Plasmodium falciparum carrying pfk13 polymorphisms harbour the SVMNT allele of pfcr in north-western Indonesia

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Abstract

Artemisinin-based combination therapy is the first-line antimalarial regimen in Indonesia. Susceptibility of Plasmodium falciparum to artemisinin is falling in the Greater Mekong sub-Region, but it is not known whether the efficacy of current combinations is also threatened in nearby Sumatera. We evaluated the genetic loci pfcr, pfmdr1 and pfk13, considered to be under selection by artemisinin combination therapy, among 404 P. falciparum infections identified by PCR detection in a cross-sectional survey of 3,731 residents of three Regencies. The pfcr haplotype SVMNT (codons 72-76) was the most prevalent and displayed significant linkage disequilibrium with the pfmdr1 haplotype YY (codons 86, 184) (OR 26.7, 95% CI 5.96 - 239.4; P<0.001). This contrasts with Mekong countries, where the CVIET haplotype of pfcr predominates. Among 231 evaluable isolates, only nine (3.9%) showed any evidence of non-synonymous gene variants in the propeller domain of pfk13. The Thr474Ala variant was seen in six individuals, and Cys580Tyr identified with low confidence in only a single isolate from an asymptomatic individual. Among a subset of 117 symptomatic P. falciparum-infected individuals randomized to receive either dihydroartemisinin-piperaquine or artemether-lumefantrine, treatment outcome was not associated with pre-treatment genotype. However, sub-microscopic persistent parasites at day 28 or day 42 of follow-up were significantly more likely to harbor the pfmdr1 haplotype NF (codons 86, 184) than were pre-treatment isolates (P<0.001 for both treatment groups). Current ACT regimens appear to be effective in Sumatera, but evidence of persistent sub-microscopic infection in some patients suggests further detailed studies of drug susceptibility should be undertaken.

249 words

Running title: Drug resistance markers in Indonesian P. falciparum
INTRODUCTION

Successful strategies for elimination of malaria require effective first-line chemotherapies. Failure of the antimalarials chloroquine and sulfadoxine-pyrimethamine compromised malaria control strategies in many malaria endemic countries and contributed to a significant increase in morbidity and mortality through the 1990s (1, 2). WHO currently recommends the use of artesinin-based combination therapy (ACT) for the treatment of uncomplicated *Plasmodium falciparum* infection, a strategy which has contributed to reductions in malaria mortality in the last two decades (3). Nevertheless, decreased susceptibility of *P. falciparum* parasites to artesinin and partner drugs has emerged in the Greater Mekong sub-Region (GMS), as evidenced by slow parasite clearance and an increased frequency of recrudescence in patients treated with the ACT dihydroartesinin-piperine (DP) (4, 5). The continued progression of clinically-relevant parasite resistance in this region may be slowed or prevented by deploying a more flexible treatment policy, informed by regular monitoring of candidate resistance-associated alleles of key genes in *P. falciparum* parasites, to identify genotypes with a selective advantage in parasites exposed to antimalarial drugs.

The marked reduction in *in vivo* parasite susceptibility to artesinin was first observed in the GMS over a decade ago (6). This is caused by mutations in the *P. falciparum* gene *pfk13* which affect the propeller domain of the kelch-13 protein (7, 8). Amplification of *plasmepsin* II gene copy number is linked to piperine resistance in the same region (9). Resistance to aminoquinolines is known to be mediated by the putative transporter *pfcr* (10), with specific haplotypes at codons 72-76 associated with resistance to chloroquine (CVIET) and amodiaquine (SVMNT) (11, 12). The degree of resistance to aminoquinolones and to artesinin is further modulated by additional variation in other genes including *pfmdr1*, encoding P-glycoprotein H1. Polymorphisms in *pfmdr1* have been associated with differential susceptibility to lumefantrine and amodiaquine (13). *In vitro* studies show that the codon 86 Tyr variant (86Y), which developed under aminoquinolone pressure in previous decades, has greater *in vitro* susceptibility to artesinin than the wild-type 86N (14, 15). Further, the haplotype NFD at codons 86, 184 and 1246 of this locus is associated with parasite persistence in ACT-treated African patients (16, 17). Thus, understanding genetic changes in parasite populations where resistance is emerging can provide timely warning of threats to current therapies.

