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## Intrahost Selection of *Plasmodium falciparum* *pfmdr1* Alleles after Antimalarial Treatment on the Northwestern Border of Thailand

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### Abstract

**Background**—Increased *pfmdr1* copy number is associated with reduced susceptibility to structurally unrelated antimalarial drugs. We assessed how administration of different antimalarial drugs altered *pfmdr1* polymorphism in parasites from patients who experienced treatment failure.

**Methods**—In studies conducted on the northwestern border of Thailand, amplifications and single-nucleotide polymorphisms in *pfmdr1* were compared before and after antimalarial drug treatment.

**Results**—Intrahost changes in *pfmdr1* copy number were observed in 20% (26/132) of patients with recurrent infections. Among infections that recrudesced after mefloquine-containing regimens, increases in *pfmdr1* copy number occurred in 68% (95% confidence interval [CI], 46%–85%), and decreases occurred in 2% (95% CI, 0.4%–11%) of isolates; corresponding proportions after artemether-lumefantrine were 25% (2/8) and 11% (2/19); after quinine, 50% (1/2) and 40% (4/10); and after artemisinins alone, 0% (0/10) and 19% (3/16) of isolates (overall  $P < .001$ ).

**Conclusions**—Intrahost selection based on *pfmdr1* copy number occurs frequently in parasite populations within individual patients. Amplification confers multidrug resistance but probably imposes a significant fitness cost to the parasites.

Antimalarial drug resistance poses a major threat to the control of malaria. In *Plasmodium falciparum* infection, resistance has evolved to all classes of antimalarial drugs with the exception of the artemisinin derivatives. Resistance mechanisms underlying this mainly involve mutations in the genes encoding target enzymes or transporters [1, 2]. Despite tight

control of its use, significant resistance to mefloquine (MFQ) emerged within 6 years after it was first deployed in Thailand in 1984 [3]. MFQ resistance is largely mediated by a P-glycoprotein pump (Pgh), which is encoded by the *pfmdr1* gene [4, 5] on chromosome 5. Pgh affects the intraparasitic concentrations of several important antimalarial drugs. Point mutations in *pfmdr1* are associated with resistance to chloroquine and are negatively associated with resistance to MFQ [6, 7]. Amplification of wild-type *pfmdr1* is associated with reduced susceptibility in vitro to structurally unrelated antimalarial drugs, such as MFQ, artesunate (ART), lumefantrine, and quinine (QU) [5, 8]. In vivo, increased *pfmdr1* copy number accounts for more than half of the treatment failures after MFQ, MFQ plus artesunate (MAS), and artemether-lumefantrine (AL) [5, 8].

Over the past 20 years, prospective studies of antimalarial drug efficacy have been conducted on the northwestern border of Thailand. We have utilized samples taken during these studies from patients who experienced treatment failure to investigate how administration of different antimalarial drugs selects for point mutations and amplification in *pfmdr1*.

## SUBJECTS, MATERIALS, AND METHODS

### Study sites

From December 1990 to August 2002, patients with *P. falciparum* infection were recruited into a series of large chemotherapeutic studies conducted by the Shoklo Malaria Research Unit (SMRU) [9-17]. The majority of patients in these investigations were part of clinical trials of antimalarial drugs, whereas the remainder were treated in SMRU outpatient clinics and identified on presentation with clinical symptoms. Patients came from 3 sites in Tak Province on the northwestern border of Thailand: Maela, Shoklo (both camps for displaced Karen people; 60 and 120 km to the north of the Thai provincial town of Mae Sot, respectively), and Mawker Tai (an area hosting migrant workers from neighboring Burma; 60 km to the south of Mae Sot). The area is one of low and unstable transmission of *P. falciparum* and *P. vivax* malaria [18, 19]. All *P. falciparum* and most *P. vivax* infections are symptomatic. Patients were included in these trials on the condition that they or their accompanying relatives provided fully informed consent. The project was part of clinical drug studies that were individually approved by the ethics committees of the Faculty of Tropical Medicine (Mahidol University, Bangkok), the Karen Refugee Committee (Mae Sot), and the Oxford Tropical Research Ethics Committee (United Kingdom). Molecular studies were approved by the Wandsworth Local Research Ethics Committee (United Kingdom).

