Corrected version of the paper. Corrections highlighted

Polymorphisms in *pfcrt* and *pfmdr1*: parasite risk factors that affect treatment outcomes for falciparum malaria after artemether-lumefantrine and artesunate-amodiaquine

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Abstract

Adequate clinical and parasitological cure by Artemisinin Combination Therapies (ACTs) relies on both the artemisinin component and the partner drug. Polymorphisms in *pfcrt* and *pfmdr1* are associated with decreased sensitivity to amodiaquine and lumefantrine, but effects of these polymorphisms on therapeutic responses to artesunate-amodiaquine and artemether-lumefantrine have not been clearly defined. Individual patient data from 30 clinical trials were harmonized and pooled, using standardized methodology from the WorldWide Antimalarial Resistance Network. Data from more than 7000 patients were analyzed to assess relationships between parasite polymorphisms in *pfcrt* and *pfmdr1* and clinically relevant outcomes after treatment with AL or ASAQ. Presence of *pfmdr1* N86 was a significant risk factors for recrudescence in patients treated with AL, but elevated *pfmdr1* copy number was not. AL and ASAQ exerted opposing selective effects on SNPs in *pfcrt* and *pfmdr1*. Monitoring selection and responding to emerging signs of drug resistance are critical tools for preserving ACT efficacy.

Introduction

Recent successes in malaria control have depended on the use of highly efficacious artemisinin combination therapies (ACTs) for first-line treatment of uncomplicated *Plasmodium falciparum* malaria. Adequate clinical and parasitological cure by ACTs relies on the rapid reduction in parasite biomass by the potent, short-acting artemisinin component[1-3] and the subsequent elimination of residual parasites by the longer-acting partner drug. The two most commonly used ACTs worldwide are artemether-lumefantrine (AL) and artesunate-amodiaquine (ASAQ).[4] PCR-adjusted efficacy for both combinations remains high in most regions.[5-7] However, there have been some reports of decreasing AL cure rates in Africa[8-11] and Asia[12], and reports of high levels of treatment failures of ASAQ.[13-18] Resistance to ACT partner drugs has historically manifested before that of artemisinins, whose short half-lives result in the exposure of residual parasites to sub-therapeutic levels of the partner drug alone. Response to the partner drug is therefore a key component of overall ACT efficacy.

Mutations in the gene encoding the *P. falciparum* chloroquine resistance transporter (*Pfcrt*) are associated with chloroquine resistance[19]; a change from lysine to threonine at codon 76 in *pfcrt* predicts responses of parasites to chloroquine.[20, 21] In the presence of *pfcrt* 76T, chloroquine resistance is modulated by point mutations in the gene that encodes the *P. falciparum* multi drug resistance transporter 1 (*pfmdr1*), primarily at codon 86 [22, 23] and also by mutations at positions 1034, 1042 and 1246. [24] Decreased susceptibility to lumefantrine has been linked to polymorphisms in these two genes.[25-35] Elevated *pfmdr1* copy number, which confers resistance to mefloquine[36], has also been associated with reduced susceptibility to lumefantrine.[37-40]

Studies of amodiaquine have demonstrated reduced *in vivo* response [41-43] and increased IC_{50} values *in vitro*, in association with the presence of both *pfmdr1* 86Y and *pfcrt* 76T alleles.[44,45] Selection of these alleles in recurrent parasites after treatment with amodiaquine alone or in combination with artesunate has been observed in a number of studies.[28,46-51] It has also been suggested that parasites that carry chloroquine-resistant *pfmdr1* alleles may be more susceptible to artesunate in classical in *vitro* assays,[24,52] an effect that could counteract the increased risk of amodiaquine failure when these drugs are combined in ASAQ.

Currently both AL and ASAQ retain high clinical efficacy with few recrudescent infections, and individual studies generally lack sufficient statistical power to assess the association between parasite genotypes and outcomes of clinical treatment. Such an assessment is a critical step in validating molecular changes in parasite populations as useful markers of early signs of changing parasite susceptibility to lumefantrine or amodiaquine. To overcome these challenges, individual patient data on *in vivo* antimalarial efficacy and molecular markers of *P. falciparum* from 30 clinical trials were standardized, pooled, and nearly 7,000 patient responses were analyzed to determine whether patients infected with parasites that carry these polymorphisms are at increased risk of treatment failure. This large data set also provided the opportunity to examine the effects of AL and ASAQ treatment on selection in parasites of particular alleles of *pfcrt* and *pfmdr1*.

Methods

Selection and inclusion of data

Prospective clinical efficacy studies of *P. falciparum* treatment with AL (6 dose regimen) or ASAQ (3 day fixed dose or loose/co-blistered regimen) with a minimum of 28 days of follow up and genotyping of *pfcrt* and/or *pfmdr1* were sought for the analysis. Studies were identified by a

systematic PubMed literature review using the search terms (artesunate AND amodiaquine) OR (artemether AND lumefantrine) OR (ACT) AND (*pfmdr1* OR *pfcrt).* Abstracts and text were screened to determine whether inclusion criteria were met. Unpublished datasets were also solicited and included in the analysis. Individual anonymized patient data including baseline characteristics, drug intake, parasite density and temperature were collected. All but one study included parasite genotyping to identify recrudescent infections of *P. falciparum*, and all studies assessed the presence of *pfcrt* and/or *pfmdr1* polymorphisms (single nucleotide polymorphisms (SNPs) and copy number variation) in parasites isolated from patients on day 0. Multiplicity of infection and molecular resistance marker data from other days including the day of microscopic recurrent parasitemia were included but were not a prerequisite for study inclusion. Metadata on study location, study design, drugs, and dosing regimens were also gathered. Figure 1 is a schematic of the patient numbers and overall flow of the study.

Data curation and generation of variables

All data sets were uploaded to the WWARN repository and standardized using the WWARN Data Management and Statistical Analysis Plans (DMSAP).[53,54] Outcome status and censoring were defined according to the Clinical DMSAP.[53] Parasites that recurred within the follow-up period were classified using WHO guidelines[55]: microscopically-detected infections during follow-up were classified as 'recurrent'; recurrent infections sharing with blood samples taken at day 0 PCR bands in polymorphic merozoite antigens or microsatellite fragment sizes were termed 'recrudescent', and recurrent infections not sharing PCR bands or microsatellite fragment sizes with blood samples taken at day 0 were classified as 're-infections' (new infections). Molecular markers were coded as either single or mixed allele genotypes in the case of SNPs and as mean copy number per sample for copy number polymorphisms. Multi-SNP haplotypes were reconstructed as described in the Molecular DMSAP.[56,57]

Statistical analysis

All statistical analyses were conducted using Stata 11 (Stata Corp, College Station, Texas). The primary endpoint was clinical efficacy, defined as the PCR-adjusted risk of *P. falciparum* recrudescent infections. The cumulative risk of recrudescence at day 28 and day 42 was computed using survival analysis [Kaplan-Meier estimates (K-M)]. Comparisons of K-M survival curves were performed using log rank tests stratified by study sites.