ACT have been used in Indonesia since 2004, after efficacy of chloroquine was severely reduced by the spread of parasites harbouring the CVIET and SVMNT haplotypes of *pfcr* (18 - 20). Two combinations were initially deployed, artesunate-amodiaquine (ASAQ) for western Indonesia and DP for eastern Indonesia (21). However, treatment failures with ASAQ were frequently documented which led to further drug policy change in 2012, putting in place country-wide deployment of DP. *In vivo* studies using ASAQ for falciparum malaria have consistently demonstrated unsatisfactory clinical efficacy in Central Java, Papua and Sumatera (22 - 25), with PCR-corrected efficacy as low as 80% in one study conducted prior to the adoption of ASAQ as the national recommendation (22). An explanation for the observed poor drug
efficacy is hindered by lack of information on parasite polymorphisms in this study. Also of great concern is that artemisinin-resistant parasites harbouring pfk13 mutants have now spread across Southeast Asia, and so with its proximity to the Mekong, and a prior history of lower parasite susceptibility to ACT treatment, genetic markers of ACT resistance in P. falciparum parasites in western Indonesia urgently require investigation.

In this study, we report the prevalence of polymorphisms of interest in the pfk13, pfcr1t and pfdm1 genes of P. falciparum isolates from a large cross-sectional survey in three Regencies in North Sumatera Province, Indonesia (26). We determined the alleles carried by P. falciparum isolates from a subset of survey participants enrolled in a randomized comparison of antimalarial efficacy of two ACT, artemether-lumefantrine (AL) and DP (27), and tested for evidence of association between variants of these three loci and treatment outcomes.
METHODS

Study sites, sample collection and patient recruitment
As previously described, we conducted a parasitological survey between January and June 2015 in Batubara, Langkat and South Nias regencies in North Sumatera province, Indonesia (26). A total of 3,731 participants were screened for *Plasmodium* species infection by microscopy and *post hoc* nested polymerase chain reaction (PCR). All microscopy-positive participants were treated with the standard 3-dose DP or 6-dose AL regimens, and those meeting inclusion criteria for a prospective efficacy trial of AL vs DP, and who gave consent, were followed up for 42 days as described elsewhere (27).

The study was approved by the Research Ethics Committees of the University of Sumatera Utara, Indonesia (ref 401/KOMET/FK USU/2014) and the London School of Hygiene and Tropical Medicine, United Kingdom (ref 8504-01).

Parasite genotyping for resistance markers
Parasite DNA was extracted from dried blood spots as described (26). We performed genotyping of *pfcrtr*, *pfdmr1* and the *pfkelch13* propeller domain using established methods with minor modifications. Polymorphisms at codons 72-76 in *pfcrtr* were determined using multiplex qPCR (28). Polymorphisms at codons 86, 184, 1034, 1042 and 1246 in *pfdmr1* were identified by direct sequencing (Humphreys et al., 2007). *Pfk13* polymorphisms were identified by nested amplification and direct sequencing of PCR products (7, 29). The prevalence of each polymorphism in the evaluated genes was estimated. Samples yielding mixed alleles contributed to the prevalence of both alleles.

Treatment outcomes
For 117 symptomatic participants with PCR-confirmed *P. falciparum* infections, randomized to receive AL or DP, *pgmet* qPCR positivity at day (D) 3 and *pfdmr1* nested PCR positivity at D28 or D42 were indicators of unsuccessful treatment (27).

Statistical analysis
Statistical analyses were performed in the STATA 11 package. Binary variables were compared across categories by estimating odds ratios (OR) with 95% confidence intervals (CI), and significance was determined using the $X^2$ distribution. Linkage disequilibrium between loci was examined in 2x2 contingency tables.
**RESULTS**

Population prevalence was estimated for each gene variant of interest by genotyping DNA from *P. falciparum* infections previously identified in our cross-sectional survey. PCR was positive for 304 tested individuals, of which 201 were identified as sub-microscopic, low density parasitaemia (26). Resistance-associated loci were amplified from among these 304 isolates.