### Drug treatments

Antimalarial treatment was classified into 5 groups: MFQ monotherapy (25 mg/kg) (the MFQ group), MFQ (25 mg/kg) plus 3 days of artesunate (12 mg/kg) (the MAS3 group), AL (4 or 6 doses) (the AL group), ART-based monotherapy with an ART derivative (artemether or artesunate 12 mg/kg over 7 days) (the ART group), and QU with or without doxycycline over 7 days (the QU group).

### Collection of blood samples

Fingerprick blood samples were obtained from patients with acute, uncomplicated falciparum malaria. Cases of malaria were confirmed by microscopy, and individuals were treated with a variety of antimalarial regimens depending on age, pregnancy status, and random allocation in the various chemotherapy trials at the time of recruitment. A small quantity (30  $\mu$ L) of whole blood from these patients was spotted onto 3MM filter paper (Whatman), airdried, and stored in plastic bags at room temperature until DNA extraction. Parasite counts were measured by microscopy on presentation with symptoms and then assessed daily until negative and weekly until day 42 or 63 in nonpregnant patients or for the entire length of gestation in pregnant women. A second blood spot was obtained from those patients who became slide positive again within the follow-up period or who presented at a later date to one of the SMRU clinics. Primary and recurrent infection sample pairs were selected randomly from the 5 treatment groups. For cases in which there were both recrudescences and new infections, equal numbers of both were chosen.

### Genotyping of *P. falciparum* by polymerase chain reaction (PCR)

Genetic comparison of *P. falciparum* in acute and recurrent infections was performed at the SMRU laboratory based in Mae Sot. Parasite DNA was extracted from blood spots using PrepMan Ultra (Applied Biosystems) or the saponin lysis/chelex extraction method [5]. Three genetic loci, which exhibit repeat-number polymorphisms, were amplified by nested PCR from the DNA of each isolate. These were the genes encoding the merozoite surface proteins 1 and 2 and the gene encoding the glutamate rich protein, located on chromosomes 9, 2, and 10, respectively. Primers and amplification conditions for all 3 loci have been described elsewhere [20]. PCR products were detected by electrophoresis of 8  $\mu$ L from each reaction on 1.7% agarose gels. The PCR products were visualized by ethidium-bromide staining and UV fluorescence and were sized against a 100-bp molecular-weight marker (Life Technologies). Clonality of infection was determined according to multiple banding at any 1 of the 3 loci assessed.

### Quantitation of *pfmdr1* copy number and detection of polymorphisms

*pfmdr1* copy number was assessed by quantitative PCR (ABI Sequence Detector 7700; Applied Biosystems) using the methods described elsewhere [5]. All reactions were performed in triplicate and were rejected if they did not conform to exponential kinetics. Copy number is reported as a continuous variable. Previous studies defined the repeatability coefficient of the copy number assay as 0.64 (viz., 95% of repeated estimates of *pfmdr1* copy number would be expected to be within  $\pm 0.32$  of the first). Single-nucleotide polymorphisms (SNPs) in *pfmdr1* and *pfprt* were detected by nested PCR–restriction fragment–length polymorphism methods, also as described elsewhere [21, 22]. All PCRs and digests included, as positive and negative controls, DNA of laboratory strains 3D7, HB3, 7G8, and Dd2, respectively.

### Statistical analyses

Data were analyzed using SPSS for Windows (version 14; SPSS). The Mann-Whitney *U* test or the Kruskal-Wallis method was used for nonparametric comparisons, and Student's *t* test

or 1-way analysis of variance was used for parametric comparisons. For categorical variables, percentages and corresponding 95% confidence intervals (CIs) were calculated using Wilson's method. Proportions were examined using the  $\chi^2$  test with Yates's correction or Fisher's exact test. Paired proportions were compared using McNemar's test. The relationship between the copy numbers of the pre- and posttreatment isolates was assessed on ordinal variables by rounding copy numbers to the nearest integer. For polyclonal infections, the assessment of copy number generates a figure representing an average of the copy numbers of genes in individual parasite clones weighted by their respective proportions. Therefore, to determine the variation in copy number attributable to the selection of resistant parasites from polyclonal infections, we also assessed pre- and posttreatment isolates' copy number as a continuous variable. Linear regression analysis was used to determine the relationship between the initial copy number and the change between the copy number of the pre- and posttreatment isolates.