Multivariable analysis of risk factors associated with PCR-adjusted recrudescence was conducted using Cox proportional hazards regression models with shared frailty parameters to adjust for site-specific effects. The risk factors that affect the clinical efficacy of AL and ASAQ have been intensively studied in pooled analyses of both ACTs. Sixty- two studies with 14,651 patients treated with AL and 39 studies including 8,337 patients treated with ASAQ were analyzed; these full analyses have been submitted for publication. The univariable and multivariable risk factors identified in those studies are shown in supplementary tables S1A and S1B. Clinical covariates in the current study were included based on the previous analyses as follows: (lumefantrine or amodiaquine dose, enrolment parasitemia, age category, and ASAQ fixed or co-blistered versus loose formulation (Table 1). Each molecular marker was then added to the model. The proportional hazard assumption was tested based on Schoenfeld residuals [58]. In the case of non-proportionality, interactions with a categorized time variable based on clinical follow-up intervals (< day 14, days 14 – 21, 21 – 28, and > day 28) were used to account for changing effects over time, and neighboring windows with similar effects of genetic covariates as determined by Wald test were

merged. Finally, other covariates (transmission intensity, region of sample origin, dose supervision, and fat intake) were included in the model if they improved model fit based on the likelihood ratio test. Multiplicity of infection was only available for 197 and 141 AL and ASAQ patients, respectively, and was excluded from further analysis. The final model was then used to estimate the adjusted hazard ratio for recrudescence in patients who carried parasites with resistant versus sensitive genotypes on day 0. The assumption of proportional hazards was tested separately for the individual covariates in the final multivariable model, and any violations were reported.

In patients who had recurrent parasitemia on or before day 42, changes in *pfcrt* and *pfmdr1* alleles between pre- and post-treatment matched pairs of samples was compared using McNemar's test. Changes in genotype, rather than presence of a particular allele, were compared between matched pairs to ensure that differences reflected selection rather than underlying differences in allele frequencies among populations. The effect of markers present at the time of recurrence on median time to PCR-adjusted re-infection (new infection) was investigated using the Wilcoxon Mann-Whitney U test. Competing risk analysis [59] was used to estimate cumulative incidence of PCR-adjusted re-infections with specific genotypes, where recrudescent and re-infections with other genotypes were treated as competing events.

The number of molecular markers used to distinguish recrudescence from re-infection varied from one to three or more loci. The effect of the number of loci genotyped on outcome classification was investigated in a regression model of predictors of recrudescence within all recurrences. No effect of this variable was observed on the number of recrudescent infections identified among recurrences in univariable or multivariable analysis, it was not further investigated.

Results

Individual patient and linked parasite genotype data from 30 studies were available (Listed individually in Supplementary Table 2). Data from 6,947 patients who were treated with AL (4,701) or ASAQ (2,246) were included in the analysis. Twenty two studies were published, representing 91% of all published clinical data on AL and ASAQ in which *pfcrt* or *pfmdr1* genotypes were determined. Baseline characteristics for patients treated with AL or ASAQ are presented in Supplementary Table S3.

Clinical efficacy of AL and ASAQ

The estimates of efficacy (defined as risk of PCR-adjusted recrudescence) of AL and ASAQ are summarized in Table 2. Of the 4,701 AL patients, 4,504 were followed up for at least one day and were included in the analysis. Similarly, of the 2,246 ASAQ patients, 2,099 were included. In total, 1,051 patients had recurrent parasitemia following AL, of which 155 (15%) were classified by PCR as recrudescent infections. The corresponding figures for ASAQ were 484 patients had recurrent parasitemia and 58 (12%) were confirmed as recrudescent. The overall clinical efficacy at day 42 was 95.3% [95%CI: 94.4-96.0] in patients treated with AL and 95.1% [95%CI: 92.3-96.7] following ASAQ treatment (Table 2). The proportion of adequate clinical and parasitological response of ASAQ was significantly higher for the fixed dose and co-blistered tablets (97.0% [95% CI: 94.4-98.4] compared to the loose formulation (93.0% [95% CI: 89.2-95.6] (p=0.003).

Baseline prevalence of genetic markers associated with resistance

The baseline prevalence of SNPs in *pfcrt* and *pfmdr1* was determined, but not all SNPs were available for all isolates. The most frequently analyzed SNPs were position 76 in *pfcrt* determined in 3,640 patients and position 86 in *pfmdr1* in 3,510, with the complete haplotype of positions 72-76 in *pfcrt*, *pfmdr1* copy number, and SNPs at positions *pfmdr1* 184 and and 1246 available in a subset of patients (Table 3).

The prevalence of *pfcrt* and *pfmdr1* alleles varied by region (Table 3). The *pfcrt* 76T allele (all in the SVMNT haplotype) was almost fixed at 96.4% (81/84) in isolates from Asia (Thailand) and Oceania (Papua New Guinea). In Africa, the only resistant haplotype observed was the CVIET allele. The 76T allele predominated: 67.6% (1155/1708) in East Africa and 73.3% (1,354/1,848) in West Africa (Table 3). Amplification of *pfmdr1* was seen in 51% (54/106) of Asian isolates examined for this genotype, but only in 2.4% (17/659) of isolates from Africa. *Pfmdr1* 86Y was found in 38%% (59/156) of isolates from Asia/Oceania; in contrast, the 86Y allele was present in 44% (896/2,033) of isolates from East Africa and 34.3% (453/1,321) of isolates from West Africa.

The SNPs at positions 184 and 1246 showed similar patterns, with *pfmdr1* Y184 and D1246 predominating in all three regions (Table 3). Almost all isolates examined carried the *pfmdr1* S1034 (703/786) and N1042 (997/1,005).

Parasite genotypes as risk factors for recrudescent infection

After controlling for age, baseline parasite density, and total lumefantrine dose (Table 1), the presence of parasites in the initial infection that carried *pfmdr1* N86 (alone or a mixed infection with *pfmdr1* 86Y) was a significant risk factor for recrudescent infection occurring between days 14 and 28 after AL treatment (Adjusted Hazards Ratio AHR = 4.06 (95% CI [1.94– 8.47]; p < 0.001) (Table 4, Figure 2A). Region of sample origin was not included as a covariate in the model because it violated the assumption of proportional hazards.

The presence of more than one copy of *pfmdr1* was not a significant risk factor for recrudescence after AL treatment and did not become a significant risk factor when the effect of region was added to the model. Once adjusted by study site, AL efficacy in patients with infections with a single copy of *pfmdr1* was not significantly different from efficacy in patients with multi-copy infections (p=0.47; Figure 2B). The interaction of region of origin with *pfmdr1* copy number could not be investigated because of insufficient multi-copy samples from Africa in the model. Because *pfmdr1* copy number was not found to be a significant risk factor for AL recrudescence, the model including both *pfmdr1* 86 and *pfmdr1* copy number is not included here or presented in Table 4.

