

Amodiaquine and Artemether-Lumefantrine Select Distinct Alleles of the *Plasmodium falciparum* *mdr1* Gene in Tanzanian Children Treated for Uncomplicated Malaria[▽]

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The artemisinin-based combination therapies artemether-lumefantrine (AL) and amodiaquine (AQ) plus artesunate have been adopted for treatment of *Plasmodium falciparum* malaria in many African countries. Molecular markers of parasite resistance suitable for surveillance have not been established for any of the component drugs in either of these combinations. We assessed *P. falciparum* *mdr1* (*Pfmdr1*) alleles present in 300 Tanzanian children presenting with uncomplicated falciparum malaria, who were enrolled in a clinical trial of antimalarial therapy. *Pfmdr1* genotype analysis was also performed with isolates from 182 children who failed AQ monotherapy and 54 children who failed AL treatment. *Pfmdr1* alleles 86Y, 184Y, and 1246Y were more common among treatment failures in the AQ group than among pretreatment infections. The converse was found in the AL-treated group. Children presenting with the 86Y/184Y/1246Y *Pfmdr1* haplotype and treated with AQ were significantly more likely to retain this haplotype if they were parasite positive during posttreatment follow-up than were children treated with AL (odds ratio, 33.25; 95% confidence interval, 4.17 to 1441; *P*, <0.001). We conclude that AL and AQ exert opposite within-host selective effects on the *Pfmdr1* gene of *P. falciparum*.

The emergence and spread of *Plasmodium falciparum* resistance to antimalarial drugs is now one of the greatest challenges facing the global effort to control malaria. In Africa, until recently, there has been a reliance on the cheap drugs chloroquine and sulfadoxine-pyrimethamine. To protect drugs from resistance, there is now clear evidence that combining them can improve their efficacy without increasing their toxicity (13), and with the development of highly effective artemisinin derivatives, there is renewed hope for the treatment of malaria in the form of artemisinin-based combination therapy (ACT). In 2001, the World Health Organization recommended ACTs as the first-line treatment for uncomplicated malaria (31).

ACTs protect the individual drugs from resistance by relying on the principle of combining two drugs with different mechanisms of action (30). The fast-acting artemisinin derivative rapidly clears the main parasite load within the few hours that it remains at therapeutic levels and thus reduces subsequent gametocyte carriage (28), while the partner drug, which is generally longer lasting, remains to clear the rest of the parasites. The combination of artesunate and mefloquine has been used with success in Southeast Asia (17). In sub-Saharan Africa, most countries have now adopted either artemether-lumefantrine (AL), which is coformulated as Coartem, or artesunate (AS)-amodiaquine (AQ) as their first-line ACT,

although the transition to widespread deployment is only just beginning. Treatment success with an ACT will depend largely on the parasite's existing level of tolerance to the partner drug, and therefore, molecular markers of drug resistance can be useful tools in the population surveillance of drug efficacy. There is significant interest in describing genetic mutations that are associated with resistance to ACT partner drugs and to the artemisinins themselves.

AQ is a 4-aminoquinoline related to chloroquine (CQ), and the mode of action of the two drugs appears to be similar; both are known to be concentrated in the parasite's lysosome (5). Although CQ resistance is now widespread across the African continent, the efficacy of AQ is variable in treatment trials (12). A mutation from lysine (K) to threonine (T) at codon 76 of the *P. falciparum* chloroquine resistance transporter (*Pfcr*) gene is associated with resistance to both CQ and AQ (1, 8, 11). However, in vitro and in vivo evidence suggests that cross-resistance between the two drugs is incomplete (12, 23). Therefore, AQ resistance is likely to be determined by additional genotypic changes found in some, but not all, CQ-resistant parasites. The identification of such changes would facilitate the rational deployment of AQ-containing combinations in settings where resistance-associated alleles were rare.

Numerous in vitro studies of polymorphisms in the *P. falciparum* multidrug-resistant 1 (*Pfmdr1*) gene have concluded that its protein product, Pgh1, plays a role in modulating levels of resistance to several structurally unrelated drugs (4, 21, 24). Five distinct single-nucleotide substitutions (SNPs), leading to amino acid changes at codons 86, 184, 1034, 1042, and 1246, have been described. The N86Y change of the *Pfmdr1* gene product is favorable for resistance to both CQ and AQ and is

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TABLE 1. PCR primer sequences and reaction conditions for the nested amplification of *Pfprt* and *Pfmdr1* fragments

Gene fragment	Primer name	Primer sequence	PCR product size (bp)	PCR cycling conditions ^a
<i>Pfprt</i> (for SNPs at codons 72 to 76)	Outer forward P1	5'-CCGTTAATAATAAATACACGCAG	546	35 cycles of 94°C for 30 s; 56°C for 30 s; and 62°C for 1 min; then 62°C for 5 min
	Outer reverse P2	5'-CGGATGTTACAAAATATAGTTACC		
	Nested forward D3	5'-AGGTTCTTGTCTTGGTAAATTGTC	164	
	Nested reverse D2	5'-CAAACTATAGTTACCAATTTTG		
<i>Pfmdr1</i> fragment 1 (for SNPs at codons 86 and 184)	Outer forward FN1/1	5'-AGGTTGAAAAAGAGTTGAAC	578	30 cycles of 94°C for 30 s; 55°C for 30 s; and 65°C for 1 min; then 65°C for 5 min
	Outer reverse REV/C1	5'-ATGACACCAACAACATAAAT		
	Nested forward MDR2/1	5'-ACAAAAAGAGTACCGCTGAAT	534	
	Nested reverse NEWREV1	5'-AAACGCAAGTAATACATAAAGTC		
<i>Pfmdr1</i> fragment 2, long (for SNPs at codons 1034, 1042, and 1246)	Outer forward MDRFR2F1	5'-GTGTATTTGCTGTAAGAGCT	958	34 cycles of 94°C for 30 s; 55°C for 1 min; and 65°C for 1.5 min; then 65°C for 5 min
	Outer reverse MDRFR2R1	5'-GACATATTAAATAACATGGGTTTC		
	Nested forward MDRFR2F2	5'-CAGATGATGAAATGTTTAAAGATC	864	
	Nested reverse MDRFR2R2	5'-TAAATAACATGGGTTCTTGACT		

