

UCSF

UC San Francisco Previously Published Works

Title

Temporal Changes in Prevalence of Molecular Markers Mediating Antimalarial Drug Resistance in a High Malaria Transmission Setting in Uganda

Permalink

<https://escholarship.org/uc/item/9db8q7n9>

Journal

American Journal of Tropical Medicine and Hygiene, 91(1)

ISSN

0002-9637

Authors

Mbogo, George W
Nankoberanyi, Sheila
Tukwasibwe, Stephen
[et al.](#)

Publication Date

2014-07-01

DOI

10.4269/ajtmh.13-0647

Peer reviewed

Temporal Changes in Prevalence of Molecular Markers Mediating Antimalarial Drug Resistance in a High Malaria Transmission Setting in Uganda

George W. Mbogo, Sheila Nankoberanyi, Stephen Tukwasibwe, Frederick N. Baliraine, Samuel L. Nsohya, Melissa D. Conrad, Emmanuel Arinaitwe, Moses Kanya, Jordan Tappero, Sarah G. Staedke, Grant Dorsey, Bryan Greenhouse, and Philip J. Rosenthal*

Infectious Diseases Research Collaboration, Kampala, Uganda; Le Tourneau University, Longview, Texas; Global AIDS Program, Centers for Disease Control and Prevention, Atlanta, Georgia; London School of Hygiene and Tropical Medicine, United Kingdom; University of California, San Francisco, California

Abstract. Standard therapy for malaria in Uganda changed from chloroquine to chloroquine + sulfadoxine-pyrimethamine in 2000, and artemether-lumefantrine in 2004, although implementation of each change was slow. *Plasmodium falciparum* genetic polymorphisms are associated with alterations in drug sensitivity. We followed the prevalence of drug resistance-mediating *P. falciparum* polymorphisms in 982 samples from Tororo, a region of high transmission intensity, collected from three successive treatment trials conducted during 2003–2012, excluding samples with known recent prior treatment. Considering transporter mutations, prevalence of the mutant *pfcr* 76T, *pfmdr1* 86Y, and *pfmdr1* 1246Y alleles decreased over time. Considering antifolate mutations, the prevalence of *pfdhfr* 51I, 59R, and 108N, and *pfdhps* 437G and 540E were consistently high; *pfdhfr* 164L and *pfdhps* 581G were uncommon, but most prevalent during 2008–2010. Our data suggest sequential selective pressures as different treatments were implemented, and they highlight the importance of genetic surveillance as treatment policies change over time.

INTRODUCTION

Despite the recent endorsement of malaria elimination as a worldwide goal, the malaria burden has not decreased notably in Uganda, where *Plasmodium falciparum* is responsible for most episodes of malaria, and millions of cases occur each year.¹ Uganda has undergone two recent changes in policy for the treatment of uncomplicated malaria, driven by the development of resistance to older antimalarials.² In 2000, chloroquine (CQ) was replaced by CQ plus sulfadoxine-pyrimethamine (SP), and in 2004 this regimen was replaced by the artemisinin-based combination therapy (ACT) artemether-lumefantrine (AL), although implementation of new treatment practices was slow. Alternate ACTs for uncomplicated malaria in Uganda are artesunate-amodiaquine (AS-AQ) and dihydroartemisinin-piperazine (DP). Each ACT consists of a potent and rapid-acting artemisinin and a longer-acting partner drug.³ Leading ACTs have shown outstanding antimalarial efficacy,^{4,5} but early signs of resistance to artemisinins, manifested as delayed parasite clearance after therapy, have been seen in parts of Southeast Asia,⁶ and resistance has been seen to most ACT partner drugs.⁷

Resistance to a number of antimalarial drugs has been linked to genetic polymorphisms in *P. falciparum*. Single nucleotide polymorphisms (SNPs) in *pfcr* and *pfmdr1*, which encode putative transport proteins, affect responses to multiple drugs. A single mutation, *pfcr* 76T, is the key mediator of resistance to CQ and AQ.⁸ In addition, polymorphisms in *pfmdr1*, which is homologous to proteins that mediate drug sensitivity in mammalian cells,⁹ modulate sensitivity of parasites to multiple drugs.^{10,11} Considering *pfmdr1* mutations that are common in Africa, 86Y and 1246Y are associated with decreased sensitivity to CQ and AQ, but wild-type (WT) sequences at the same alleles mediate decreased sensitivity to lumefantrine, mefloquine, and artemisinins.^{12–14} Another *pfmdr1* polymorphism,

Y184F, is common, but of uncertain significance, and two others, S1034C and N1042D, are seen in Asia, but rare in Africa.⁹

In Uganda and many other African countries the prevalence of the key mediator of CQ resistance, *pfcr* 76T, increased to saturation levels by the 1990s.^{15–17} With cessation of CQ use, reduction in the prevalence of *pfcr* 76T has been seen. Notably, in Malawi, over 8 years after CQ withdrawal, the prevalence of parasites with *pfcr* 76T decreased from 85% to 13%,¹⁸ and in 2005 excellent antimalarial efficacy of CQ was shown.¹⁹ Decreases in the prevalence of *pfcr* 76T have also been seen in eastern Kenya and Zanzibar, although changes have not been as dramatic as in Malawi.^{20,21} Changes have also been seen in the prevalence of key *pfmdr1* genotypes in Kenya, Tanzania, and Zanzibar, with WT N86 and D1246 genotypes increasing over time.^{11,21–23}

The SNPs in folate enzyme genes are associated with decreased sensitivity to antifolate antimalarials such as SP.²⁴ Antifolate resistance develops in a stepwise manner. Parasites containing five mutations, *pfdhfr* 51I, 59R, and 108N plus *pfdhps* 437G and 540E, which together mediate clinically relevant resistance, have recently been seen to be common in East Africa and near fixation in Uganda,^{16,25} where continued selection is presumably caused by continued use of antifolates for intermittent preventive therapy and other uses. However, mutations that mediate a higher level of resistance and are seen elsewhere, notably *pfdhfr* I164L and *pfdhps* A581G, are generally uncommon in Africa,²⁶ although some reports have noted increased prevalence in parts of east Africa.^{27,28} Notably, *pfdhfr* 164L was reported in 14% of *P. falciparum* isolates collected from symptomatic patients in southwestern Uganda in 2005.²⁹

Considering the impacts of parasite polymorphisms in both transporter and folate genes on drug sensitivity and changing malaria treatment practices over time, it was of interest to examine the prevalence of key SNPs in Uganda over time. We therefore examined sequences of interest in *P. falciparum* isolates from patients enrolled in drug efficacy trials from 2003 to 2012 in a high malaria transmission area of Uganda.