No association was observed between the *pfmdr1* 184, *pfmdr1* 1246, and *pfcrt* polymorphisms and recrudescent infections after AL treatment. The risk for parasites with the *pfmdr1* N86 + D1246 haplotype is not reported here because it represents a subset of the *pfmdr1* N86 sample set (of the samples genotyped for both SNPs, all but 17 samples with *pfmdr1* N86 also had D1246).

For patients treated with ASAQ, none of the analyzed *pfcrt* or *pfmdr1* parasite genotypes were significant risk factors for recrudescent infections in the multivariable analysis.

Post-treatment selection of genetic markers associated with resistance

To examine changes in the genotypes of parasites following drug treatment, we compared the prevalence of *pfmdr1* and *pfcrt* alleles in paired isolates from the initial and the recurrent parasites in the subset of patients in whom parasites recurred during the 42 day follow up period. Post-treatment changes among specific genotypes are presented in Table 5 for all recurrent infections. Significant selection of *pfcrt* K76 and *pfmdr1* N86 occurred in both recrudescent and re-infecting parasites after AL treatment. Selection of *pfmdr1* 184F and D1246 alleles was also observed in the recurrent parasites and *pfmdr1* D1246 in those that reinfected patients after treatment. Selection of single or multiple copies of *pfmdr1* was not observed in any of the groups (Table 5). *Pfmdr1* 86Y and 1246Y were significantly selected in recurrent and re-infections after treatment with ASAQ (Table 5).

Median time to re-infection

The genotype of parasites at the time of re-infection provides another metric of their susceptibility to a drug. This analysis indicated that in patients treated with AL, re-infecting parasites carrying *pfmdr1* N86, *pfmdr1* D1246 or *pfcrt* K76 alleles appeared earlier than those carrying *pfmdr1* 86Y, *pfmdr1* 1246Y or *pfcrt* 76T (Figure 3A). Correspondingly, in patients treated with AL, parasites carrying *pfmdr1* N86 had a median time to re-infection of 28 days (interquartile range = 21–35) compared to 35 days (interquartile range = 28-42) for those with *pfmdr1* 86Y ($p<0.001$). Similar differences in the time to re-infection were observed for patients infected with parasites that carried the *pfmdr1* 184F ($p=0.008$) or *pfcrt* K76 alleles ($p=0.001$) when compared to *pfmdr1* Y184 or *pfcrt* 76T.

In contrast, in patients treated with ASAQ, parasites carrying *pfmdr1* 86Y, *pfmdr1* 1246Y, or *pfcrt* 76T appeared earlier after treatment than those carrying *pfmdr1* N86, *pfmdr1* D1246 or *pfcrt* K76 (Figure 3B). Parasites with *pfcrt* 76T had a median re-infection day of 28 (interquartile range = 21–35) compared to day 37.5 (interquartile range = 28-42) for those carrying K76 ($p=0.053$) and those with *pfmdr1* 1246Y re-infected on a median day of 21 (interquartile range = 21-28) compared to day 28 (interquartile range = 21-35) for those with D1246 ($p=0.001$).

Discussion

This pooled analysis of data from 31 clinical studies shows clearly that the genotypes of infecting parasites influence the outcome of AL treatment. Patients infected with parasites that carried the *pfmdr1* N86 allele or increased *pfmdr1* copy number were at significantly greater risk of treatment failure than those whose parasites carried the 86Y allele or a single copy of *pfmdr1*. Analysis of the clinical outcomes after treatment with ASAQ did not link a particular genotype with treatment failure in this smaller data set, however it did show clear evidence of selection of particular parasite genotypes. Our findings are consistent with previous molecular studies in which changes in the prevalence of particular alleles of *pfcrt* or *pfmdr1* have been documented in response to introduction or increased use of lumefantrine [25-35] or amodiaquine.[15, 28, 40-51]

Our observation that parasites with the *pfmdr1* N86, D1246, and *pfcrt* K76 alleles re-infected patients earlier after AL treatment, and parasites carrying the *pfmdr1* 86Y, 1246Y, and *pfcrt* 76T alleles re-infected patients earlier after ASAQ is also congruent with the molecular studies. These differences suggest that parasites with these genotypes can withstand higher drug concentrations compared with parasites that carry the alternative alleles. Recently, Malmberg and colleagues demonstrated this effect quantitatively. After AL treatment, parasites with the *pfmdr1*

N86/184F/D1246 haplotype were able to re-infect patients whose lumefantrine blood concentrations were 15-fold higher than was the case for parasites carrying the 86Y/Y184/1246Y haplotype,[33] providing a potential pharmacological explanation for the molecular findings. Together, these observations suggest that monitoring shifts to earlier time of re-infection could provide a relatively simple warning of declining susceptibility to these drugs, especially if combined with timed measurement of drug concentrations in patients' blood.

In Southeast Asia, parasites with multi-copy *pfmdr1* are common in areas where mefloquine has been intensively deployed,[36] and increased *pfmdr1* copy number is strongly associated with artesunate-mefloquine treatment failures. Almost half of the samples in our data set from Southeast Asia region had at least two copies of the gene. In contrast, multiple copy number was rarely observed in our large sample of isolates from Africa, where populations have had little exposure to mefloquine.

The results of this study did not indicate that parasites with increased copy number of *pfmdr1* are less sensitive to lumefantrine. Our findings contrast with reports of decreased *in vitro* lumefantrine susceptibility with increased copy number[37–40], but support the conclusions of *in vivo* studies[38] which indicate that multi-copy *pfmdr1* is not a risk factor for AL treatment failure.

In our data set from Southeast Asia, the amplified alleles all carried the N86 allele of *pfmdr1*[34,36,62]. This association was not found in the few parasites from Africa in our data set that did have an increased copy number[31], indicating that either of the N86Y alleles of *pfmdr1* can apparently be amplified in this region.

The evidence of strong selection of particular alleles by both drugs in recurrent parasites, coupled with our observation that particular parasite genotypes increase risk of treatment failure, demonstrates that tracking these molecular markers can signal early declines in susceptibility to lumefantrine or amodiaquine. Both alleles of *pfmdr1 N*86Y, Y184F and D1246Y are common in African *P. falciparum* populations, and *pfcrt* K76 has increased in prevalence in recent years, so changes in the prevalence of these alleles can be a sensitive indicator of selection of parasite populations by AL and ASAQ. In turn, declining efficacy of these partner drugs exposes the artemether or artesunate component of the ACT to selective pressure and could facilitate emergence of new foci of resistance to artemisinin, as observed in the Mekong region. The recent identification of a marker correlated with slow response to artemisinin,[63] will allow molecular assessment of this trend, as well.

Application of these molecular tools is increasingly feasible both in the context of clinical trials and in community surveys of populations where AL or ASAQ are heavily used. These approaches can offer cost effective methods that detect evidence of declines in parasite susceptibility far earlier than before, allowing detailed studies of clinical responses to the drugs in areas of concern. This two stage approach can provide an opportunity for policy makers to manage emerging threats of resistance before clinical failure of a drug is manifest and preserve the useful therapeutic life of these valuable antimalarials for as long as possible.