^a Each PCR program was preceded by an initial denaturation step of 94°C for 3 min.

concomitantly associated with increased sensitivity to mefloquine and halofantrine (4). Sidhu et al. (24) have shown, using reverse genetics, that the 1042D and 1034C/1042D/1246Y alleles are associated with increased resistance to quinine and increased sensitivity to mefloquine and artemisinin drugs. This finding supported the earlier work of Reed et al. (21) using allelic replacement experiments.

Studies from Southeast Asia have demonstrated an association between the increased copy number of the *Pfmdr1* gene and treatment failure with mefloquine (15, 19), supporting in vitro studies showing that laboratory lines with amplified *Pfmdr1* loci have reduced susceptibility to mefloquine and related drugs such as halofantrine (18). Recent evidence from Thailand suggests that amplification of *Pfmdr1* can also modulate susceptibility to the artemether-lumefantrine ACT, leading to poor treatment response in some patients (20).

Two African clinical trial reports provide evidence for the selection of particular *Pfmdr1* alleles after AL treatment. In Zanzibar, the posttreatment prevalence of 86N was significantly higher than pretreatment levels in parasites that reinfect patients within the 42-day follow-up period (25). This finding is supported by evidence from Uganda (2), where AL treatment for uncomplicated malaria selected newly infecting parasites carrying the 86N, 184F, and 1246D alleles. Dokomajilar et al. found no evidence for an increased *Pfmdr1* copy number in either pre- or posttreatment infections, suggesting that amplification of the *Pfmdr1* locus may be less important in African parasite populations (2).

To further investigate the effect of antimalarial treatment on different alleles of the *Pfmdr1* gene, we present genetic analyses of samples from a randomized four-arm antimalarial effectiveness trial in Muheza, Tanzania (10). In this trial, AQ monotherapy exhibited a high treatment failure rate of 76%, whereas AL was efficacious but permitted the establishment of new infections in 18% of subjects toward the end of the 28-day follow-up period. The prevalence of polymorphisms at *Pfprt* codon positions 72 to 76 and *Pfmdr1* changes N86Y, Y184F, S1034C, N1042D, and D1246Y were found in pretreatment parasite isolates and in a matched pair analysis of pre- and

posttreatment samples from the AQ monotherapy and AL treatment arms of the trial.

MATERIALS AND METHODS

Subjects. Briefly, a randomized four-arm drug effectiveness trial was carried out between September 2002 and October 2004. A total of 1,811 children (ages 4 to 59 months) were recruited from the Muheza district, Tanzania, and followed for 28 days. The drug groups consisted of those undergoing AQ monotherapy (terminated after 1 year due to a high level of treatment failure), AQ+sulfadoxine-pyrimethamine (SP), AQ+AS, and AL. Apart from the first dose, drug administration was not supervised, in order to provide an estimation of drug effectiveness in a more "real-world" setting. Full details of the trial, including recruitment, randomization, and follow-up have been published previously (10). After PCR detection of the polymorphic merozoite surface protein 2 gene (*msp2* genotyping) to distinguish recrudescences from new infections, the percentage of parasitological treatment failures in each drug group was AQ, 48.5%; AQ+SP, 34.8%; AQ+AS, 12.1%; and AL, 2.7% (10).

Molecular analysis of filter paper-preserved blood samples collected during year 1 of the trial was carried out with two groups, as follows: (i) a block-randomized pretreatment subset of samples selected to provide a baseline estimate of allele prevalences, irrespective of drug group or treatment outcome ($n = 300$); and (ii) in patients who had parasitological treatment failure (i.e., those with recurrent parasites, with or without symptoms, during the 28-day follow-up) after AQ monotherapy or AL, samples were tested for the selection of alleles between day 0 (D0; pretreatment) and day of treatment failure (Dfail; from day 3 up to day 28). A total of 182 (AQ) and 54 (AL) treatment failures were analyzed as matched pairs, D0/Dfail.

Genotyping. DNA was extracted from blood spots on filter paper by using the Chelex method (16).

To distinguish recrudescence from new infections in parasitological treatment failures, the block 3 region of *msp2*, which exists in three allelic forms, was amplified using allele-specific primers, and size polymorphisms were detected by agarose gel electrophoresis (26). Where 50% or more of the alleles present in the Dfail sample were new, compared to the D0 sample, this was classed as a new infection.

SNPs that resulted in an amino acid change at codons in the *Pfprt* and *Pfmdr1* genes were determined by PCR amplification, followed by a sequence-specific oligonucleotide probe assay adapted from Pearce et al. (14). The *Pfmdr1* gene codons tested were N86Y (i.e., denoting an encoded amino acid change from asparagine to tyrosine at codon 86), F184Y, S1034C, D1042N, D1246Y, and in *Pfprt* the codons tested were C72S, M74I, N75E, and K76T.