* Address correspondence to Philip J. Rosenthal, Box 0811, University of California, San Francisco, CA 94143. E-mail: prosenthal@medsfgh.ucsf.edu

MATERIALS AND METHODS

Clinical trials. We analyzed 982 archived *P. falciparum* isolates obtained from treatment trials in Tororo District. Details of the clinical studies have been published (Table 1).^{30–33} For the first study, which enrolled patients ≥ 6 months of age with uncomplicated *P. falciparum* malaria from December 2002 to May 2004 and compared the efficacies of CQ+SP, AQ+SP, and AS+AQ,³¹ we analyzed 198 pretreatment DNA samples collected from September to November 2003 for *pfmdr1* and *pfdhps* 581 polymorphisms, and included data on other polymorphisms that were already published.¹⁶ For the second study, which enrolled patients 1–10 years of age that presented with uncomplicated *P. falciparum* malaria from December 2004 to July 2005 and compared the efficacies of AS+AQ, and AL,³² we analyzed 201 pretreatment DNA samples for *pfdhfr* and *pfdhps* polymorphisms, and included data on *pfcr1* and *pfmdr1* SNPs that were already published.³⁴ For the third study, a longitudinal trial in which 351 children 4–12 months of age were enrolled and randomized to receive either AL or DP for each episode of uncomplicated malaria,³³ we analyzed 584 samples, including 204 from all first episodes of falciparum malaria and 380 from all recurrent episodes presenting 84 or more days after a prior treatment. For this trial, in a subset of samples that were genotyped, $< 1\%$ of recurrent infections within 28 days were caused by recrudescence; we anticipate that no infections occurring ≥ 84 days after a prior infection were the result of recrudescence.^{33,35}

Target gene amplification and mutation analysis. Parasite DNA was extracted from dried filter paper blood spots using Chelex, as previously described.³⁶ The DNA from control strains 3D7, Dd2, 7G8, FCR3, V1/S, K1, and Peru was obtained from the Malaria Research and Reference Reagent Resource Center. Genes of interest were amplified, and polymorphisms in *pfcr1*, *pfmdr1*, *pfdhfr*, and *pfdhps* were analyzed by nested polymerase chain reaction (PCR) followed by restriction fragment length polymorphism (RFLP) analysis, as previously described.^{37–39} Target sequences were amplified (see Supplemental Table 1 for primers), and PCR products were treated with polymorphism-specific restriction endonucleases (*ApoI* for *pfcr1* K76T; *AflIII*, *DraI*, and *BglII* for *pfmdr1* N86Y, Y184F, and D1246Y, respectively; *MluCI*, *XmnI*, *BsrI* and *DraI* for *pfdhfr* N51I, C59R, S108N and I164L, respectively; and *AvaII*, *FokI* and *BstUI* for *pfdhps* A437G, K540E and A581G, respectively). Reaction products were resolved on 2.5% agarose gels, and electrophoretic band patterns were categorized as WT, mixed, or mutant genotypes by visual inspection of gels and comparison with DNA from control strains.

Data analysis. Data were entered into a Microsoft (Microsoft Corp., Redmond, WA) access database and exported into Statistical Package for the Social Sciences (SPSS Inc., Chicago, IL) software 17.0, which was used for analysis. For these analyses, mixed genotypes were grouped with either mutant or WT, as indicated. Graphs were generated with GraphPad Prism 5.0 (GraphPad Prism, La Jolla, CA). Temporal changes in SNP frequencies were analyzed using logistic regression with date of collection as a continuous or categorical variable, as indicated, to evaluate for trends over time or differences between discrete time periods, respectively. A *P* value < 0.05 was considered statistically significant.

RESULTS

We evaluated the prevalence of polymorphisms of interest in *pfcr1*, *pfmdr1*, *pfdhfr*, and *pfdhps* from *P. falciparum* isolates collected in three clinical trials, all conducted in Tororo District over the course of a decade during which standard antimalarial treatment in Uganda underwent major changes. To minimize the influence of prior therapies on parasite genetics, samples were collected before treatment in the two older trials, and in the newer longitudinal trial, samples were from first episodes of malaria and from episodes ≥ 84 days since a prior episode. The SNPs of interest were categorized as WT, mixed, or mutant, based on sequences of the reference 3D7 strain (Table 2).

For *pfcr1* K76T, the prevalence of mutant parasites was nearly 100% throughout the early years of observation, consistent with other reports from Uganda,^{15,16,40,41} and likely due in part to widespread use of chloroquine even after the national treatment policy changed in 2004. The prevalence of WT and mixed K76T genotypes increased modestly from 2008 to 2011, and then a marked change was seen in 2012, with 16% mixed and 17% pure WT genotypes seen (Figure 1). Considering the period from 2007 to 2012, the probability of being infected with a WT or mixed (versus mutant) genotype increased significantly over time ($P < 0.001$).

For *pfmdr1*, the N86Y, Y184F, and D1246Y alleles were highly polymorphic throughout the period of observation. Although results varied from year to year, clear patterns were seen at the N86Y and D1246Y alleles, with increasing prevalence of WT genotypes over time (Figure 1). At Y184F, increasing prevalence of the mutant genotype was seen. Considering the entire period of observation, statistically significant increases in the probability of infection with a WT or mixed genotype for the N86Y ($P < 0.001$) and D1246Y ($P < 0.001$) alleles, and with a mutant (versus mixed or WT) genotype for the Y184F allele ($P < 0.001$) were seen. Prevalence of

TABLE 1
Description of trials that provided samples for the molecular study*

Baseline study characteristics	Antimalarial regimens studied		
	CQ+SP vs. AQ+SP vs. AS+AQ†	AL vs. AS+AQ†	AL vs. DP‡
Study period	2002–04	2004–05	2007–12
Age at enrollment	≥ 6 months	1–10 years	4–12 months
Number of participants	347	403	351
Median age (years)	1.27	1.83	0.98
Geometric mean parasite density	18,484/ μ L	22,071/ μ L	16,349/ μ L
Samples successfully genotyped	189/198 (95%)	188/201 (93%)	561/584 (96%)

* CQ = chloroquine; SP = sulfadoxine-pyrimethamine; AQ = amodiaquine; AL = artemether-lumefantrine; DP = dihydroartemisinin-piperazine.