## RESULTS

Between December 1992 and June 2002, a total of 1289 paired isolates were identified from patients whose parasitemia reappeared within the follow-up period. In total, 230 pairs (18%) were subjected to molecular analysis of *pfmdr1* and genotyping of merozoite antigens (table 1), of which 79 (34%) came from pregnant women (29 after MFQ, 33 after ART, and 17 after QU). *pfmdr1* copy number was successfully determined in 199 pairs (87%), and SNPs in 169 (73%); these proportions did not differ significantly between treatment groups or between pregnant and nonpregnant patients. Polyclonal infections were detected for 73 (32%) pretreatment isolates and for 52 (23%) posttreatment isolates ( $P = .04$ ).

### *pfmdr1* copy number

After MAS3 treatment, increased copy number in the primary isolate was significantly associated with PCR-confirmed recrudescences; PCR-confirmed recrudescences occurred for 81% (26/32) of the primary isolates with an increased *pfmdr1* copy number, compared with 44% (10/23) for those with single copies (relative risk, 1.9 [95% CI, 1.1–3.1];  $P = .009$ ).

### PCR-confirmed recrudescences

Among the 151 paired isolates from patients with PCR-confirmed recrudescences, copy number could be assessed in both the primary and the recurrent infection isolates in 132, of which 93 (70%) had a primary isolate with a single *pfmdr1* copy. Detection of intrahost changes in copy number were frequent, occurring in 52% (69/132) of patients, of which 41 (59%) were increases in copy number, and 28 (41%) were decreases (figure 1). In total, amplification from a single to >1 copy number occurred in 12% (16/132) of patients, and deamplification from multiple to a single copy number occurred in 7.5% (10/132). The change in copy number from the pre- to posttreatment isolate differed significantly between treatment groups (table 2). Although 68% (13/19) of isolates from patients with recrudescence infections after MFQ or MAS3 had an increase in *pfmdr1* copy number, this occurred in only 25% (2/8) of patients after AL ( $P = .09$ ) and in 0% (0/10) of patients after ART ( $P < .001$ ). These proportions were similar when selecting only monoclonal infections.

There was also a significant difference between treatment groups in the rates at which recurrent isolates showed a *decrease* in *pfmdr1* copy number, although by definition this analysis was confined to pairs in which the primary isolate had an increased *pfmdr1* copy number. Only 2% (1/48) of isolates from patients experiencing treatment failure after MFQ or MAS3 deamplified from multiple to a single copy number, compared with 19% (3/16) after ART ( $P = .045$ ), 40% (4/10) after QU ( $P = .002$ ), and 11% (2/19) after AL ( $P = .19$ ).

When copy number was quantified as a continuous variable, the mean change between the primary and recurrent isolate was +0.58 (95% CI, 0.1–1.04) after MAS3, +0.46 (95% CI, 0.09–0.83) after AL, +0.32 (95% CI, 0.01–0.66) after MFQ, –0.16 (95% CI, –0.45 to 0.13) after ART, and –0.46 (95% CI, –0.97 to 0.05) after QU (overall  $P = .029$ ). The change in copy number was correlated with the copy number of the primary isolate ( $R_s = -0.48$ ;  $P < .001$ ), and this was apparent for each treatment group (figure 2). Hence, isolates with a low pretreatment copy number had the greatest increase after recrudescence, whereas those starting with a high copy number were more likely to decrease. In a linear regression model, there was no significant difference in the change in copy number between MFQ and MAS3 nor between ART and QU. After combining treatment groups with long terminal elimination half-life (MFQ + MAS3), there was a significant difference in the linear regression model with treatment regimens that were eliminated rapidly (ART + QU) ( $P < .001$ ).