Two fragments from the *Pfmdr1* gene and one from the *Pfprt* gene, designed to encompass all SNP sites under investigation, were amplified using the primers and conditions shown in Table 1. All fragments were amplified by nested PCR in a 96-well plate format. The 25- μ l PCR mixes contained primers at 200 nM final concentration, 1.5 mM MgCl₂, 250 μ M each deoxynucleotide triphosphate, and

TABLE 2. Oligonucleotide probes used to detect sequence polymorphisms in genes *Pfcr1* and *Pfmdr1*

Probe name ^a	Probe sequence
<i>Pfcr1</i>	
72C	5'-TAT TTA AGT GTA TGT GTA
72S	5'-TAT TTA AGT GTA AGT GTA
74-76 IET	5'-TA ATT GAA ACA ATT TTT G
74-76 MNK	5'-TA ATG AAT AAA ATT TTT G
74-76 MNT	5'-TA ATG AAT ACA ATT TTT G
<i>Pfmdr1</i>	
86 N	5'-AG AAC ATG AAT TTA GGT G
86 Y	5'-AG AAC ATG TAT TTA GGT G
184 Y	5'-A GGT TTA TAT ATT TGG TC
184 F	5'-A GGT TTA TTT ATT TGG TC
1034 S	5'-A TGG GGA TTC AGT CAA AG
1034 C	5'-A TGG GGA TTC TGT CAA AG
1042 N	5'-TA TTT ATT AAT AGT TTT G
1042 D	5'-TA TTT ATT GAT AGT TTT G
1246 D	5'-AC TTA AGA GAT CTT AGA A
1246 Y	5'-AC TTA AGA TAT CTT AGA A

^a The probe name indicates the polymorphic codon position and encoded amino acid. Nucleotide substitutions associated with drug resistance are in bold face type.

1 U Bioline TaqI polymerase (Bioline, London, United Kingdom). Extracted DNA (5 µl) was added to each first-round PCR mixture. One microliter of the first-round product was then used as a template in a 25-µl nested amplification. DNA samples extracted from parasite lines 3D7, W2, and 7G8 were used as positive controls, as these lines carry all the *Pfcr1* and *Pfmdr1* mutations under examination.

The denatured, nested PCR products were UV cross-linked to nylon membranes. After blocking, one membrane was exposed to an 18-bp 3'-digoxigenin-labeled oligonucleotide probe specific to the wild-type sequence. A second identical membrane was exposed to a similar probe bearing the mutant sequence (Table 2). High-stringency washing in 3 M tetramethylammonium chloride ensured that only probes with the exact complementary sequence remained hybridized to the immobilized PCR product; those with a single nucleotide mismatch at the SNP site were washed off (14). Probes that remained bound were detected by incubation with antidigoxigenin Fab fragments conjugated to alkaline phosphatase, followed by exposure to the substrate disodium 3-(4-methoxyphosphoryl)-5-iodo-4-yl phenyl phosphate. The resulting chemiluminescence was detected by autoradiography and scored visually by two readers.

Statistical analysis. Statistical analysis was carried out using STATA (version 9) software. A nonparametric test for matched pair data (McNemar's test) was

used to analyze the selection of SNPs at each locus for D0 and the day of recurrent parasitemia (Dfail).

RESULTS

Baseline allele prevalence. A baseline sample of 300 isolates for genotyping was generated by block randomization of pretreatment samples from all four arms of the trial, representing 27.3% of all participants in year 1 of the trial. Only the CVIET and CVMNK alleles of *Pfcr1* at codon positions 72 to 76 were identified in the pretreatment baseline. All of the analyzed samples contained the 1034S or 1042N alleles of the *Pfmdr1* gene. Where both wild-type and mutant alleles were detected in the same sample, they were scored as mixed infections and were included in subsequent analyses as described. The prevalence of each allele at the three polymorphic loci in *Pfmdr1* is shown in Fig. 1. The number of successful PCR amplifications is shown for each locus on the x axis.

Presentation alleles and treatment outcomes. Using parasitological failure by day 28 as the outcome measure, we analyzed the baseline samples stratified by treatment group. Within the subset of subjects who had received AQ monotherapy, a significant association with parasitological failure was found, with the presence on day 0 of either the 86Y allele (Fisher's exact test, $P = 0.032$; $n = 40$) or the 184Y allele ($P = 0.0054$; $n = 42$). Conversely, pretreatment carriage of either the 86N allele ($P = 0.005$; $n = 52$) or the 184F allele ($P = 0.0262$; $n = 52$) was associated with treatment failure in the AL treatment group.

Allele prevalence among treatment failures. *Pfcr1* genotypes were examined in the AQ treatment group only. All infections persisting after AQ treatment harbored the CVIET allele at amino acid positions 72 to 76, and 5% also carried mixed infections with the CVMNK allele.

Within both the AQ and AL treatment groups, all the children who failed treatment by day 28 had both their pre- and posttreatment samples analyzed, and the allele prevalence at each locus, generated from all available genotyping results at each time point, is shown in Fig. 2. The number of samples involved in each calculation is shown in the legend for each graph. The 86Y allele prevalence increased after treatment