† Pretreatment samples obtained from clinical studies.^{31,32}

‡ Samples from all first episodes and recurrent episodes ≥ 84 days after prior treatment.³³

TABLE 2
Proportions of patients infected with *Plasmodium falciparum* parasites containing polymorphisms in *pfprt*, *pfmdr1*, *pfdhfr*, and *pfdhps**

Genotype	Year							
	2003–2004†	2005‡	2007	2008	2009	2010	2011	2012
	<i>pfprt</i> K76T							
WT	0	0	0	0	4 (4%)	0	0	13 (17%)
Mixed	1 (1%)	0	0	3 (2%)	2 (2%)	4 (4%)	9 (8%)	12 (16%)
Mutant	79 (99%)	201 (100%)	38 (100%)	144 (98%)	87 (94%)	104 (96%)	102 (92%)	52 (67%)
	<i>Pfmdr1</i> N86Y							
WT	20 (10%)	19 (9%)	12 (32%)	34 (23%)	23 (26%)	48 (44%)	52 (47%)	40 (51%)
Mixed	136 (71%)	76 (38%)	7 (19%)	88 (61%)	27 (30%)	41 (37%)	41 (37%)	32 (41%)
Mutant	36 (19%)	106 (53%)	18 (49%)	23 (16%)	39 (44%)	21 (19%)	18 (16%)	6 (8%)
	<i>Pfmdr1</i> Y184F							
WT	141 (75%)	169 (85%)	25 (68%)	103 (71%)	75 (83%)	41 (37%)	63 (59%)	50 (68%)
Mixed	43 (23%)	28 (14%)	7 (19%)	22 (15%)	6 (7%)	45 (41%)	7 (7%)	4 (6%)
Mutant	3 (2%)	3 (1%)	5 (13%)	20 (14%)	9 (10%)	25 (22%)	36 (34%)	19 (26%)
	<i>Pfmdr1</i> D1246Y							
WT	3 (2%)	32 (17%)	9 (23%)	35 (24%)	16 (17%)	22 (19%)	22 (20%)	42 (53%)
Mixed	89 (48%)	80 (40%)	12 (31%)	50 (34%)	22 (23%)	55 (50%)	53 (48%)	27 (34%)
Mutant	93 (50%)	86 (43%)	18 (46%)	61 (42%)	58 (60%)	34 (31%)	35 (32%)	10 (13%)
	<i>Pfdhfr</i> N511							
WT	0	1 (1%)	0	1 (1%)	1 (1%)	0	0	0
Mixed	5 (6%)	5 (2%)	0	0	0	1 (1%)	0	0
Mutant	75 (94%)	185 (97%)	34 (100%)	144 (99%)	92 (99%)	110 (99%)	109 (100%)	79 (100%)
	<i>Pfdhfr</i> C59R							
WT	24 (7%)	13 (7%)	3 (8%)	4 (3%)	6 (7%)	10 (9%)	5 (4%)	6 (8%)
Mixed	122 (37%)	60 (31%)	1 (3%)	9 (6%)	3 (3%)	12 (11%)	14 (13%)	5 (6%)
Mutant	187 (56%)	117 (62%)	33 (89%)	132 (91%)	84 (90%)	87 (80%)	92 (83%)	65 (86%)
	<i>Pfdhfr</i> S108N							
WT	0	1 (1%)	0	0	0	1 (1%)	0	0
Mixed	0	4 (2%)	20 (59%)	29 (23%)	10 (11%)	18 (16%)	10 (9%)	2 (2%)
Mutant	80 (100%)	186 (97%)	14 (41%)	99 (77%)	83 (89%)	92 (83%)	101 (91%)	77 (98%)
	<i>Pfdhfr</i> I164L							
WT	80 (100%)	152 (99%)	34 (100%)	137 (99%)	83 (91%)	98 (88%)	105 (100%)	77 (100%)
Mixed	0	2 (1%)	0	1 (1%)	7 (8%)	12 (11%)	0	0
Mutant	0	0	0	0	1 (1%)	1 (1%)	0	0
	<i>Pfdhps</i> A437G							
WT	5 (2%)	3 (2%)	0	1 (1%)	1 (1%)	1 (1%)	1 (1%)	0
Mixed	48 (14%)	24 (12%)	0	1 (1%)	1 (1%)	2 (2%)	1 (1%)	4 (5%)
Mutant	280 (84%)	170 (86%)	33 (100%)	142 (98%)	91 (98%)	108 (97%)	108 (98%)	75 (95%)
	<i>Pfdhps</i> K540E							
WT	8 (2%)	2 (1%)	0	0	0	1 (1%)	1 (1%)	0
Mixed	86 (26%)	25 (13%)	0	6 (4%)	4 (4%)	4 (4%)	14 (13%)	3 (4%)
Mutant	239 (72%)	165 (86%)	33 (100%)	138 (96%)	89 (96%)	106 (95%)	95 (86%)	76 (96%)
	<i>Pfdhps</i> A581G							
WT	193 (100%)	195 (98%)	37 (100%)	129 (88%)	83 (89%)	97 (87%)	95 (88%)	76 (98%)
Mixed	0	1 (1%)	0	17 (11%)	9 (10%)	4 (4%)	9 (8%)	1 (2%)
Mutant	0	2 (1%)	0	1 (1%)	1 (1%)	10 (9%)	4 (4%)	0

WT = wild-type.

*Data shown are numbers (proportions in parentheses) of samples with each genotype.

†Data from 2003–2004 (samples obtained from November 2003 to May, 2004) are combined.

‡Data obtained from December 2004 to July 2005 are included for 2005; 98% of samples from this trial were collected in 2005.

the 86Y/Y184/1246Y haplotype decreased markedly over time, with increases in haplotypes including WT sequences at positions 86 and 1246 (Table 3). Based on known selective effects of antimalarial drugs that have been commonly used in Uganda, these results suggest decreasing selective pressure of CQ and/or increasing selective pressure of AL over time in Uganda.

For mutations associated with resistance to antifolate anti-malarials, five well-described mutations in *pfdhfr* (N511, C59R, S108N) and *pfdhps* (A437G, K540E) were all very common throughout the period of observation (Table 2). Considering uncommon genotypes associated with high-level resistance, both *pfdhfr* 164L and *pfdhps* 581G were nearly absent early and late in our study, but more common from 2008 to 2010, with peak prevalence of mixed or mutant sequences at *pfdhfr* 164 at 9–12% in 2009 and 2010, and at *pfdhps* position 581 at 11–13% in 2008–2011 (Figure 2). The

prevalence of the *pfdhfr* 164 WT genotype was significantly lower in 2009–10 than in other years ($P < 0.001$) and the prevalence of the *pfdhps* 581 WT genotype was significantly lower in 2009–12 than in earlier years ($P = 0.01$).

DISCUSSION

We examined changes in the prevalence of *P. falciparum* polymorphisms associated with drug resistance over a decade during which standard antimalarial treatment in Uganda underwent major changes. All evaluated samples were from the same region of Uganda, and from patients without known recent antimalarial treatment before analysis, limiting selective pressure from prior therapy, and thus providing a reasonable assessment of the prevalence of polymorphisms in parasites circulating in Tororo each year. Important changes were seen in the prevalence of a number of SNPs of interest.