In patients treated with MFQ or MAS3, the change in copy number between primary and recurrent isolates was correlated with the time to recrudescence ( $R = 0.3$ ;  $P = .01$ ); hence, infections with a low copy number in the primary isolate had the greatest increase after recrudescence, whereas those infections starting with a high copy number were more likely to decrease. However, patients with an initially higher copy number also tended to recrudescence earlier ( $R = -0.3$ ;  $P = .01$ ). After controlling for the copy number of the initial isolate, there was no effect of the change in copy number on the time to recrudescence, age of the patient, clonality of infection, or initial parasitemia.

### New infections

Of the 55 paired isolates from reinfections, copy number could be assessed in both primary and recurrent isolates in 48 (87%) (table 3). Overall, 70% (19/27) of patients with isolates with a single *pfmdr1* copy number were reinfected with isolates with an increased copy number, compared with 19% (4/21) of patients with isolates with increased copy number being reinfected with isolates with a single copy number ( $P = .003$ ). However, after stratifying by treatment group, individual group numbers were small, and the difference did not reach significance.

### SNPs

*pfmdr1* SNPs were detected at codon 86 in 2.4% (4/169) of pretreatment isolates, at codon 184 in 32% (39/121), at codon 1034 in 0.6% (1/169), and at codon 1042 in 8.9% (15/169). After MAS3, all (10/10) of the 1042D alleles present in the pretreatment isolate had reverted to 1042N (wild type), whereas the reverse process (1042N to 1042D) occurred only in 2.6% (1/39) ( $P = .01$ ) (figure 3). No significant difference was observed after any of the other treatments or for SNPs at codons 86, 184, or 1034.

## DISCUSSION

Our results demonstrate that *pfmdr1* copy number often changes during the course of a recurrent infection after antimalarial therapy. Overall, 54% (38/71) of primary isolates with a single *pfmdr1* copy number had an increased copy number in the recurrent isolate, and this figure did not differ when selecting only single clone infections according to 3-loci genotyping. There are 3 possible explanations for these findings. First, there could be expansion of an existing subpopulation with *pfmdr1* amplification either present in a multiclonal infection or at undetectable levels in a monoclonal primary infection. Second, an infection could be newly acquired in the presence of declining concentrations of antimalarial drugs. Third, there could be de novo amplification and subsequent growth of a resistant parasite population. It was not possible to quantify the parasite subpopulations within apparently clonal infections, and, therefore, we are unable to determine the exact frequency or distribution of de novo *pfmdr1* amplification from the current data. Amplification could have occurred through unequal crossover at either mitosis or meiosis in the mosquito vector, but the ability to select this in vitro [23, 24], the high frequency of changes in *pfmdr1* copy number observed within patients, and recent genetic data analyzing amplicon size, sequence, and break points strongly suggest that, in the human host, amplification events occur relatively frequently and do so during mitotic DNA replication in the blood-stage infection. In the presence of appropriate drug pressure favoring survival of the amplified genotype, increased copy number will, therefore, be readily selected.

Drug pressure is largely determined by the elimination half-life of the antimalarial drug used [25], which, in this study, varied considerably, with average values of 1 h for the active metabolite of artesunate, 16 h for QU, 4 days for lumefantrine, and 14 days for MFQ. Although increased *pfmdr1* copy number is associated with reduced susceptibility to ARTs [5], QU, and lumefantrine [8], when recrudescences followed these rapidly eliminated drugs, amplification was uncommon. In fact, if the primary isolate already had increased *pfmdr1* copy number, deamplification to a single copy predominated. This suggests that, in the absence of the drug-derived selective pressure, there is a parasite fitness disadvantage to having increased *pfmdr1* copy number and that this competitive growth disadvantage is sufficient for these unfit parasites to be outgrown by their fitter rivals in 8–12 asexual cycles (the time between elimination of the drug and recrudescence of the parasite).