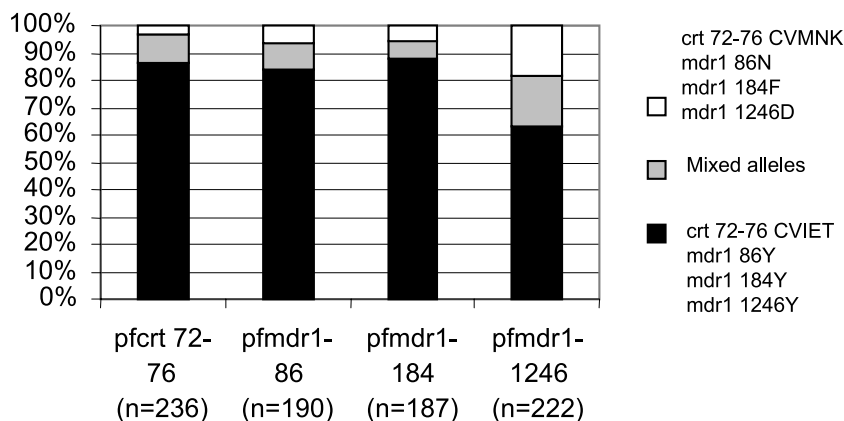


FIG. 1. Pretreatment random baseline prevalence of *Pfcr1* and *Pfmdr1* alleles. The white portion of the bars shows the prevalence of the alleles associated with CQ sensitivity. The black portion shows the prevalence of alleles associated with CQ resistance. The gray portion shows the prevalence of samples where a mixed infection containing both alleles was detected.

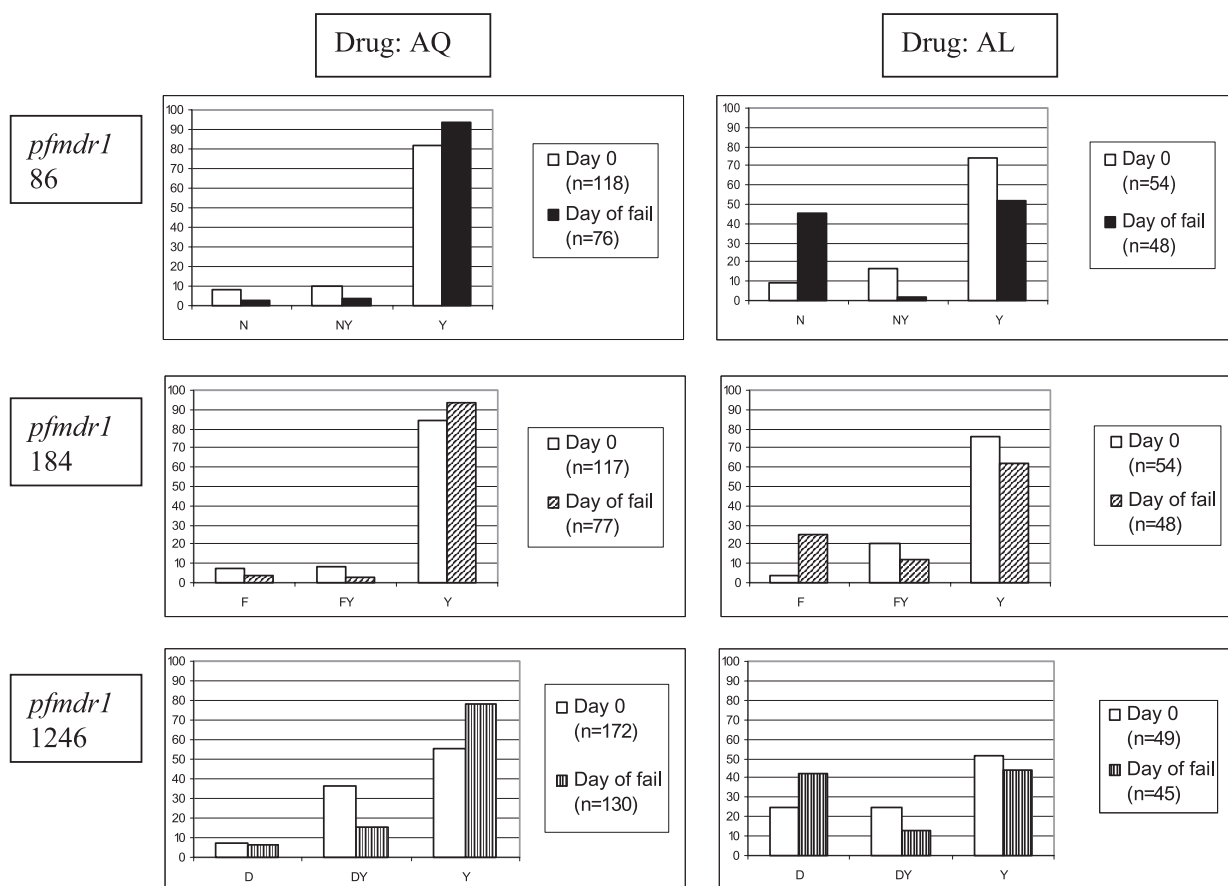


FIG. 2. Prevalence of pre- and posttreatment *Pfmdr1* alleles following therapy with either AQ or AL. Mixed infections are shown as separate columns in the middle of each graph. The number of samples involved in each prevalence calculation is shown in the legend in each graph.

with AQ from 81.4% to 93.4% and decreased after treatment with AL from 74.1% to 52.1%. Similarly, the 184Y allele was present in 93.5% of posttreatment infections from children who failed AQ treatment and in only 62.5% of those from children who failed AL treatment. For the 1246 locus, the posttreatment proportions of Y alleles were 78.5% and 44.4% in the AQ and AL arms, respectively. The pattern of *Pfmdr1* allele prevalence change observed across the AL group is very

similar to that reported recently by Dokomajilar et al. (2) from their study of Ugandan children, confirming that the alleles of interest in this group are *Pfmdr1* 86N, 184F, and 1246D. In contrast, the alleles of interest in the AQ treatment groups are *Pfmdr1* 86Y, 184Y, and 1246Y.