Finally, these results suggest that both AL and ASAQ interact with the proteins encoded by *pfcrt* and *pfmdr1*, but the two drugs select alternative alleles. This opposing selection of parasite genotypes by the partner drugs could influence the choice of an ACT in regions with different

patterns of *pfcrt* and *pfmdr1* polymorphisms. For example, if a particular allele is rapidly increasing under intensive use of AL, introduction of AQ might be introduced to counteract that trend. Concurrent use of these two ACTs that exert opposing selective pressures on recurrent parasites could provide a counterbalance and prevent strong directional selection in both *pfcrt* and *pfmdr1*, maintaining the overall efficacy of both AL and ASAQ for a long period. Despite logistical challenges, the simultaneous use of multiple first line therapies is supported by mathematical models,[64-66] and concurrent availability of AL and ASAQ, as implemented in some West African countries [4] may provide a practical means to test this strategy directly.

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Tables And Figures

Table 1. Multivariable risk factors for PCR-adjusted recrudescent infections of artemether-lumefantrine and artesunate-amodiaquine at day 42 based upon previous studies (references will be listed at publication)

Treatment	Variable	Adjusted HR [95% CI]	p-value
AL (N=4,433; 150 recrudescences)			
	Age category: ≥ 12 years (reference)		
	< 1 year	1.96 [0.73 – 5-32]	0.184
	1 to < 5 years	**2.05 [1.23 – 3.39]**	**0.006**
	5 to < 12 years	1.23 [0.68 – 2.21]	0.488
	Enrolment parasite density (log-scale)	**1.13 [1.05 – 1.23]**	**0.002**
	Lumefantrine dose (mg/kg)	0.99 [0.98 – 1.00]	0.086
ASAQ (N=7,652; 220 recrudescences)			
	Age category: ≥ 12 years (reference)		
	< 1 year	**2.20 [1.01 - 4.78]**	**0.047**
	1 to < 5 years	**2.27 [1.13 - 4.55]**	**0.021**
	5 to < 12 years	1.51 [0.72 - 3.17]	0.140
	Enrolment parasite density (log-scale)	**1.50 [1.16 - 1.93]**	**0.002**
	Amodiaquine dose (mg/kg)	0.92 [0.82 - 1.04]	0.180
	Drug formulation: Fixed dose (reference)		
	Co-blistered	0.98 [0.41 - 2.32]	0.960
	Loose	**2.94 [1.58 - 5.48]**	**0.001**

Table 2. PCR-adjusted adequate clinical and parasitological response (ACPR) of artemether-lumefantrine and artesunate-amodiaquine after 42 days of follow up

		AL	ASAQ fixed dose and co-blistered	ASAQ loose
At risk (N)		4,763	1,113	986
ACPR by group (%, [95% CI])				
Age Category				
	< 1 year	96.7 [92.7-98.5]	100	85.2 [70.5-93.0]
	1-<5 years	93.6 [92.1-94.8]	96.4 [93.2-98.1]	93.8 [90.0-96.2]
	5-12 years	96.7 [94.9-97.8]	98.8 [91.6-99.8]	99 [96.1-99.8]
	>= 12 years	96.6 [95.2-97.6]	-	-
Region				
	Asia/Oceania	97.0 [95.6-97.9]	-	-
	East Africa	93.8 [92.4-95.0]	100*	91.2 [88.0-94.7]
	West Africa	96.2 [94.6-97.3]	96.9 [94.2-98.3]	99.2 [96.8-99.8]*
Overall		95.3 [94.4-96.0]	97.0 [94.4-98.4]	93.0 [89.2-95.6]

*Followed up to day 28

Table 3. Baseline (pre-treatment) prevalence of genetic markers associated with drug resistance

Markers	Asia/Oceania	East Africa	West Africa
***pfcrt* 76**			
Sample size	84	1708	1848
K	3 (4%)	553 (32%)	494 (27%)
K/T	2 (2%)	125 (7%)	249 (13%)
T	79 (94%)	1030 (60%)	1105 (60%)
***pfcrt* 72-76**			
Sample size	84	155	84
CVMNK	3 (4%)	37 (24%)	14 (17%)
CVIET	0	117 (75%)	53 (63%)
SVMNT	79 (94%)	0	0
mixed	2 (2%)	1 (1%)	17 (20%)
***pfmdr1* 86**			
Sample size	156	2033	1321
N	97 (62%)	759 (37%)	678 (51%)
N/Y	0	378 (19%)	190 (14%)
Y	59 (38%)	896 (44%)	453 (34%)
***pfmdr1* 184**			
Sample size	158	1275	686
Y	133 (84%)	803 (63%)	287 (42%)
Y/F	7 (4%)	130 (10%)	77 (11%)
F	18 (11%)	342 (27%)	322 (47%)
***pfmdr1* 1246**			
Sample size	64	1017	687
D	54 (84%)	454 (45%)	526 (77%)
D/Y	10 (16%)	309 (30%)	86 (13%)
Y	0	254 (25%)	75 (11%)
***pfmdr1* 86 + 1246**			
Sample size	56	1000	685
N D	2 (3%)	129 (13%)	263 (38%)
N Y	0	9 (1%)	2 (0%)
Y D	47 (84%)	248 (25%)	199 (29%)
Y Y	0	220 (22%)	71 (10%)
mixed	7 (13%)	394 (39%)	150 (22%)
***pfmdr1* copy number**			
Sample size	106	659	0
1	52 (29%)	642 (98%)	0
2	36 (34%)	16 (2%)	0
>2	18 (17%)	1 (0%)	0

Table 4. Multivariable risk factors for PCR-adjusted recrudescent infections of artemether-lumefantrine on day 42

Marker	Variable	Adjusted Hazard Ratio [95% CI]	P-value
pfmdr1* 86 (N=2,474; 117 recrudescent infections)			
	pfmdr1 N86 or N/Y:		
	in recrudescence up to day 14	0.90 [0.28 - 2.89]	0.858
	in recrudescence between day 14-28	**4.06 [1.94– 8.47]**	**< 0.001**
	in recrudescence after day 28	0.92 [0.46 - 1.81]	0.805
	Enrolment parasite density (log_e-scale)	1.12 [0.97 - 1.30]	0.116
	Age category (reference: < 1 year)		
	1 to < 5 years	1.08 [0.41 - 2.82]	0.882
	5 to < 12 years	0.80 [0.28 – 2.26]	0.671
	>= 12 years	0.74 [0.23 - 2.35]	0.611
	Lumefantrine dose (mg/kg)	0.99 [0.98 - 1.00]	0.219
***pfmdr1* copy number(N=739; 54 recrudescent infections)**			
	pfmdr1 copy number > 1:**		
	in recrudescence up to day 14	1.15 [0.11 – 11.95]	0.907
	in recrudescence between day 14-21	2.18 [0.58 – 8.13]	0.247
	in recrudescence after day 21	0.94 [0.29 – 3.11]	0.922
	Region (reference: Africa)		
	Asia/Oceania	**11.94 [2.33 – 61.06]**	**0.003**
	Enrolment parasite density (log_e-scale)	1.04 [0.85 - 1.26]	0.707
	Age category (reference: < 5 years)		
	5 to < 12 years	0.86 [0.34 – .21]	0.761
	>= 12 years	0.30 [0.09- 1.07]	0.064
	Lumefantrine dose (mg/kg)	0.98 [0.96 - 1.00]	0.092