**Polymorphisms in Pfcrtr**

*K* genotype at codons 72 to 76 was successful for 183 isolates (60.2%). We observed the *pfcrt*-SVMNT haplotype as the dominant allele, being present in 140 of these (76.5% of evaluable isolates), either alone (68.6% of these) or mixed with CVMNK or CVIET haplotypes (31.4%) (Figure 1A). The prevalence of parasites harboring the wild-type haplotype CVMNK, alone or mixed, was 34.9%. CVIET occurred in 20.2% of isolates. Parasites carrying the SVMNT haplotype, alone or mixed, were the most prevalent in each of the three sites, comprising 42/49 in Batubara regency (85.7%), 33/39 in Langkat regency (84.6%), and 65/95 in South Nias regency (68.4%). In South Nias, the CVIET haplotype was observed more commonly than in the other regencies, occurring in 28/95 of isolates (29.5%).

**Polymorphisms in Pfmdr1**

Codons 25-201 of *pfmdr1* were successfully amplified and sequenced for 267 isolates (66.1%). The prevalence of *Pfmdr1* N86 (Asn) wild-type allele was predominant overall (174/267, 65.2%), but did vary among sites 37% to 79%. The 86Y (Tyr) variant, associated with chloroquine and amodiaquine resistance, occurred in 93/267 (34.8%), and two rare mutations, 86F (Phe) and 86S (Ser), were also observed, each in two individuals. The wild-type Y184 was highly prevalent, occurring in more than 90% of isolates in Batubara regency, and over 80% in Langkat and South Nias regencies (Figure 2). We did not observe any mutation in the *Pfmdr1* codons 1034, 1042 or 1246 alleles among 73, 74 and 69 evaluable sequences, respectively, and no further analysis of these codons was conducted.

The combined haplotype at *pfmdr1* codons 86 and 184 was determined for each isolate. The NF haplotype is known to be selected by artemether-lumefantrine, while the YY haplotype is selected by amodiaquine (13). We also included samples with mixed alleles at only one of the two positions, such that two haplotypes could be unambiguously assumed to occur in that isolate. We noted the haplotype YY (91/261, 34.9%) was almost three times more prevalent in the population than the parasites carrying haplotype NF (34/261, 13.0%). However, this ratio differed by site, with YY predominant over NF in Batubara and Langkat but equally distributed in South Nias (Figure 1B).

**Population prevalence of polymorphisms in Pfk13**

*Kelch13* propeller domain sequence was determined on at least one DNA strand for *P. falciparum* isolates from 231 participants, with the wild-type genotype present in the majority. Previous surveys of allele
prevalence at this locus have sampled among clinical malaria cases, whereas the majority of our 231 sequences came from asymptomatic individuals tested as part of our cross-sectional survey (26). Parasite densities were therefore usually low, and sequencing quality was not always adequate to confirm genotypes on both DNA strands of the pfk13 amplicon. Nine isolates were considered to harbor non-synonymous polymorphisms with low, moderate or high confidence (Table 1). The previously described amino acid substitution T474A was the most prevalent, occurring in six individuals and at least once in each Regency, and the CS80Y substitution was identified at low confidence in a single isolate from South Nias. The other common Southeast Asian mutant-alleles R539T and F446I were not observed among our isolates (29). Although K13 polymorphisms occurred in all 3 sites, prevalence was uniformly low: 4 of 66 in Batubara, 3 of 60 in Langkat and 2 of 106 in South Nias (Figure 1C).

**Associations between Pfcr1, Pfmdr1 and Pfk13 polymorphisms in the P. falciparum population**

We investigated any evidence of linkage disequilibrium between the pfcr1 and pfmdr1 polymorphisms among isolates in our cross-sectional survey. Isolates carrying the SVMNT pfcr1 haplotype were significantly more likely to carry the pfmdr1 YY-haplotype (OR 26.7, 95% CI 5.96 - 239.4; P<0.001). Conversely, only 8 of 116 isolates harbouring pfcr1 SVMNT also carried the pfmdr1 haplotype NF (7.0%), compared to 11 of 26 harbouring other pfcr1 genotypes (OR 0.101, 95% CI 0.031 - 0.333; P<0.001). We observed that pfk13 propeller domain variant alleles were present in a background of pfcr1 SVMNT (all four evaluable) and pfmdr1 YY or NY (four and three evaluable, respectively), but it was not possible to test these associations statistically as we had too few isolates successfully typed at all three loci.