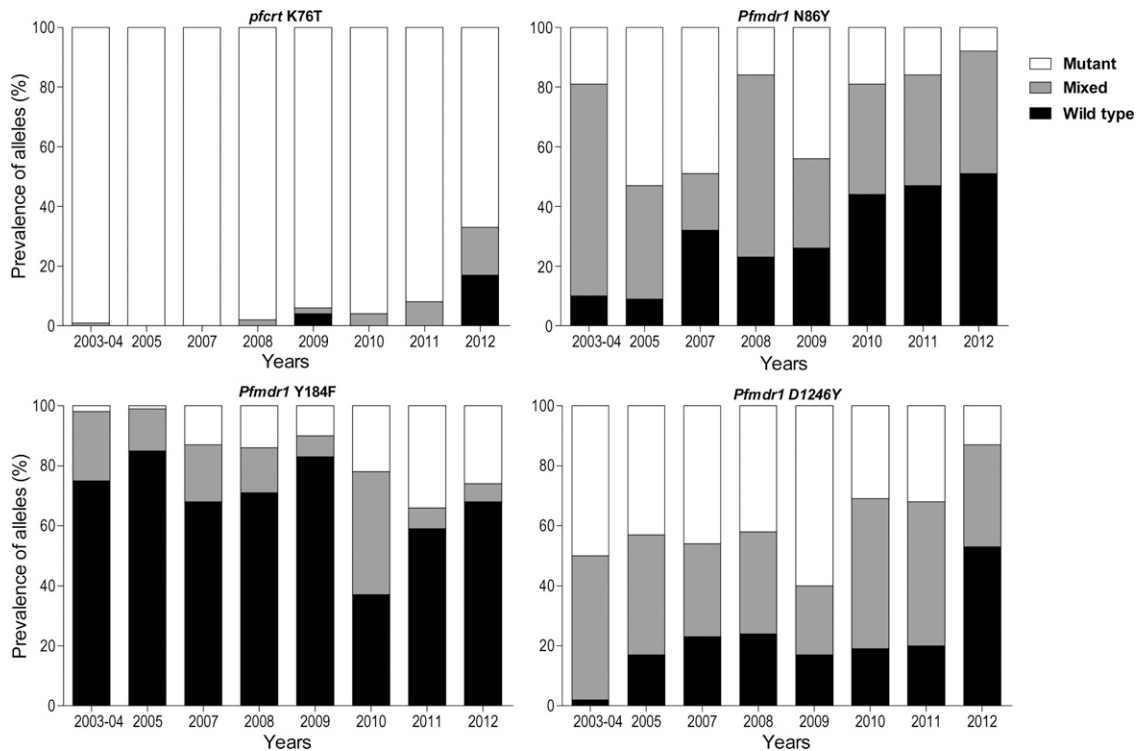


FIGURE 1. Prevalence of polymorphisms over time at codon 76 of *pfprt*, and codons 86, 184, and 1246 of *pfmdr1*.

For *pfprt* K76T, which is known to mediate CQ and AQ resistance, WT parasites were nearly absent in the early years of observation, but these became much more common by 2012. For *pfmdr1* N86Y and D1246Y, which impact upon sensitivity to multiple drugs, the prevalence of WT alleles increased steadily over time. For polymorphisms in *pfdhfr* and *pfdhps*, which mediate resistance to antifolates, five well-described mutations remained common over time, and mutations at two additional alleles associated with high level resistance showed modest prevalence only from 2008–11. Overall, the identified changes in prevalence seem to be consistent with differing selective drug pressures caused by changes in treatment practices in Uganda over time.

How can we explain the observed changes in the prevalence of *P. falciparum* drug resistance-mediating SNPs in Uganda over time? The mutant *pfprt* 76T genotype persisted in Uganda at high (> 90%) prevalence levels through 2011, long after it had decreased elsewhere in East Africa. For example, the prevalence of the 76T genotype was 63% in eastern Kenya in 2008,²² 63% in Zanzibar in 2010,²¹ and 32% in western

Kenya in 2011.¹¹ The persistence of the mutant *pfprt* 76T genotype in Uganda suggests that continued selective pressure from CQ persisted long after the national treatment policy changed in 2004, when the standard therapy for malaria was changed from CQ + SP to AL. The CQ remains available in Uganda, and in fact it continues to be recommended for regular chemoprophylaxis in children with sickle cell disease.⁴² Very recently, the prevalence of parasites with the CQ-sensitive *pfprt* K76 genotype increased markedly in Tororo, suggesting a decrease in community use of CQ.

For *pfmdr1*, the prevalence of mutant 86Y and 1246Y genotypes gradually decreased in Uganda from 2003 to 2012. These changes were likely due both to decreased use of CQ and gradual establishment of AL as the standard antimalarial drug in Tororo, because both decreasing CQ exposure and increased lumefantrine exposure will select for WT sequences at these alleles.^{3,43,44} In other parts of East Africa, the prevalence of *pfmdr1* N86 and D1246 have been observed to increase following widespread use of ACTs.^{11,23,45} Of note, *pfmdr1* WT genotypes have been associated with decreased sensitivity to lumefantrine.^{46,47} Taken together, available data suggest that, although the efficacies of leading ACTs appear to remain excellent, parasites in East Africa are undergoing changes rendering them less sensitive to lumefantrine. Continued selection may lead to highly resistant parasites, jeopardizing the antimalarial efficacy of AL, the first-line therapy for malaria in Uganda and surrounding countries.

Considering changes in the prevalence of SNPs that mediate antifolate resistance, five well-characterized mutations in *pfdhfr* (N51I, C59R, and S108N) and *pfdhps* (A437G, K540E) were very common in Tororo throughout the last 10 years. These data suggest continued strong selective pressure despite the withdrawal of SP from treatment guidelines in 2004. Of interest was whether selection of additional polymorphisms in

TABLE 3
Pfmdr1 haplotype distribution over time

Year	86Y/1246Y	N86/D1246	N86/184F/D1246	86Y/Y184/1246Y
2003	15/180 (8%)	0/180 (0%)	0/197 (0%)	70/197 (36%)
2005	70/198 (35%)	15/198 (8%)	2/197 (1%)	67/197 (34%)
2007	13/37 (35%)	7/37 (19%)	1/37 (3%)	12/37 (32%)
2008	13/144 (9%)	19/144 (13%)	8/150 (5%)	11/150 (7%)
2009	24/89 (30%)	9/89 (10%)	3/87 (4%)	23/87 (26%)
2010	9/110 (8%)	14/110 (13%)	0/110 (0%)	3/110 (3%)
2011	9/110 (8%)	10/110 (9%)	4/105 (4%)	5/105 (5%)
2012	2/78 (3%)	22/78 (28%)	7/73 (10%)	0/73 (0%)

Data from 2003–2004 and late 2004–2005 were combined as explained in Table 2. The results shown represent the proportion and percentage of each haplotype found among all those with successful analysis for each allele, considering only results with pure wild-type (WT) or mutant genotypes.