*Region not included as a covariate or interaction term with *pfmdr1* 86 genotype because proportional hazards assumption was not met

**Sparse data on *pfmdr1* copy number in Africa prevented the inclusion of region as an interaction term

Table 5. Selection of *pfcrt* and *pfmdr1* genotypes after treatment with artemether-lumefantrine and artesunate-amodiaquine

		Recurrence		Recrudescence		Re-infection	
Marker	**Genotype**	**AL**	**ASAQ**	**AL**	**ASAQ**	**AL**	**ASAQ**
pfcrt 76	K --> T [a]	16% (89/571)	10% (25/237)	5% (4/73)	20% (7/35)	17% (82/493)	9% (17/196)
	T --> K	**30% (171/571)**	8% (18/237)	**25% (18/73)**	11% (4/35)	**31% (152/493)**	7% (14/196)
	no change	54% (311/571)	82% (194/237)	70% (51/73)	69% (24/35)	53% (259/493)	84% (165/196)
		<0.001	0.286	**0.004 (exact)**	0.366	**<0.001**	0.590
pfmdr1 86	N --> Y	13% (95/692)	**27% (92/341)**	11% (10/87)	18% (5/28)	14% (85/603)	**28% (87/308)**
	Y --> N	**41% (285/692)**	16% (54/341)	**36% (31/87)**	14% (4/28)	**42% (254/603)**	16% (49/308)
	no change	45% (312/692)	57% (195/341)	53% (46/87)	68% (19/28)	44% (264/603)	56% (172/308)
		<0.001	**0.002**	**0.001**	0.739	**<0.001**	**0.001**
pfmdr1 184	Y--> F	**25% (73/291)**	12% (37/303)	26% (14/55)	12% (3/25)	25% (59/236)	12% (34/273)
	F --> Y	18% (51/291)	17% (50/303)	18% (10/55)	4% (1/25)	17% (41/236)	18% (49/273)
	no change	57% (167/291)	71% (216/303)	56% (31/55)	84% (21/25)	58% (136/236)	70% (190/273)
		0.048	0.163	0.414	0.625	0.072	0.100
pfmdr1 1246	D --> Y	14% (38/273)	**32% (102/317)**	11% (5/44)	39% (11/28)	15% (33/227)	**32% (90/284)**
	Y --> D	**32% (86/273)**	19% (60/317)	30% (13/44)	14% (4/28)	**32% (73/227)**	20% (56/284)
	no change	54% (149/273)	49% (155/317)	59% (26/44)	46% (13/28)	53% (121/227)	48% (138/284)
		<0.001	**0.001**	0.059	0.119	**<0.001**	**0.005**
pfmdr1 copy number	1 --> 2 or more	0.5% (1/247)	--	3% (1/37)	--	0	--
	2 or more --> 1	1% (2/247)	--	0	--	1% (2/210)	--
	no change	98.5% (244/247)	--	97% (36/37)	--	99% (208/210)	--
		1.000 (exact)		0.317 (exact)		0.500 (exact)	

Changes in bold indicate statistically significant selection ($p < 0.05$) using McNemar's paired test. Those marked (exact) were tested using the exact distribution for small sample sizes. A small number of recurrent infections (4 for AL and 6 for ASAQ) were not PCR-adjusted and were excluded from the analysis of recrudescent and re-infections.

[a] Each category includes all changes from one allele to another. For example, K → T includes K→ T, K→ K/T and K/T → T changes

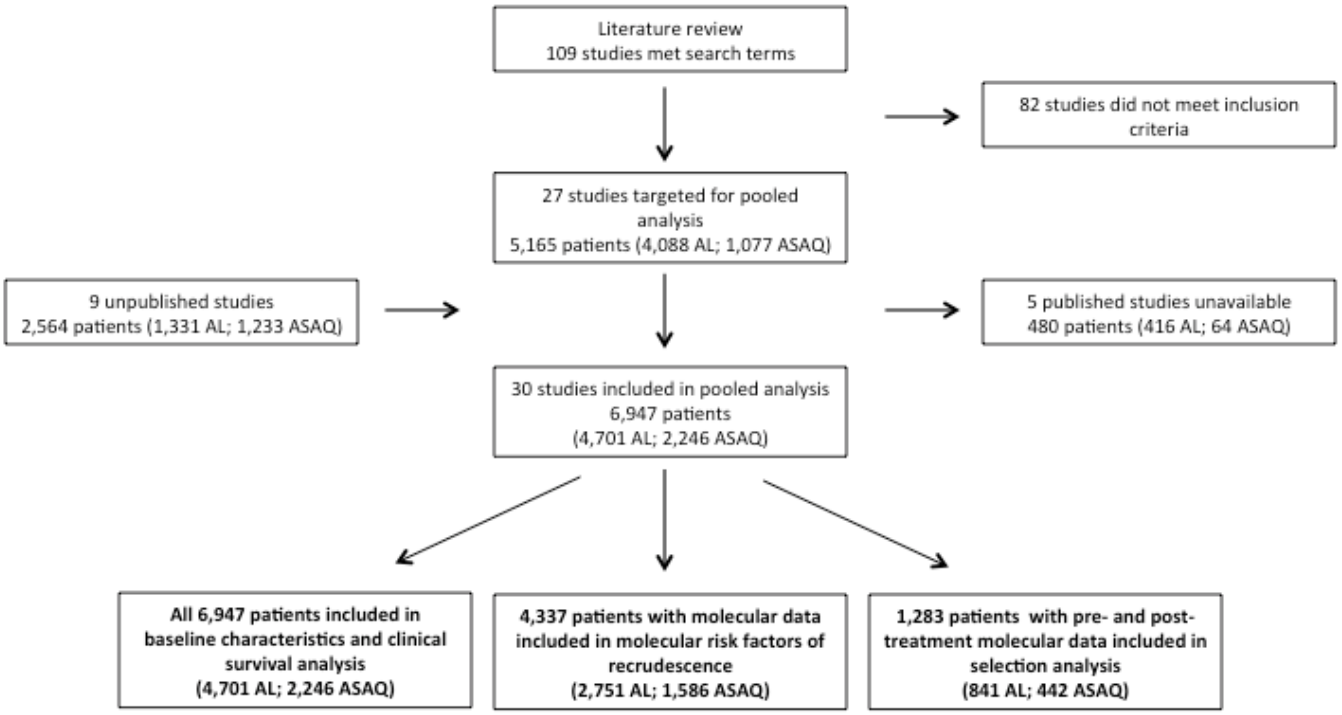
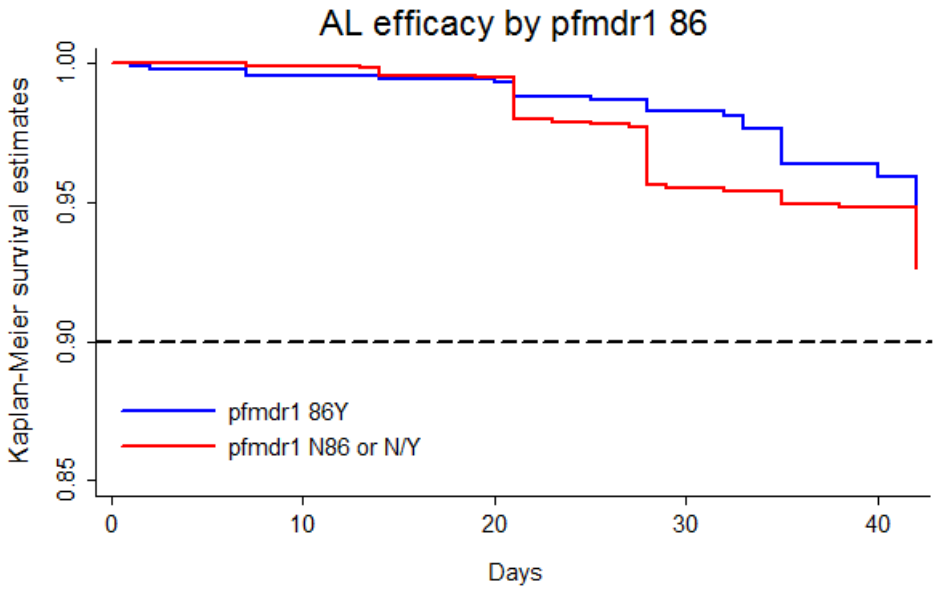