In our previous studies, we have demonstrated that increased *pfmdr1* copy number is the major determinant of treatment response to MFQ as well as to MAS3. These observations were confirmed in recent laboratory studies, which demonstrated a key role of *pfmdr1* copy number and expression in determining MFQ susceptibility in vitro [26]. In the present study, we highlight that gene amplification is selected very commonly in patients treated with either regimen. Overall recrudescence rates after MAS3 have averaged 5%, of which 70% (i.e., net 3.5%) showed amplification [5]. However, this unstable trait conferring a survival advantage in the presence of a drug is readily lost when the selection pressure is removed, even in a single host.

Recent studies from Africa have demonstrated high rates of reversion of SNPs to wild-type *pfmdr1* in recurrent isolates after AL therapy [27–29]. This generally represents selection of

newly acquired infections in the presence of residual drug, rather than de novo mutations in recrudescence infections. The N86Y and N1042D SNPs of *pfmdr1* are associated with decreased susceptibility to chloroquine but increased susceptibility to lumefantrine, MFQ, and artesunate [8, 22]. In Thailand, we observed an increase in the prevalence of the 1042N wild-type allele after MFQ and MAS3 therapy, although this was not apparent for any of the other drugs or SNPs tested. We also observed an increase in the prevalence of *pfmdr1* amplification after reinfection following treatment with MAS3 (32%–63%) and AL (59%–88%), but numbers were small, and this increase did not reach significance.

Among field isolates, *pfmdr1* amplification occurs almost exclusively in isolates with the wild-type allele at codons 86, 1034, and 1042 [5]. Hence, reversion to 86N and 1042N may represent the first stage of the process leading to *pfmdr1* amplification in the field. Once present, clinical resistance is associated with increased gametocyte carriage and, thus, increased transmissibility [30], which, under appropriate drug pressure, translates into a survival advantage for parasites with increased *pfmdr1* copy number, fueling the spread of antimalarial drug resistance. The force that drives this selection is determined by the relative treatment failure rate and consequent transmission advantage of the resistant over the sensitive genotype. Although increased *pfmdr1* copy number confers a major selective advantage when MFQ alone is used for treatment (cure rates decrease from almost 100% to 50%), when MAS is given, the advantage is lessened as efficacy remains high. This reduces the differential survival advantage and, thus, the force driving the spread of resistance. The greatest increment in MFQ resistance is an increase from 1 to 2 copies, which is associated with a 2.4-fold decrease (95% CI, 1.3–2.5-fold decrease) in in vitro susceptibility [5]. Further increments confer proportionally less resistance but may incur further fitness costs. This probably explains why MFQ resistance has stabilized in this area over the past 10 years [31, 32]. A state of relative equilibrium has been reached in which the survival advantage of *pfmdr1* amplification is balanced by its fitness disadvantage.