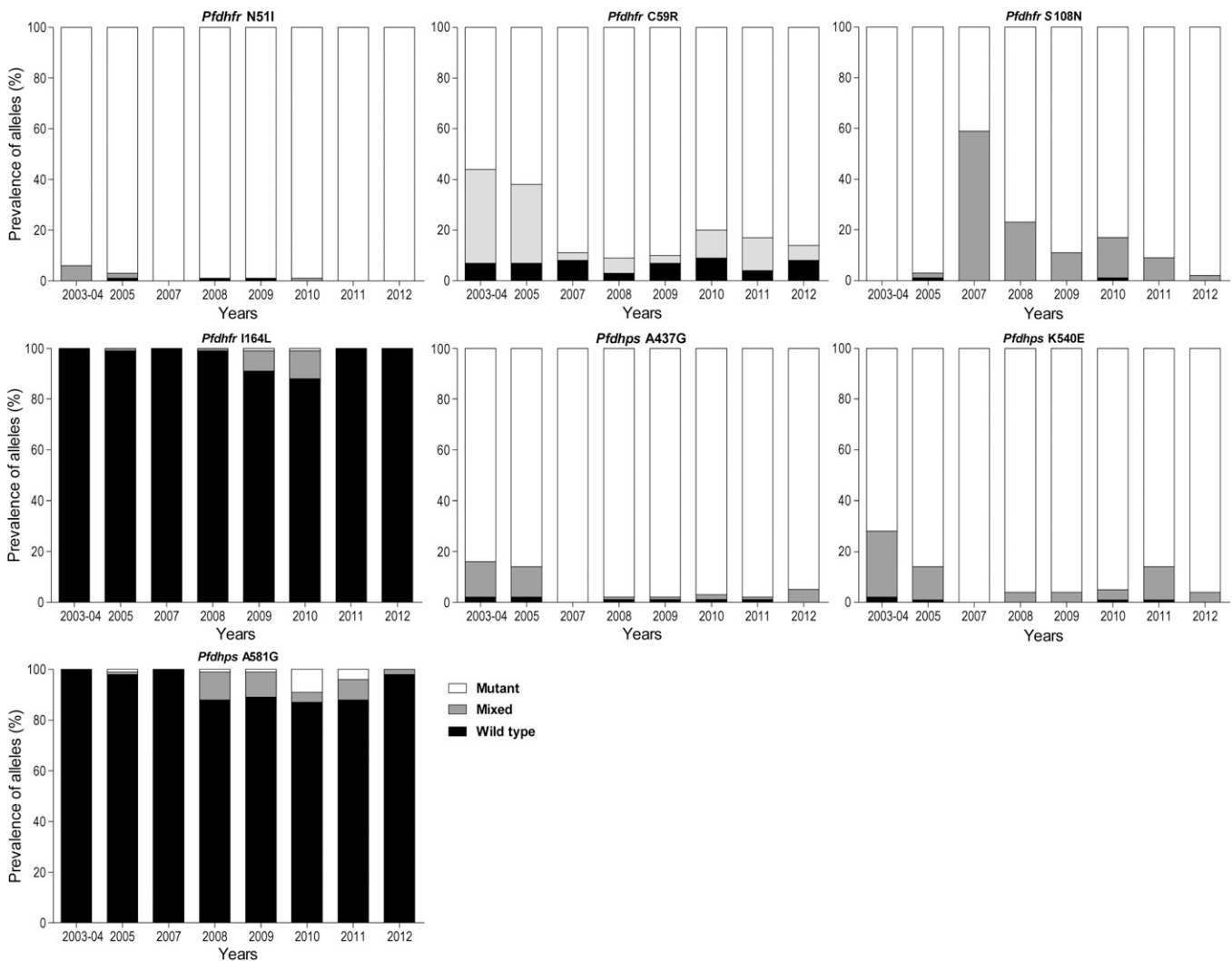


FIGURE 2. Prevalence of polymorphisms over time in the indicated alleles of *pfdhfr* and *pfdhps*.

pfdhfr or *pfdhps* was seen, in particular the *pfdhfr* 164L and *pfdhps* 581G mutations that are associated with high-level antifolate resistance.²⁴ In Kenya, an increase in prevalence of the five common mutations, and also emergence of *dhps* 581G, was reported over a 13-year period of observation,⁴⁸ despite withdrawal of SP for the treatment of malaria in 2006. Other reports have noted a fairly high prevalence of SNPs that mediate high-level antifolate resistance in some settings, including a *pfdhfr* 164L prevalence of 14% in *P. falciparum* from an area of Uganda with low malaria transmission intensity²⁹ and modest prevalence of both the *pfdhfr* 164L and *pfdhps* 581G mutations in human immunodeficiency virus (HIV)-infected Ugandan children receiving regular trimethoprim-sulfamethoxazole prophylaxis.^{49,50} In other recent studies from East Africa, the prevalence of *pfdhps* 581G was > 50% in samples from eastern Kenya and Tanzania.^{27,28} In our results from Tororo, the *pfdhfr* 164L and *pfdhps* 581G mutations were very uncommon, except for increased prevalence from 2008 to 2011. Reasons for continued antifolate selective pressure likely include continued use of SP to treat malaria, treatment of bacterial infections with antifolates, use of SP in intermittent preventive treatment in pregnant women, which remains the World Health Organization (WHO) policy, and widespread

use of another antifolate, trimethoprim-sulfamethoxazole, as prophylaxis against opportunistic infections in HIV-infected individuals. The decreased prevalence of both the *pfdhfr* 164L and *pfdhps* 581G mutations in Tororo in the most recent years suggests decreasing use of SP to treat malaria quite recently and fitness disadvantages of genotypes that mediate the highest levels of antifolate resistance; these fitness disadvantages may most effectively prevent selection in areas with very high malaria transmission intensity, such as Tororo.⁷

Our study had some limitations. First, samples were collected from studies with different designs. In the two older treatment efficacy trials subjects were enrolled and samples obtained immediately before treatment of malaria, but information on prior treatment before enrollment was necessarily limited. The more recent trial was a longitudinal trial in which children were treated with the same regimen, either AL or DP, for every episode of malaria over 5 years. In this trial, to limit impacts of prior treatments on infecting parasites, we studied only parasites causing first infections in the study and those without prior therapy within 84 days. Another limitation was that data were derived from a combination of older and recent assays, and for the oldest trial different numbers of samples were assessed for different alleles. Although we used

the same RFLP methods for all assessments, assays done at different points in time after sample collection may have led to somewhat different results, in particular in the case of minority strains within populations.