Figure 1. Patient flowchart

A



B

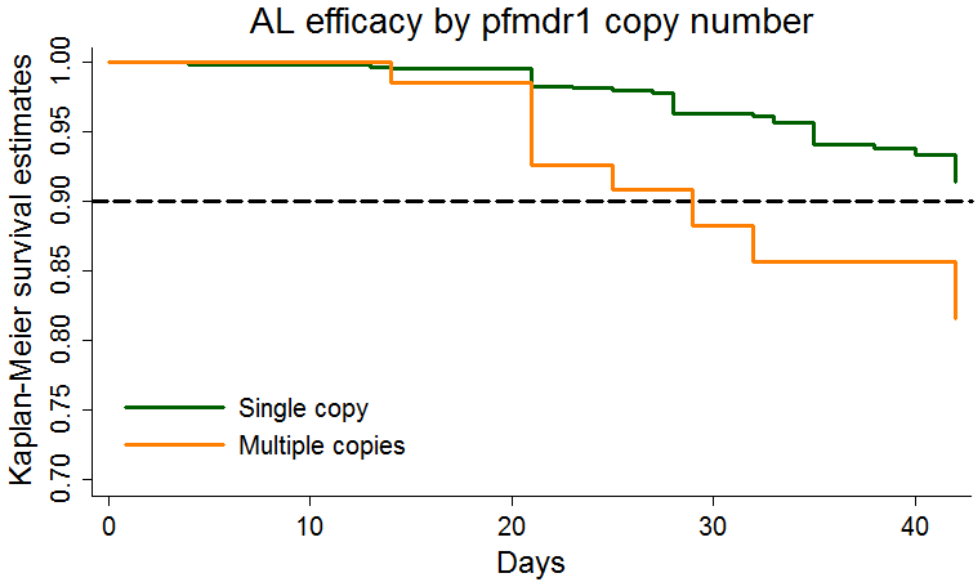
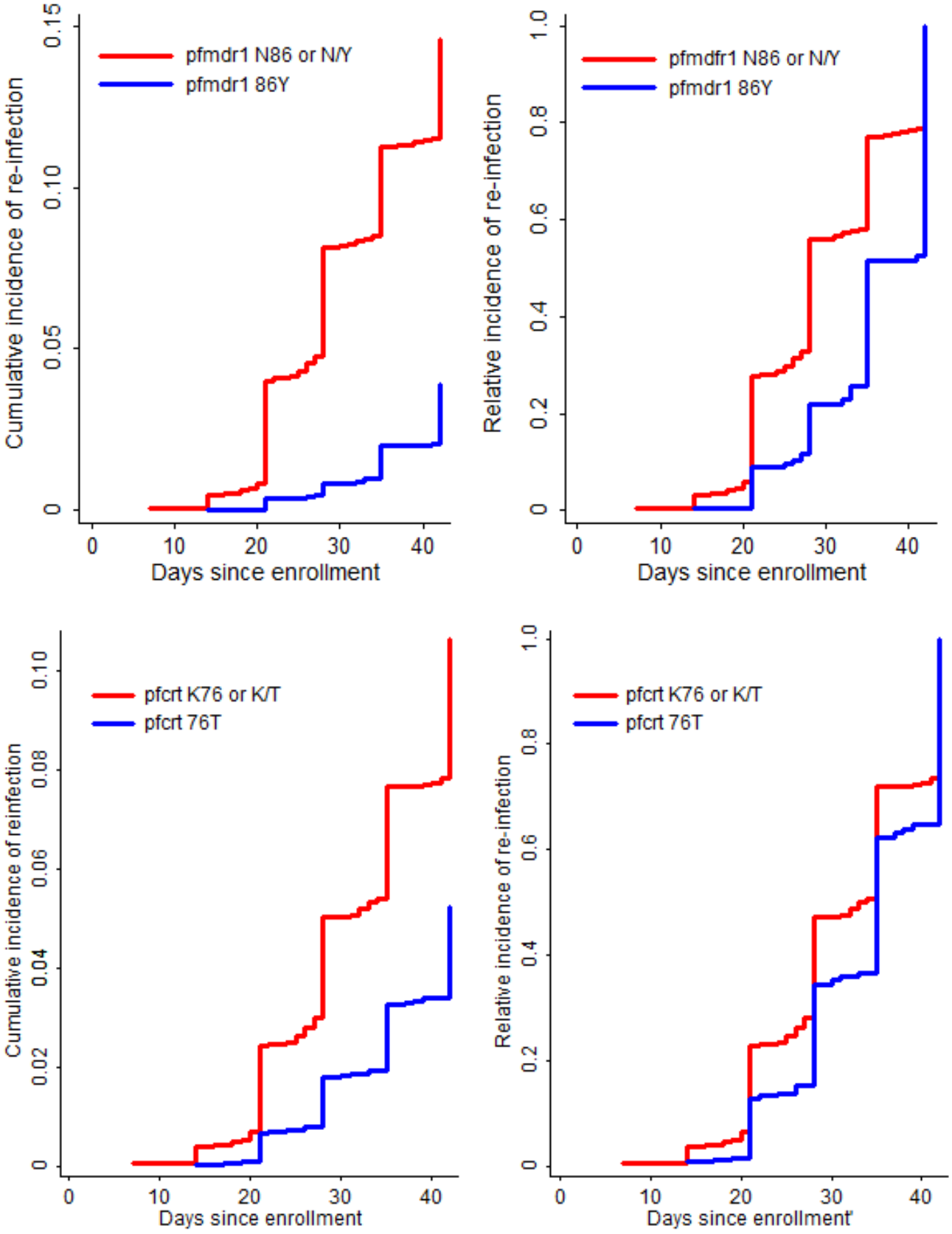