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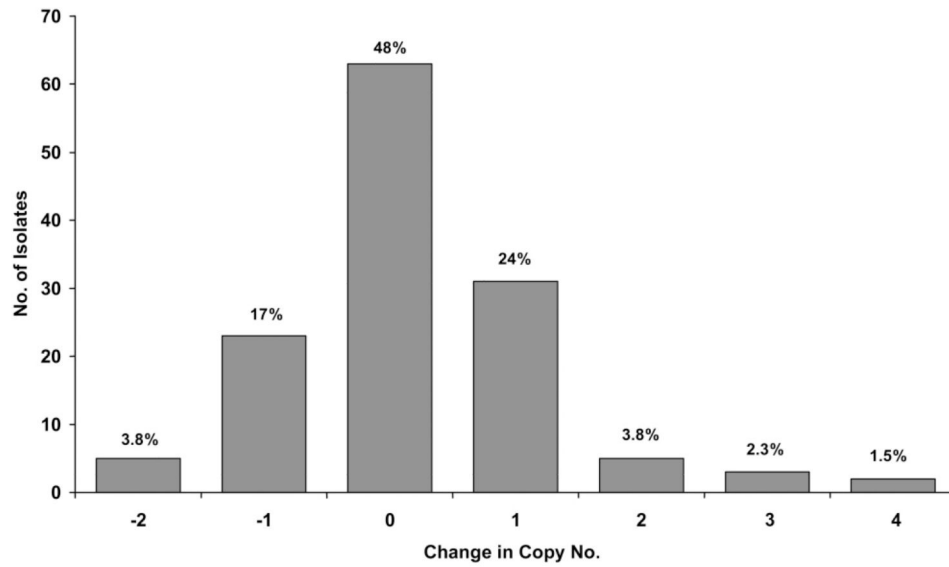
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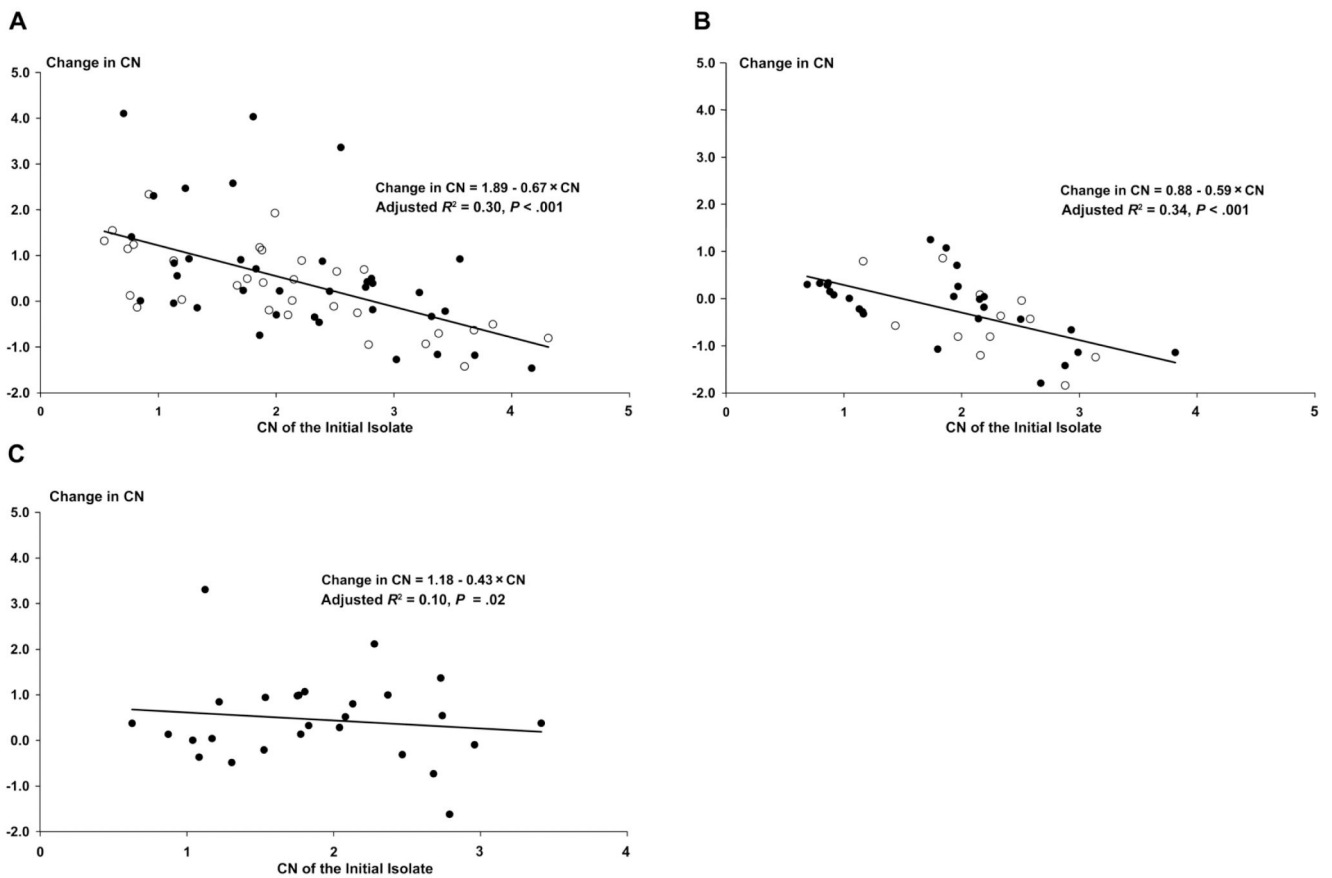
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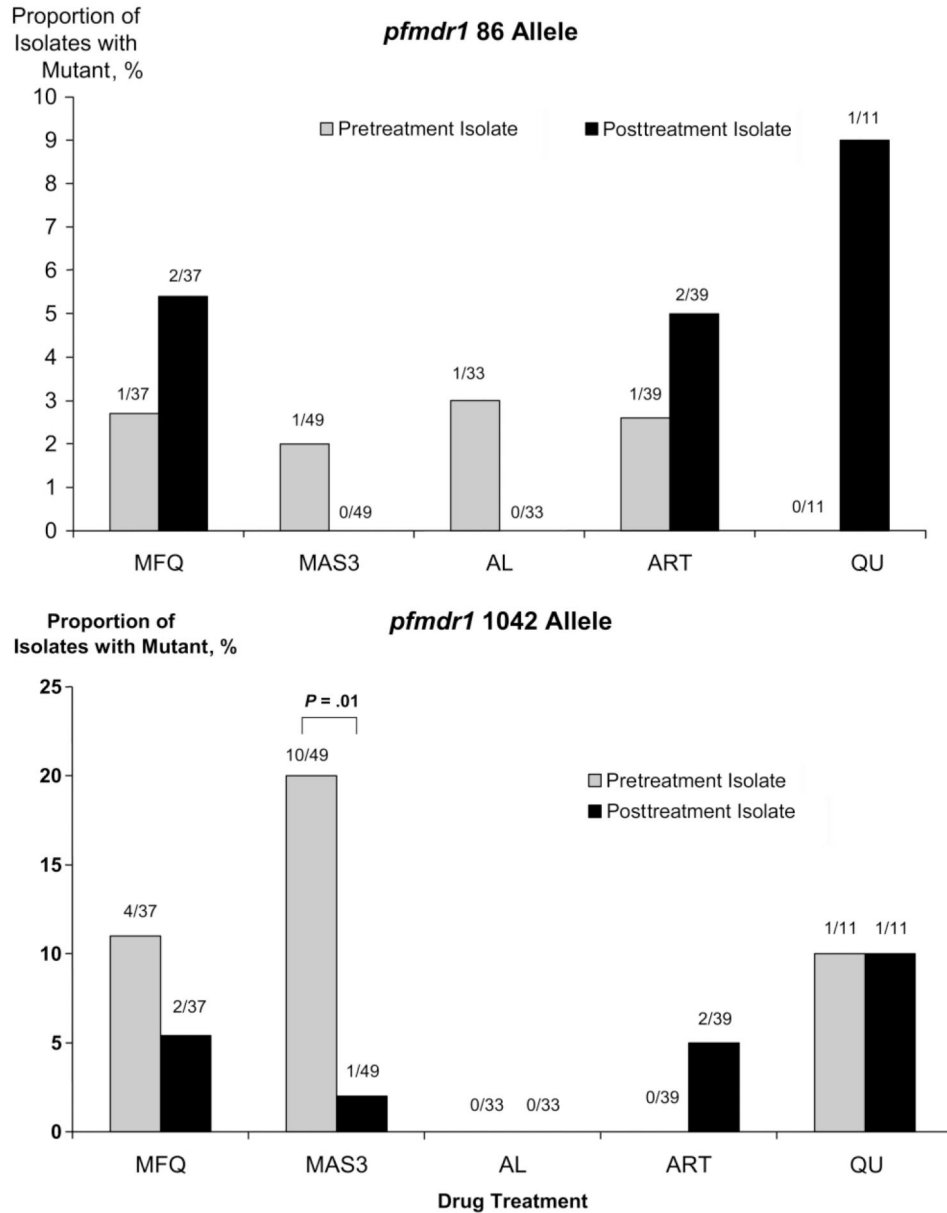
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**Figure 1.** Histogram of change in copy no. (rounded to nearest integer) between the posttreatment isolate and the pretreatment isolate (shown as the former minus the latter), in polymerase chain reaction–confirmed recrudescence infections ( $n = 132$ ).