Matched pair analysis of AQ monotherapy. There were a total of 182 treatment failures in the AQ monotherapy arm of the trial. Of the 137 for which *msp2* genotyping data were

TABLE 3. Analysis of directional selection acting on the single locus alleles at *Pfmdr1* positions 86, 184, and 1246 from children with parasites following therapy with either AQ or AL^a

AQ treatment group			AL treatment group		
Allele present at D0 (n)	No. of alleles changed on Dfail	McNemar's χ^2 test (P value)	Allele present at D0 (n)	No. of alleles changed on Dfail	McNemar's χ^2 test (P value)
<i>Pfmdr1</i> 86Y (43)	1	0.1797	<i>Pfmdr1</i> 86Y (43)	17	0.0017
<i>Pfmdr1</i> 86N (5)	4		<i>Pfmdr1</i> 86N (5)	3	
<i>Pfmdr1</i> 184Y (52)	0	0.1573	<i>Pfmdr1</i> 184Y (30)	8	0.0196
<i>Pfmdr1</i> 184F (4)	2		<i>Pfmdr1</i> 184F (1)	1	
<i>Pfmdr1</i> 1246Y (62)	0	0.0253	<i>Pfmdr1</i> 1246Y (15)	8	0.2482
<i>Pfmdr1</i> 1246D (9)	5		<i>Pfmdr1</i> 1246D (10)	4	

^a The numbers of samples tested with each allele present on D0 (n) are shown with the allele name in columns one and four. The number of samples in which the other allele was detected (i.e., allele changed) in the Dfail sample is shown in columns two and five. The direction of the allele changes from D0 to Dfail were assessed using McNemar's χ^2 test for paired samples. Significant P values are in boldface. Mixed infections harboring both alleles were omitted for this analysis.

TABLE 4. Analysis of directional selection acting on the three-locus haplotype YYY at *Pfmdr1* positions 86, 184, and 1246 from children with parasites following therapy with either AQ or AL^a

Drug	Haplotype on D0	Haplotype on Dfail				P value	Comment
		Any YYY	No YYY	Any NFD	No NFD		
AQ	Any YYY	38	1			0.0339	Directional selection for YYY
	No YYY	7	1				
	Any NFD			0	3	0.3173	No evidence of directional selection
	No NFD			1	44		
AL	Any YYY	16	14			0.0389	Directional selection against YYY
	No YYY	5	6				
	Any NFD			2	6	0.5930	No evidence of directional selection
	No NFD			8	25		

^a The direction of the haplotype changes from D0 to Dfail were assessed using McNemar's χ^2 test for paired samples. Only the samples where a change occurs (in bold type) contribute to this analysis. Significant *P* values are in bold type. Mixed alleles YN, YF, and YD are coded as "Y" in this analysis.

obtained, 45 (33%) were classed as new infections after PCR correction. Matched pre- and posttreatment data for the same individual were analyzed at *Pfmdr1* loci 86, 184, and 1246 using a stringent two-by-two approach, with the omission of mixed infections harboring both alleles. After treatment with AQ, there was no evidence of directional selection for *Pfmdr1* 86Y or 184Y, but there was significant directional selection of *Pfmdr1* 1246Y ($P = 0.0253$) (Table 3).

Matched pair analysis of AL treatment. There were a total of 54 treatment failures in the AL combination treatment arm of the trial (year 1 only). By *msp2* genotyping, 39 of 45 interpretable treatment failure cases were classed as new infections (87%). Using the same stringent two-by-two tables as for the AQ arm, we found evidence for significant directional selection of *Pfmdr1* 86N ($P = 0.0017$) and 184F ($P = 0.0196$) after treatment with AL (Table 3).

Haplotype analysis. Genotyping data for all three *Pfmdr1* loci were combined to determine whether the 86Y/184Y/1246Y haplotype was present in each pre- and posttreatment sample in both treatment groups. If a sample contained one mixed allele (e.g., if *Pfmdr1* 86Y, 184Y, 1246Y, and 1246D were detected), we assumed that the haplotype of interest, YYY, must have occurred in one parasite genome within this sample (along with haplotype YYD) and therefore we included it in the analysis. If two or all three alleles were mixed, we have included these also, but it should be noted that there is a possibility that the YYY haplotype did not actually occur within a single genome in these samples. This effectively collapses each isolate into one of two states at each locus.

Pre- and posttreatment genotype data were available at all three *Pfmdr1* loci (86, 184, and 1246) for 47 of those children who failed treatment in the AQ treatment group. Of those, 39 individuals harbored the YYY haplotype. In six of these isolates, mixed alleles were present at more than one of the three loci, but these were kept in the analysis. In 38 of these 39 individuals, the YYY haplotype remained in the treatment failure sample (Table 4). However, among AL treatment failures with available matched genotype data at all three loci, only 16 out of 30 samples that carried YYY at presentation harbored the YYY haplotype after treatment (Table 4). Therefore, children presenting with the 86Y/184Y/1246Y *Pfmdr1* haplotype and treated with AQ were significantly more likely to retain this haplotype if they were found to be parasite positive during posttreatment follow-up than were children

treated with AL (OR, 33.25; 95% CI, 4.17 to 1441; $P < 0.001$). The converse analysis, that is, analysis of the NFD haplotype, did not indicate a difference between the two treatment groups in persistence of infections harboring this genotype. However, as the NFD haplotype was rare in presentation infections, comprising only 2.4% of infections in the random baseline (total $n = 164$), this analysis lacked sufficient power to detect even moderate directional selection. Interestingly, the NFD haplotype was more common among pretreatment isolates of AL patients who later failed treatment (8 out of 49) than among pretreatment isolates of AQ-treated patients who later failed treatment (7 out of 107; OR, 2.79; 95% CI, 0.818 to 9.61; $P = 0.0543$). This is consistent with the single-locus analyses presented above.