Despite some limitations, our data offer the best available description of changes in the prevalence of key resistance-mediating *P. falciparum* polymorphisms over time in Uganda. Indeed, parasites changed notably from 2003 to 2012. Our results suggest continued use of CQ and SP well beyond the establishment of AL as the national treatment regimen in 2004. However, our most recent results suggest decreasing selective pressure from both CQ and SP, with increasing prevalence of the *pfprt* K76 WT genotype and loss of folate gene polymorphisms associated with high level resistance. In addition, increasing prevalence of the WT *pfmdr1* N86 and D1246 alleles has been seen over time. These results are reassuring, in suggesting that recommended use of AL to treat uncomplicated malaria has increased. However, the results also raise the concern that continued heavy use of AL may select for parasites with decreased lumefantrine sensitivity, potentially leading to AL treatment failures. Continued surveillance of *P. falciparum* polymorphisms will be important, both to provide insight into the evolution of drug resistance and to offer feedback regarding national treatment practices. In future surveillance higher throughput assays,⁵¹ and deep sequencing methods to identify minority strains,⁵² may improve our ability to assess changes in allele prevalence over time.

Received November 5, 2013. Accepted for publication February 26, 2014.

Published online May 5, 2014.

Note: Supplemental table appears at www.ajtmh.org.

Acknowledgments: We thank the participants in all the clinical trials that provided samples for our analyses, the parents or guardians of study children, and clinical and laboratory personnel for each trial.

Financial support: This study was funded by an International Center of Excellence in Malaria Research grant (AI089674) and a Fogarty International Center training grant (TW007375), both from the National Institutes of Health. Some study samples were from a trial supported by the Doris Duke Charitable Foundation; the U.S. President's Emergency Plan for AIDS Relief; and Cooperative Agreement U62P024421 from the Centers for Disease Control and Prevention (CDC); the National Center for HIV, Viral Hepatitis, STD, and TB Prevention; and the Global AIDS Program. The funders were not involved with study design, data analysis, or manuscript preparation.

Disclaimer: The contents of the manuscript are solely the responsibility of the authors and do not necessarily represent the official views of the CDC.

Authors' addresses: George W. Mbogo, Sheila Nankoberanyi, Stephen Tukwasibwe, Samuel L. Nsohya, Emmanuel Arinaitwe, and Moses Kanya, Infectious Diseases Research Collaboration, Kampala, Uganda, E-mails: mbgeorge@yahoo.co.uk, nankshila@yahoo.com, stephentukwasibwe@yahoo.com, samnsohya@yahoo.co.uk, earinaitwe@idrc-uganda.org, and mkanya@infocom.co.ug. Frederick N. Baliraine, Le Tourneau University, Longview, TX, E-mail: FredBaliraine@letu.edu. Melissa D. Conrad, Grant Dorsey, Bryan Greenhouse, and Philip J. Rosenthal, Department of Medicine, University of California, San Francisco, CA, E-mails: ConradM@medsfgh.ucsf.edu, gdorsey@medsfgh.ucsf.edu, bgreenhouse@medsfgh.ucsf.edu, and prosenthal@medsfgh.ucsf.edu. Jordan Tappero, Global AIDS Program, Centers for Disease Control and Prevention, Atlanta, GA, E-mail: jtappero@cdc.gov. Sarah G. Staedke, London School of Hygiene and Tropical Medicine, UK, E-mail: sarah.staedke@lshtm.ac.uk.

Reprints requests: Philip J. Rosenthal, Department of Medicine, Box 0811, University of California, San Francisco, CA 94143-0811. E-mail: prosenthal@medsfgh.ucsf.edu.

REFERENCES

1. Yeka A, Gasasira A, Mpimbaza A, Achan J, Nankabirwa J, Nsohya S, Staedke SG, Donnelly MJ, Wabwire-Mangen F, Talisuna A, Dorsey G, Kanya MR, Rosenthal PJ, 2012. Malaria in Uganda: challenges to control on the long road to elimination: I. Epidemiology and current control efforts. *Acta Trop* 121: 184–195.
2. Nanyunja M, Nabyonga Orem J, Kato F, Kaggwa M, Katureebe C, Saweka J, 2011. Malaria treatment policy change and implementation: the case of Uganda. *Malar Res Treat* 2011: 683167.
3. Nosten F, White NJ, 2007. Artemisinin-based combination treatment of falciparum malaria. *Am J Trop Med Hyg* 77: 181–192.
4. Dorsey G, Staedke S, Clark TD, Njama-Meya D, Nzarubara B, Maiteki-Sebuguzi C, Dokomajilar C, Kanya MR, Rosenthal PJ, 2007. Combination therapy for uncomplicated falciparum malaria in Ugandan children: a randomized trial. *JAMA* 297: 2210–2219.
5. Yeka A, Dorsey G, Kanya MR, Talisuna A, Lugemwa M, Rwakimari JB, Staedke SG, Rosenthal PJ, Wabwire-Mangen F, Bukirwa H, 2008. Artemether-lumefantrine versus dihydroartemisinin-piperaquine for treating uncomplicated malaria: a randomized trial to guide policy in Uganda. *PLoS ONE* 3: e2390.
6. Dondorp AM, Nosten F, Yi P, Das D, Phyto AP, Tarning J, Lwin KM, Ariey F, Hanpithakpong W, Lee SJ, Ringwald P, Silamut K, Imwong M, Chotivanich K, Lim P, Herdman T, An SS, Yeung S, Singhasivanon P, Day NP, Lindegardh N, Socheat D, White NJ, 2009. Artemisinin resistance in *Plasmodium falciparum* malaria. *N Engl J Med* 361: 455–467.
7. Rosenthal PJ, 2013. The interplay between drug resistance and fitness in malaria parasites. *Mol Microbiol* 89: 1025–1038.
8. Fidock DA, Nomura T, Talley AK, Cooper RA, Dzekunov SM, Ferdig MT, Ursos LM, Sidhu AB, Naude B, Deitsch KW, Su XZ, Wootton JC, Roepe PD, Welles TE, 2000. Mutations in the *P. falciparum* digestive vacuole transmembrane protein PfCRT and evidence for their role in chloroquine resistance. *Mol Cell* 6: 861–871.
9. Valderramos SG, Fidock DA, 2006. Transporters involved in resistance to antimalarial drugs. *Trends Pharmacol Sci* 27: 594–601.
10. Mwai L, Diriyé A, Masseno V, Muriithi S, Feltwell T, Musyoki J, Lemieux J, Feller A, Mair GR, Marsh K, Newbold C, Nzila A, Carret CK, 2012. Genome wide adaptations of *Plasmodium falciparum* in response to lumefantrine selective drug pressure. *PLoS ONE* 7: e31623.
11. 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 *Pfprt* in changing chloroquine, amodiaquine, mefloquine and lumefantrine susceptibility in western-Kenya *P. falciparum* samples during 2008–2011. *PLoS ONE* 8: e64299.
12. Nsohya SL, Kiggundu M, Nanyunja S, Joloba M, Greenhouse B, Rosenthal PJ, 2010. *In vitro* sensitivities of *Plasmodium falciparum* to different antimalarial drugs in Uganda. *Antimicrob Agents Chemother* 54: 1200–1206.
13. Pickard AL, Wongsrichanalai C, Purfield A, Kamwendo D, Emery K, Zalewski C, Kawamoto F, Miller RS, Meshnick SR, 2003. Resistance to antimalarials in southeast Asia and genetic polymorphisms in *pfmdr1*. *Antimicrob Agents Chemother* 47: 2418–2423.
14. Woodrow CJ, Krishna S, 2006. Antimalarial drugs: recent advances in molecular determinants of resistance and their clinical significance. *Cell Mol Life Sci* 63: 1586–1596.
15. Dorsey G, Kanya MR, Singh A, Rosenthal PJ, 2001. Polymorphisms in the *Plasmodium falciparum* *pfprt* and *pfmdr1* genes and clinical response to chloroquine in Kampala, Uganda. *J Infect Dis* 183: 1417–1420.
16. Francis D, Nsohya SL, Talisuna A, Yeka A, Kanya MR, Machekano R, Dokomajilar C, Rosenthal PJ, Dorsey G, 2006. Geographic differences in antimalarial drug efficacy in Uganda are explained by differences in endemicity and not by known molecular markers of drug resistance. *J Infect Dis* 193: 978–986.