Figure 2. Polymerase chain reaction-adjusted efficacy as assessed by Kaplan-Meier survival estimates for artemether-lumefantrine (AL) by *pfmdr1* genotype of initial parasites. Dotted line indicates WHO-recommended 90% efficacy cutoff for antimalarials. Clinical response of patients with parasites that carry A) *pfmdr1* 86Y (blue) versus 86N or N/Y (red); N = 2,474 patients at risk B) *pfmdr1* copy number > 1 (yellow) versus single copy (green); N = 739 patients at risk.

Figure 3A.


pfmdr1 N86 or N/Y
pfmdr1 86Y
Cumulative incidence of re-infection
0
0.05
0.10
0.15
0
10
20
30
40
Days since enrollment
pfmdfr1 N86 or N/Y
pfmdr1 86Y
Relative incidence of re-infection
0
0.2
0.4
0.6
0.8
1.0
0
10
20
30
40
Days since enrollment
pfcrt K76 or K/T
pfcrt 76T
Cumulative incidence of reinfection
0
0.02
0.04
0.06
0.08
0.10
0
10
20
30
40
Days since enrollment
pfcrt K76 or K/T
pfcrt 76T
Relative incidence of re-infection
0
0.2
0.4
0.6
0.8
1.0
0
10
20
30
40
Days since enrollment

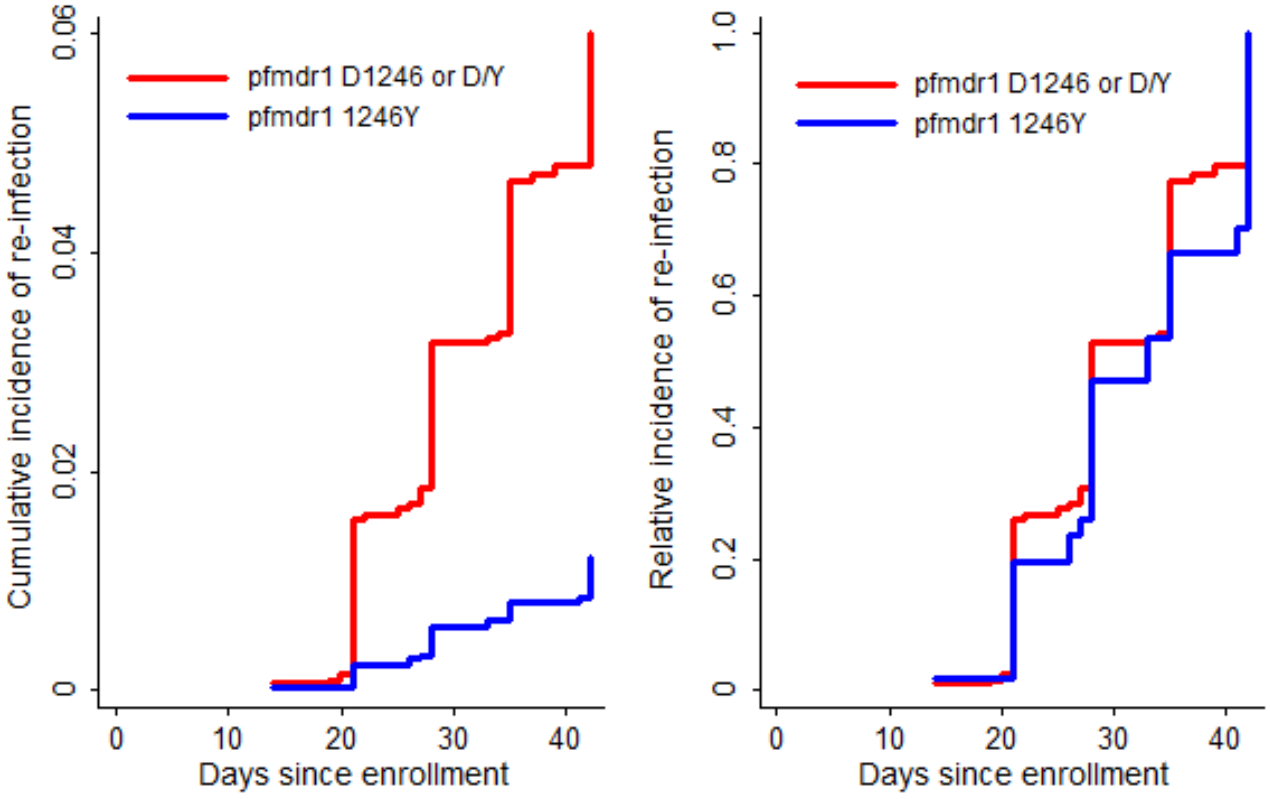
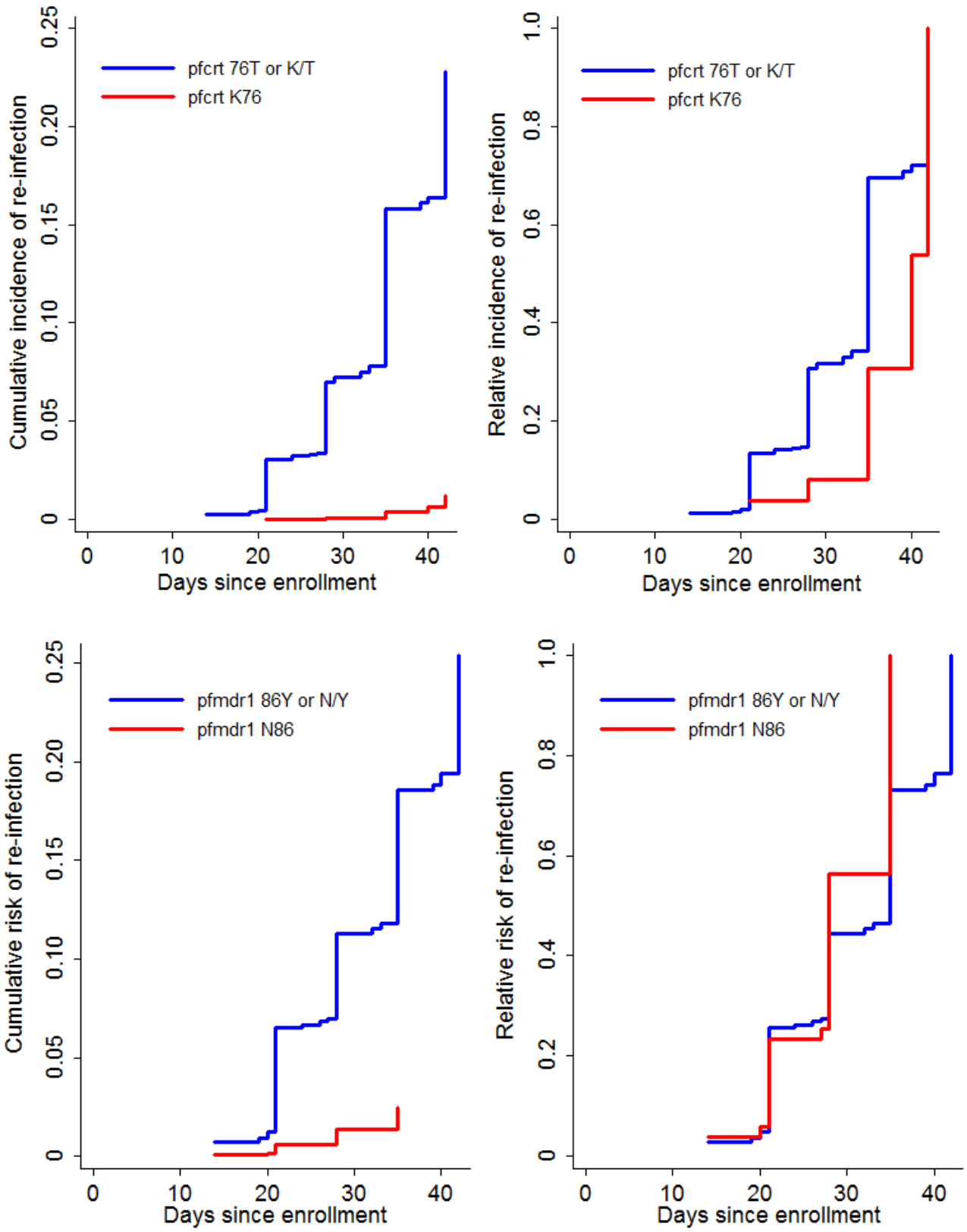