Linkage tests. The YYY and NFD haplotypes of *Pfmdr1* may be maintained in the parasite population by linkage disequilibrium. To investigate linkage disequilibrium in the population, pairwise associations between loci were examined in the baseline sample. Loci 86 and 184 of *Pfmdr1* were significantly associated ($n = 165$; OR, 35.8; 95% CI, 6.30 to 201; $P < 0.0001$), as were loci 86 and 1246 ($n = 129$; OR, 11.6; 95% CI, 2.47 to 71.6; $P < 0.0001$), but positions 184 and 1246 were not strongly associated ($n = 127$; OR, 2.83; 95% CI, 0.518 to 14.1; $P = 0.127$). Only samples where single alleles were detected at both loci were included in the test. However, to confirm our estimations of haplotype and linkage analysis, full gene sequence data would be required.

Copy number of *Pfmdr1*. The copy number of *Pfmdr1* was determined in paired samples (day 0, day of treatment failure) from the AQ treatment group ($n = 8$ pairs) and the AL group ($n = 15$ pairs). The estimated gene copy number was close to 1.0 in all isolates tested (data not shown). The method used was adapted from Price et al. (19) for use on a Rotorgene 3000 (Corbett, Australia).

DISCUSSION

In this study, we examined the carriage of alleles of the *Pfmdr1* gene in Tanzanian children with uncomplicated falciparum malaria before and after treatment with AQ or AL. Analysis of within-host selection at each locus and of the survival of specific three-locus haplotypes after treatment provided clear evidence that different *Pfmdr1* alleles were selected in the two treatment groups. Our data suggest that AQ treat-

ment positively selects for parasites harboring the *Pfmdr1* 86Y/184Y/1246Y haplotype, whereas AL treatment selects against such parasites, leading to enhanced survival of parasites with the *Pfmdr1* 86N/184F/1246D haplotype. We note that, as shown by the *msp2* genotype results for the matched pair analysis, each treatment appears to exert its selective effect in different ways; AQ treatment failures are predominantly recrudescence infections in which AQ-tolerant parasites present in the D0 sample have survived treatment, whereas AL treatment failures are predominantly new infections, indicating that while most malaria parasites are cleared by AL, drug-tolerant genotypes have been able to reinfect hosts soon after treatment.

Among AL-treated children, the YYY *Pfmdr1* haplotype tended to be replaced by the NFD haplotype in posttreatment infections, although the latter form of the gene was uncommon in the population as a whole. Our results are consistent with those from a recent study in Zanzibar (25), where the prevalence of 86N increased from 15% at day 0 to 41% at day of failure in children who failed AL treatment, and with the results of Dokomajilar et al. (2), who observed significant accumulation of *Pfmdr1* alleles 86N, 184F, and 1246D among Ugandan children who were parasite positive after treatment with AL. We have extended the findings of these studies with regard to AL-treated cases by examining within-host selection and presenting new evidence that AL selects for the three-locus NFD haplotype. Furthermore, in contrast to the findings of Dokomajilar et al. (2), we found that the *Pfmdr1* alleles carried at baseline were associated with different treatment outcomes in the two treatment groups.

This is the first study to systematically examine the role of *Pfmdr1* haplotypes in AQ treatment failures in vivo. It is clear that multiple genes are responsible for AQ resistance. Previous studies of *Pfcr1* alleles in AQ treatment failures have found an incomplete association with the chloroquine-resistant K76T mutation (8, 11), in that this mutation alone is clearly not sufficient to confer in vivo resistance to AQ. In vitro studies show that the *Pfcr1* codon 72-to-76 SVMNT haplotype is associated with greatly reduced sensitivity to CQ and AQ in cultured parasites (23, 29). In contrast, parasite lines with the "African" *Pfcr1* CQ-resistant haplotype of CVIET show a range of in vitro sensitivities to AQ (23). We hypothesize that in African parasites carrying *Pfcr1* CVIET, such as the majority of isolates studied here, *Pfmdr1* mutations also contribute to AQ resistance.

The prevalence of the 184Y allele of *Pfmdr1* observed in this study is very different from that which we observed in West Africa, where the baseline prevalence of 184Y was 26% (9). The role of the 184 mutation in drug resistance remains unclear, suggesting that the use of CQ has selected different haplotypes of *Pfmdr1* in different locations, depending on the predominant alleles of *Pfcr1* and other genes present in these populations. Analysis of the genetic diversity around the *Pfmdr1* gene may provide evidence that a selective sweep has favored the spread of these genes, as has been shown for the alleles of *Pfdhfr* and *Pfdhps* associated with resistance to SP (22).

The increase in prevalence of alleles 86N, 184F, and 1246D after AL treatment can be explained in a number of ways. First, in vitro studies suggest that those parasites carrying "wild-type" 86N alleles have a higher fitness after removal of the

selective drug pressure of CQ (7). Thus, the increase in prevalence may reflect a rebound in fitter, CQ-sensitive parasites. Second, in AL-treated patients, extended exposure of parasites to lumefantrine after clearance of artemether occurs and this may select for new infections emerging from the liver, with parasites carrying alleles 86N, 184F, and 1246D. The latter hypothesis is supported by in vitro studies demonstrating the increased tolerance of laboratory parasite clones to the related drugs mefloquine and halofantrine, if they carry the 86N allele (4) or the 1034S/1042N/1246D allele (24). If correct, this hypothesis might predict that in African settings where high transmission occurs and reinfection is common and these *Pfmdr1* alleles are circulating, ACT based on AL may not have the success it has enjoyed in Southeast Asia. We and others have found no evidence that copy number of the *Pfmdr1* gene contributes to either AL treatment failure or to AQ resistance, which suggests that sequence alteration rather than amplification is currently the predominant *Pfmdr1*-dependent mechanism of drug evasion in African parasite populations.