**Figure 2.** Change in copy no. (CN; posttreatment isolate minus pretreatment isolate) in polymerase chain reaction–confirmed recrudescence infections. *A*, Mefloquine (*white circles*) and mefloquine plus 3 days of artesunate (*black circles*). *B*, Artemisinin monotherapy (*white circles*) and quinine (*black circles*). *C*, Artemether-lumefantrine.



**Figure 3.**

Proportion of isolates with mutant *pfmdr1* alleles in pre- and posttreatment isolates after antimalarial treatment (including reinfections and recrudescences). AL, artemether-lumefantrine; ART, artemisinin monotherapy; MAS3, mefloquine plus 3 days of artesunate; MFQ, mefloquine monotherapy; QU, quinine.

**Table 1**  
**Classification according to 3-loci genotyping (merozoite surface proteins 1 and 2 and the gene encoding the glutamate rich protein) of the 230 parasite isolates subjected to *pfmdrl* genotyping in the 5 treatment groups**

<b>Total</b>	<b>Mefloquine</b>	<b>Mefloquine plus artesunate</b>	<b>Artemether-lumefantrine</b>	<b>Artemisinin monotherapy<sup>a</sup></b>	<b>Quinine</b>	<b>Total</b>
Novel infections	1	21	18	14	1	55
Recrudescence	33	40	32	29	17	151
Recrudescence + novel infections	5	6	0	4	0	15
Indeterminate	3	5	0	1	0	9
<b>Total</b>	<b>42</b>	<b>72</b>	<b>50</b>	<b>48</b>	<b>18</b>	<b>230</b>

<sup>a</sup> Artesunate or artemether at 12 mg/kg over 7 days.

**Table 2**  
**Change in *pfmdrl* copy no. (CN) between pretreatment and posttreatment isolates after polymerase chain reaction–confirmed recrudescence**

Pretreatment CN, posttreatment CN	Mefloquine	Mefloquine plus artesunate	Artemether-lumefantrine	Artemisinin monotherapy <sup>a</sup>	Quinine
Single					
Single	3 (33)	3 (30)	6 (75)	10 (100)	1 (50)
Increased	6 (67)	7 (70)	2 (25)	0 (0)	1 (50)
Total	9	10	8	10	2
Increased					
Increased	22 (100)	25 (96)	17 (89)	13 (81)	6 (60)
Single	0 (0)	1 (3.8)	2 (11)	3 (19)	4 (40)
Total	22	26	19	16	10

NOTE. Data are no. (%) of isolates.

<sup>a</sup> Artesunate or artemether at 12 mg/kg over 7 days.

**Table 3**  
**Population prevalence of copy no. in the pretreatment and reinfecting (posttreatment) isolates**

Category	Mefloquine	Mefloquine plus artesunate <sup>a</sup>	Artemether-lumefantrine <sup>b</sup>	Artemisinin monotherapy <sup>c,d</sup>
Pretreatment				
Single	1 (100)	13 (68)	7 (41)	6 (55)
Increased	0 (0)	6 (32)	10 (59)	5 (45)
New infections				
Single	0 (0)	7 (37)	2 (12)	3 (27)
Increased	1 (0)	12 (63)	15 (88)	8 (73)
Total	1	19	17	11

*NOTE.* Data are no. (%) of isolates. Paired proportions for each treatment group were compared using McNemar's test to determine the rates of change in copy no. (single to amplified vs. amplified to single).

<sup>a</sup>P = .15.

<sup>b</sup>P = .06.

<sup>c</sup>P = .38.

<sup>d</sup> Artesunate or artemether at 12 mg/kg over 7 days.