection for resistance was highest immediately following

Table 2. Annual Prevalence of *pfmdr1* Polymorphisms in *Plasmodium falciparum* Isolates From Kampala, Uganda, 2004–2008

Year	*pfmdr* 86N	*pfmdr* 86N/Y	*pfmdr* 86Y	*pfmdr* 184F	*pfmdr* 184Y/F	*pfmdr* 184Y	*pfmdr* 1246D	*pfmdr* 1246D/Y	*pfmdr* 1246Y
2004	3/32 (9%)	7/32 (22%)	22/32 (69%)	4/32 (13%)	4/32 (13%)	24/32 (75%)	5/31 (16%)	7/31 (23%)	19/31 (61%)
2005	34/280 (12%)	46/280 (16%)	200/280 (71%)	27/279 (10%)	30/279 (11%)	222/279 (80%)	55/280 (20%)	71/280 (25%)	154/280 (55%)
2006	25/210 (12%)	32/210 (15%)	153/210 (73%)	11/102 (11%)	20/210 (10%)	166/210 (79%)	42/210 (20%)	35/210 (17%)	133/210 (63%)
2007	20/103 (19%)	7/103 (7%)	76/103 (74%)	11/102 (11%)	8/102 (8%)	83/102 (81%)	25/103 (24%)	22/103 (21%)	56/103 (54%)
2008	7/35 (20%)	3/35 (9%)	25/35 (71%)	4/35 (12%)	5/35 (14%)	26/35 (74%)	9/35 (26%)	9/35 (26%)	17/35 (48%)

drug administration, but, consistent with our results, the selective pressure lasted far longer than would be expected based on the half-life of pyrimethamine [14].

Why does AL, a combination whose components are principally eliminated within days, continue to select for polymorphisms for a few months? First, lumefantrine or an active metabolite may have a longer terminal half-life than is generally appreciated, providing a wider window of selection for resistance than would be expected for a drug with a half-life of 3–4 days [1]. Second, due to genetic variations or other factors, some individuals may exhibit longer artemether or lumefantrine half-lives than others, allowing longer periods of selection. However, in a recent study of Ugandan children with uncomplicated malaria [2], none of the subjects had a lumefantrine half-life >49 hours (S. Parikh, personal communication). Third, the inherent features of malaria may also allow continued selection long after drug levels have decreased to negligible levels. Many new malaria illnesses in semi-immune children may follow extended periods of asymptomatic infection. In this event, the clinical impacts of selection might be seen long after the selection took place.

The strong and prolonged selective pressure of AL for polymorphisms that alter drug sensitivity is concerning. However, there are reasons for reassurance that, for now, AL remains an excellent therapy for uncomplicated malaria in Africa. First, AL has demonstrated outstanding efficacy for the treatment of falciparum malaria in many trials [11, 12]. Second, in vitro drug sensitivities of *P. falciparum* to artemisinins and lumefantrine have generally remained high [15]. Third, over the duration of our longitudinal trial in Kampala, the prevalence of key polymorphisms did not change noticeably (Table 2). However, AL use was not yet common in Kampala during the course of our study, but it has greatly increased across Africa in the last few years. Of concern are reports of uncommon recrudescences after therapy with AL [4, 5, 11, 12] and occasional African isolates with diminished in vitro response to artemisinins or lumefantrine [8]. Because of difficulties in assigning clinical outcomes and complexities of in vitro drug sensitivity assays, it is uncertain whether early signs of diminished efficacy of AL have been seen in Africa. In any event, as most countries in Africa now rely on AL as the mainstay of malaria therapy, careful surveillance of the clinical efficacy of this drug and of the in vitro activity of both artemisinins and lumefantrine is an urgent priority.

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