A third explanation for our observations for AL-treated patients is that posttreatment parasitemia was caused by recrudescence of parasite subpopulations that were at very low densities in the pretreatment population and thus not identified in the *msp2* PCR genotyping assay. These may be remnants of the starting parasite population that survived the artemether concentrations present in the host in the first few days of treatment, and therefore, these genotypes have not only survived lumefantrine but may also bear a signal of artemisinin selection. Evidence to support this comes from our previous in vivo study of the combination CQ plus artesunate (CQ+AS) (27). Molecular analysis of the transmission potential of different genotypes demonstrated a fourfold reduction in transmissibility to mosquitoes of parasites harboring the 86Y allele compared to those with the 86N allele among children treated with CQ+AS. In contrast, among children receiving CQ monotherapy, the 86Y allele was associated with a 14-fold advantage (6). Thus the artesunate component was responsible for a 56-fold reduction of transmissibility of parasites carrying the 86Y allele compared to those carrying the 86N allele. This is also consistent with in vitro studies that show artemisinin sensitivity increasing in *Pfmdr1* 86Y allele-carrying parasites (4, 21, 24). We therefore hypothesize that part of the selective effect of AL is derived from the artemisinin component, which acts more strongly against parasites carrying *Pfmdr1* 86Y alleles than against those with 86N alleles.

There is now growing evidence for allelic selection after treatment with AL, demonstrating the importance of monitoring this combination closely in high transmission areas. The NFD haplotype at codon positions 86, 184, and 1246, as well as *Pfmdr1* copy number (20), should be considered in future drug resistance studies with AL. Furthermore, we have shown that AQ monotherapy can select for the *Pfmdr1* YYY haplotype at these codons. Thus, AQ and AL exert opposing selective effects, and the possibility of a balanced deployment of AL treatment for clinical malaria in children, together with AQ-containing combinations such as AQ+AS or AQ+SP for cases in older patients, should be considered in areas where AQ remains efficacious, such as the West African Sahel.