17. Kyosiimire-Lugemwa J, Nalunkuma-Kazibwe AJ, Mujuzi G, Mulindwa H, Talisuna A, Egwang TG, 2002. The Lys-76-Thr mutation in PfCRT and chloroquine resistance in *Plasmodium falciparum* isolates from Uganda. *Trans R Soc Trop Med Hyg* 96: 91–95.
18. 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.
19. Laufer MK, Thesing PC, Eddington ND, Masonga R, Dzinjalumala FK, Takala SL, Taylor TE, Plowe CV, 2006. Return of chloroquine antimalarial efficacy in Malawi. *N Engl J Med* 355: 1959–1966.
20. Mwai L, Ochong E, Abdirahman A, Kiara SM, Ward S, Kokwaro G, Sasi P, Marsh K, Borrmann S, Mackinnon M, Nzila A, 2009. Chloroquine resistance before and after its withdrawal in Kenya. *Malar J* 8: 106.
21. Froberg G, Jornhagen L, Morris U, Shakely D, Msellem MI, Gil JP, Bjorkman A, Martensson A, 2012. Decreased prevalence of *Plasmodium falciparum* resistance markers to amodiaquine despite its wide scale use as ACT partner drug in Zanzibar. *Malar J* 11: 321.
22. Mang'era CM, Mbai FN, Omedo IA, Mireji PO, Omar SA, 2012. Changes in genotypes of *Plasmodium falciparum* human malaria parasite following withdrawal of chloroquine in Tiwi, Kenya. *Acta Trop* 123: 202–207.
23. Malmberg M, Ngasala B, Ferreira PE, Larsson E, Jovel I, Hjalmarsson A, Petzold M, Premji Z, Gil JP, Bjorkman A, Martensson A, 2013. Temporal trends of molecular markers associated with artemether-lumefantrine tolerance/resistance in Bagamoyo district, Tanzania. *Malar J* 12: 103.
24. Gregson A, Plowe CV, 2005. Mechanisms of resistance of malaria parasites to antifolates. *Pharmacol Rev* 57: 117–145.
25. Dorsey G, Dokomajilar C, Kiggundu M, Staedke SG, Kanya MR, Rosenthal PJ, 2004. Principal role of dihydropteroate synthase mutations in mediating resistance to sulfadoxine-pyrimethamine in single-drug and combination therapy of uncomplicated malaria in Uganda. *Am J Trop Med Hyg* 71: 758–763.
26. Nzila A, Ochong E, Nduati E, Gilbert K, Winstanley P, Ward S, Marsh K, 2005. Why has the dihydrofolate reductase 164 mutation not consistently been found in Africa yet? *Trans R Soc Trop Med Hyg* 99: 341–346.
27. Gesase S, Gosling RD, Hashim R, Ord R, Naidoo I, Madebe R, Moshia JF, Joho A, Mandia V, Mrema H, Mapunda E, Savael Z, Lemnge M, Moshia FW, Greenwood B, Roper C, Chandramohan D, 2009. High resistance of *Plasmodium falciparum* to sulphadoxine/pyrimethamine in northern Tanzania and the emergence of *dhps* resistance mutation at Codon 581. *PLoS ONE* 4: e4569.
28. Spalding MD, Eyase FL, Akala HM, Bedno SA, Prigge ST, Coldren RL, Moss WJ, Waters NC, 2010. Increased prevalence of the *pfdhfr*/*phdhps* quintuple mutant and rapid emergence of *pfdhps* resistance mutations at codons 581 and 613 in Kisumu, Kenya. *Malar J* 9: 338.
29. Lynch C, Pearce R, Pota H, Cox J, Abeku TA, Rwakimari J, Naidoo I, Tibenderana J, Roper C, 2008. Emergence of a *dhfr* mutation conferring high-level drug resistance in *Plasmodium falciparum* populations from southwest Uganda. *J Infect Dis* 197: 1598–1604.
30. Yeka A, Banek K, Bakyaaita N, Staedke SG, Kanya MR, Talisuna A, Kironde F, Nsoby SL, Kilian A, Slater M, Reingold A, Rosenthal PJ, Wabwire-Mangen F, Dorsey G, 2005. Artemisinin versus nonartemisinin combination therapy for uncomplicated malaria: randomized clinical trials from four sites in Uganda. *PLoS Med* 2: e190.
31. Bakyaaita N, Dorsey G, Yeka A, Banek K, Staedke SG, Kanya MR, Talisuna A, Kironde F, Nsoby S, Kilian A, Reingold A, Rosenthal PJ, Wabwire-Mangen F, 2005. Sulfadoxine-pyrimethamine plus chloroquine or amodiaquine for uncomplicated falciparum malaria: a randomized, multisite trial to guide national policy in Uganda. *Am J Trop Med Hyg* 72: 573–580.
32. Bukirwa H, Yeka A, Kanya MR, Talisuna A, Banek K, Bakyaaita N, Rwakimari JB, Rosenthal PJ, Wabwire-Mangen F, Dorsey G, Staedke SG, 2006. Artemisinin combination therapies for treatment of uncomplicated malaria in Uganda. *PLoS Clin Trials* 1: e7.
33. Arinaitwe E, Sandison TG, Wanzira H, Kakuru A, Homby J, Kalanya J, Kanya MR, Vora N, Greenhouse B, Rosenthal PJ, Tappero J, Dorsey G, 2009. Artemether-lumefantrine versus dihydroartemisinin-piperaquine for falciparum malaria: a longitudinal, randomized trial in young Ugandan children. *Clin Infect Dis* 49: 1629–1637.
34. Nsoby SL, Dokomajilar C, Joloba M, Dorsey G, Rosenthal PJ, 2007. Resistance-mediating *Plasmodium falciparum* *pfcr* and *pfmdr1* alleles after treatment with artesunate-amodiaquine in Uganda. *Antimicrob Agents Chemother* 51: 3023–3025.
35. Conrad MD, LeClair N, Arinaitwe E, Wanzira H, Kakuru A, Bigira V, Muhindo M, Kanya MR, Tappero JW, Greenhouse B, Dorsey G, Rosenthal PJ, 2014. Comparative impacts over 5 years of artemisinin-based combination therapies on *P. falciparum* polymorphisms that modulate drug sensitivity in Ugandan children. *J Infect Dis*. First published online March 8, 2014, doi:10.1093/infdis/jiu141.
36. 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.
37. Djimde A, Doumbo OK, Cortese JF, Kayentao K, Doumbo S, Diourte Y, Dicko A, Su XZ, Nomura T, Fidock DA, Wellems TE, Plowe CV, Coulbaly D, 2001. A molecular marker for chloroquine-resistant falciparum malaria. *N Engl J Med* 344: 257–263.
38. Duraisingh MT, Curtis J, Warhurst DC, 1998. *Plasmodium falciparum*: detection of polymorphisms in the dihydrofolate reductase and dihydropteroate synthetase genes by PCR and restriction digestion. *Exp Parasitol* 89: 1–8.
39. Duraisingh MT, Jones P, Sambou I, von Seidlein L, Pinder M, Warhurst DC, 2000. The tyrosine-86 allele of the *pfmdr1* gene of *Plasmodium falciparum* is associated with increased sensitivity to the anti-malarials mefloquine and artemisinin. *Mol Biochem Parasitol* 108: 13–23.
40. Baliraine FN, Rosenthal PJ, 2011. Prolonged selection of *pfmdr1* polymorphisms after treatment of falciparum malaria with artemether-lumefantrine in Uganda. *J Infect Dis* 204: 1120–1124.
41. Kamugisha E, Jing S, Minde M, Kataraihya J, Kongola G, Kironde F, Swedberg G, 2012. Efficacy of artemether-lumefantrine in treatment of malaria among under-fives and prevalence of drug resistance markers in Igombe-Mwanza, north-western Tanzania. *Malar J* 11: 58.
42. Nakibuuka V, Ndezi G, Nakiboneka D, Ndugwa CM, Tumwine JK, 2009. Presumptive treatment with sulphadoxine-pyrimethamine versus weekly chloroquine for malaria prophylaxis in children with sickle cell anemia in Uganda: a randomized controlled trial. *Malar J* 8: 237.
43. Dokomajilar C, Nsoby SL, Greenhouse B, Rosenthal PJ, Dorsey G, 2006. Selection of *Plasmodium falciparum* *pfmdr1* alleles following therapy with artemether-lumefantrine in an area of Uganda where malaria is highly endemic. *Antimicrob Agents Chemother* 50: 1893–1895.
44. 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.
45. Thomsen TT, Ishengoma DS, Mmbando BP, Lusingu JP, Vestergaard LS, Theander TG, Lemnge MM, Bygbjerg IC, Alifrangis M, 2011. Prevalence of single nucleotide polymorphisms in the *Plasmodium falciparum* multidrug resistance gene (*Pfmdr-1*) in Korogwe District in Tanzania before and after introduction of artemisinin-based combination therapy. *Am J Trop Med Hyg* 85: 979–983.
46. Mwai L, Kiara SM, Abdirahman A, Pole L, Rippert A, Diriye A, Bull P, Marsh K, Borrmann S, Nzila A, 2009. *In vitro* activities of piperaquine, lumefantrine, and dihydroartemisinin in Kenyan *Plasmodium falciparum* isolates and polymorphisms