Figure 3A. Cumulative (left panels) and relative (right panels) risks of PCR-adjusted re-infection for baseline *pfcrt* and *pfmdr1* genotypes after artemether-lumefantrine treatment, where recrudescent and re-infections with other genotypes were treated as competing events.

Figure 3B.


pfcrt 76T or K/T
pfcrt K76
Cumulative incidence of re-infection
0
0.05
0.10
0.15
0.20
0.25
0
10
20
30
40
Days since enrollment
pfcrt 76T or K/T
pfcrt K76
Relative incidence of re-infection
0
0.2
0.4
0.6
0.8
1.0
0
10
20
30
40
Days since enrollment
pfmdr1 86Y or N/Y
pfmdr1 N86
Cumulative risk of re-infection
0
0.05
0.10
0.15
0.20
0.25
0
10
20
30
40
Days since enrollment
pfmdr1 86Y or N/Y
pfmdr1 N86
Relative risk of re-infection
0
0.2
0.4
0.6
0.8
1.0
0
10
20
30
40
Days since enrollment

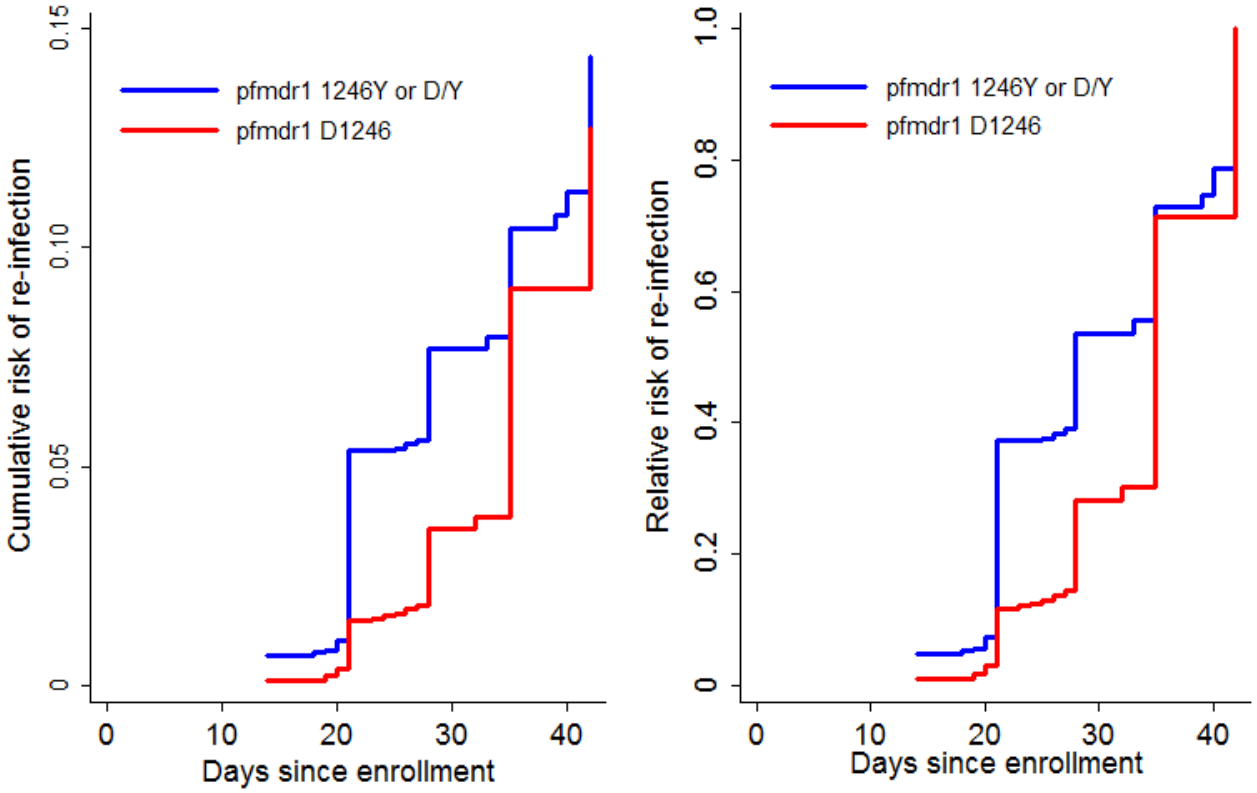


Figure 3B. Cumulative (left panels) and relative (right panels) risks of PCR-adjusted re-infection for baseline *pfcrt* and *pfmdr1* genotypes after artesunate-amodiaquine treatment, where recrudescent and re-infections with other genotypes were treated as competing events