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REFERENCES

- Djimde, A., O. K. Doumbo, J. F. Cortese, K. Kayentao, S. Doumbo, Y. Diourte, A. Dicko, X. Z. Su, T. Nomura, D. A. Fidock, T. E. Wellems, C. V. Plowe, and D. Coulibaly. 2001. A molecular marker for chloroquine-resistant falciparum malaria. *N. Engl. J. Med.* **344**:257–263.
- Dokomajilar, C., S. L. Nsohya, B. Greenhouse, P. J. Rosenthal, and G. Dorsey. 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.
- Reference deleted.
- Duraisingh, M. T., P. Jones, I. Sambou, L. von Seidlein, M. Pinder, and D. C. Warhurst. 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.
- Famin, O., and H. Ginsburg. 2002. Differential effects of 4-aminoquinoline-containing antimalarial drugs on hemoglobin digestion in *Plasmodium falciparum*-infected erythrocytes. *Biochem. Pharmacol.* **63**:393–398.
- Hallett, R. L., C. J. Sutherland, N. Alexander, R. Ord, M. Jawara, C. J. Drakeley, M. Pinder, G. Walraven, G. A. T. Targett, and A. Allouche. 2004. Combination therapy counteracts the enhanced transmission of drug-resistant malaria parasites to mosquitoes. *Antimicrob. Agents Chemother.* **48**:3940–3943.
- Hayward, R., K. J. Saliba, and K. Kirk. 2005. *Pfmdr1* mutations associated with chloroquine resistance incur a fitness cost in *Plasmodium falciparum*. *Mol. Microbiol.* **55**:1285–1295.
- Holmgren, G., J. P. Gil, P. M. Ferreira, M. I. Veiga, C. O. Obonyo, and A. Björkman. 2006. Amodiaquine resistant *Plasmodium falciparum* malaria in vivo is associated with selection of *pfcr1* 76T and *pfmdr1* 86Y. *Infect. Genet. Evol.* **6**:309–314.
- Meerman, L., R. Ord, J. T. Bousema, M. van Niekerk, E. Osman, R. Hallett, M. Pinder, G. Walraven, and C. J. Sutherland. 2005. Carriage of chloroquine-resistant parasites and delay of effective treatment increase the risk of severe malaria in Gambian children. *J. Infect. Dis.* **192**:1651–1657.
- Mutabingwa, T. K., D. Anthony, A. Heller, R. Hallett, J. Ahmed, C. Drakeley, B. M. Greenwood, and C. J. M. Whitty. 2005. Amodiaquine alone, amodiaquine+sulfadoxine-pyrimethamine, amodiaquine+artesunate, and artemether-lumefantrine for outpatient treatment of malaria in Tanzanian children: a four-arm randomised effectiveness trial. *Lancet* **365**:1474–1480.
- Ochong, E. O., I. V. van den Broek, K. Keus, and A. Nzila. 2003. Short report: association between chloroquine and amodiaquine resistance and allelic variation in the *Plasmodium falciparum* multiple drug resistance 1 gene and the chloroquine resistance transporter gene in isolates from the upper Nile in southern Sudan. *Am. J. Trop. Med. Hyg.* **69**:184–187.
- Olliaro, P., and P. Mussano. 2003. Amodiaquine for treating malaria. *Cochrane Database Syst. Rev.* **2**:CD000016.
- Olliaro, P. L., and W. R. Taylor. 2004. Developing artemisinin based drug combinations for the treatment of drug resistant falciparum malaria: a review. *J. Postgrad. Med.* **50**:40–44.
- Pearce, R. J., C. Drakeley, D. Chandramohan, F. Mosha, and C. Roper. 2003. Molecular determination of point mutation haplotypes in the dihydrofolate reductase and dihydropteroate synthase of *Plasmodium falciparum* in three districts of northern Tanzania. *Antimicrob. Agents Chemother.* **47**:1347–1354.
- Pickard, A. L., C. Wongsrichanalai, A. Purfield, D. Kamwendo, K. Emery, C. Zalewski, F. Kawamoto, R. Scott Miller, and S. R. Meshnick. 2003. Resistance to antimalarials in Southeast Asia and genetic polymorphisms in *pfmdr1*. *Antimicrob. Agents Chemother.* **47**:2418–2423.
- Plowe, C. V., A. Djimde, M. Bouare, O. Doumbo, and T. E. Wellems. 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.
- Price, R., J. A. Simpson, P. Teja-Isavatharm, M. M. Than, C. Luxemburger, D. G. Heppner, T. Chongsuphajaisiddhi, F. Nosten, and N. J. White. 1999. Pharmacokinetics of mefloquine combined with artesunate in children with acute falciparum malaria. *Antimicrob. Agents Chemother.* **43**:341–346.
- Price, R. N., C. Cassar, A. Brockman, M. Duraisingh, M. van Vugt, N. J. White, F. Nosten, and S. Krishna. 1999. The *pfmdr1* gene is associated with a multidrug-resistant phenotype in *Plasmodium falciparum* from the western border of Thailand. *Antimicrob. Agents Chemother.* **43**:2943–2949.
- Price, R. N., A. C. Uhlemann, A. Brockman, R. McGready, E. Ashley, L. Phaipun, R. Patel, K. Laing, S. Looareesuwan, N. J. White, F. Nosten, and S. Krishna. 2004. Mefloquine resistance in *Plasmodium falciparum* and increased *pfmdr1* gene copy number. *Lancet* **364**:438–447.
- Price, R. N., A. C. Uhlemann, M. van Vugt, A. Brockman, R. Hutagalung, S. Nair, D. Nash, P. Singhasivanon, T. J. C. Anderson, S. Krishna, N. J. White, and F. Nosten. 2006. Molecular and pharmacological determinants of the therapeutic response to artemether-lumefantrine in multidrug-resistant *Plasmodium falciparum* malaria. *Clin. Infect. Dis.* **42**:1570–1577.
- Reed, M. B., K. J. Saliba, S. R. Caruana, K. Kirk, and A. F. Cowman. 2000. *Pgh1* modulates sensitivity and resistance to multiple antimalarials in *Plasmodium falciparum*. *Nature* **403**:906–909.
- Roper, C., R. Pearce, S. Nair, B. Sharp, F. Nosten, and T. Anderson. 2004. Intercontinental spread of pyrimethamine-resistant malaria. *Science* **305**:1124.
- Sidhu, A. B., D. Verdier-Pinard, and D. A. Fidock. 2002. Chloroquine resistance in *Plasmodium falciparum* malaria parasites conferred by *pfcr1* mutations. *Science* **298**:210–213.
- Sidhu, A. B., S. G. Valderramos, and D. A. Fidock. 2005. *Pfmdr1* mutations contribute to quinine resistance and enhance mefloquine and artemisinin sensitivity in *Plasmodium falciparum*. *Mol. Microbiol.* **57**:913–926.
- Sisowath, C., J. Strömberg, A. Mårtensson, M. Mselle, C. Obondo, A. Björkman, and J. P. Gil. 2005. In vivo selection of *Plasmodium falciparum* *pfmdr1* 86N coding alleles by artemether-lumefantrine (Coartem). *J. Infect. Dis.* **191**:1014–1017.
- Snounou, G., X. Zhu, N. Siripoon, W. Jarra, S. Thaithong, K. N. Brown, and S. Viriyakosol. 1999. Biased distribution of *msp1* and *msp2* allelic variants in *Plasmodium falciparum* populations in Thailand. *Trans. R. Soc. Trop. Med. Hyg.* **93**:369–374.
- Sutherland, C. J., C. J. Drakeley, U. Obisike, R. Coleman, M. Jawara, G. A. T. Targett, P. Milligan, M. Pinder, and G. Walraven. 2003. The addition of artesunate to chloroquine for the treatment of *Plasmodium falciparum* malaria in Gambian children delays, but does not prevent treatment failure. *Am. J. Trop. Med. Hyg.* **69**:19–25.
- Sutherland, C. J., R. Ord, S. Dunyo, M. Jawara, C. J. Drakeley, N. Alexander, R. Coleman, M. Pinder, G. Walraven, and G. A. T. Targett. 2005. Reduction of malaria transmission to Anopheles mosquitoes with a six-dose regimen of co-artemether. *PLoS Med.* **2**:e92.
- Warhurst, D. C. 2003. Polymorphism in the *Plasmodium falciparum* chloroquine-resistance transporter protein links verapamil enhancement of chloroquine sensitivity with the clinical efficacy of amodiaquine. *Malaria J.* **2**:31.
- White, N. 1999. Antimalarial drug resistance and combination chemotherapy. *Philos. Trans. R. Soc. Lond. B.* **354**:739–749.
- World Health Organization. 2001. Antimalarial drug combination therapy: report of a WHO technical consultation. WHO, Geneva, Switzerland. www.rbm.who.int/cmc_upload/0/000/015/082/use_of_antimalarials2.