- in *pfprt* and *pfmdr1*. *Antimicrob Agents Chemother* 53: 5069–5073.
47. Happi CT, Gbotosho GO, Folarin OA, Sowunmi A, Hudson T, O'Neil M, Milhous W, Wirth DF, Oduola AM, 2009. Selection of *Plasmodium falciparum* multidrug resistance gene 1 alleles in asexual stages and gametocytes by artemether-lumefantrine in Nigerian children with uncomplicated falciparum malaria. *Antimicrob Agents Chemother* 53: 888–895.
 48. Iriemenam NC, Shah M, Gatei W, van Eijk AM, Ayisi J, Kariuki S, Vanden Eng J, Owino SO, Lal AA, Omosun YO, Otieno K, Desai M, ter Kuile FO, Nahlen B, Moore J, Hamel MJ, Ouma P, Slutsker L, Shi YP, 2012. Temporal trends of sulphadoxine-pyrimethamine (SP) drug-resistance molecular markers in *Plasmodium falciparum* parasites from pregnant women in western Kenya. *Malar J* 11: 134.
 49. Gasasira AF, Kanya MR, Ochong EO, Vora N, Achan J, Charlebois E, Ruel T, Kateera F, Meya DN, Havlir D, Rosenthal PJ, Dorsey G, 2010. Effect of trimethoprim-sulphamethoxazole on the risk of malaria in HIV-infected Ugandan children living in an area of widespread antifolate resistance. *Malar J* 9: 177.
 50. Sandison TG, Homsy J, Arinaitwe E, Wanzira H, Kakuru A, Bigira V, Kalamya J, Vora N, Kublin J, Kanya MR, Dorsey G, Tappero JW, 2011. Protective efficacy of co-trimoxazole prophylaxis against malaria in HIV exposed children in rural Uganda: a randomized clinical trial. *BMJ* 342: d1617.
 51. LeClair NP, Conrad MD, Baliraine FN, Nsanjabana C, Nsohya SL, Rosenthal PJ, 2013. Optimization of a ligase detection reaction-fluorescent microsphere assay for characterization of resistance-mediating polymorphisms in African samples of *Plasmodium falciparum*. *J Clin Microbiol* 51: 2564–2570.
 52. Taylor SM, Parobek CM, Aragam N, Ngasala BE, Martensson A, Meshnick SR, Juliano JJ, 2013. Pooled deep sequencing of *Plasmodium falciparum* isolates: an efficient and scalable tool to quantify prevailing malaria drug-resistance genotypes. *J Infect Dis* 208: 1998–2006.