**Pfcr1, Pfmdr1 and Pfk13 polymorphisms in parasites before and after ACT treatment**

A subset of individuals with symptomatic *P. falciparum* infections were enrolled in a prospective treatment efficacy study, randomized to receive either AL or DP (27). We observed an unexpected high proportion of ACT- treated patients with persisting sub-patent *P. falciparum* parasites, and so we explored whether pfcr1, pfmdr1 and pfk13 genotypes in the pre-treatment parasite population contributed to trial outcomes. Among 71 evaluable PCR-confirmed *P. falciparum* isolates with remaining DNA samples available, the amodiaquine-resistant SVMNT haplotype of pfcr1 (at codons 72 – 76) dominated in both treatment groups (28 of 34 in the DP group (82.4%); 35 of 37 in the AL group (94.6%) (Fig. 2). The chloroquine-resistant CVIET and drug-sensitive CVMNK pfcr1 haplotypes were both less common, together accounting for 11/34 and 8/37 of pre-treatment isolates in the DP and AL treatment groups, respectively, including a number of mixed infections in which SVMNT was also present. The relative proportions of SVMNT differed according to site, with the highest in Batubara and the lowest in South Nias (Fig. 2).

For pfmdr1, the YY haplotype at codons 86 and 184 was predominant in the pre-treatment population for both ACT groups (32 of 49, 65.3% for DP; 36 of 47, 76.6% for AL), reflecting the high prevalence of this haplotype observed in the cross-sectional population survey (Suppl. Fig. 1). The rare 86S
allele was also identified in two individuals (Fig. 2). The 86N allele was common only in South Nias, and rare in pre-treatment isolates from the other 2 Regencies. For pfk13, wild-type genotypes (96%, 72 of 75) dominated in the propeller domain. The T474A polymorphism was detected in 3 (4.0%) pre-treatment isolates in the AL group, in each case mixed with wild-type sequence. All parasite isolates harbouring pfk13 mutations also carried the SVMNT haplotype of pfcrt. We found no evidence of slow clearance by qPCR during the first 72h following treatment with either ACT, except in a single DP-treated patient that exhibited PCR-confirmed early treatment failure (27).

An unexpected finding of our clinical study was that a significant number of persistent PCR-detectable P. falciparum infections remained 28 or 42 days after treatment (27). We therefore attempted to genotype pfmdr1 in these recurrent isolates and compare to those of the baseline isolates. Successful amplification of the pfmdr1 amplicon containing codons 86 and 184 was achieved for 31 and 30 samples at day 28 and 42, respectively. We observed a significant selection for N86 and 184F at days 28 and 42 in both treatment arms, but found no evidence that presence of the NF haplotype before treatment was associated with persistent parasitaemia in follow-up (P = 0.62). The proportion of patients in the DP and AL groups carrying the pfmdr1 haplotype NF increased from 6.1% and 4.6% at baseline to 58.8% (10/17) and 50.0% (7/14) at day 28 (OR 21.9, 95% CI 4.0-143.8, P<0.001; OR 21.0, 95% CI 2.9-227.5, P<0.001 for DP and AL, respectively). Corresponding figures for day 42 were 42.1% (8/19) and 53.3% (8/15) (OR 11.2, 95% CI 2.1-72.5, P=0.003; OR 24, 95% CI 3.5-254.7, P<0.001, respectively). Paired analysis of pre- and post-treatment pfmdr1 genotypes by McNemar’s test of asymmetry confirmed directional selection favouring the pfmdr1 NF haplotype at both day 28 (21 evaluable participants pooled across DP and AL groups, P < 0.001) and day 42 (23 evaluable participants, P = 0.002) (Fig. 3). There were insufficient data to stratify this analysis by treatment group.

Unfortunately, parasite densities were very low in the sub-patent parasite infections at day 28 and day 42, and insufficient material was available to perform qPCR-based genotyping of pfcrt or direct sequencing of pfk13 amplicons in this group of isolates.
DISCUSSION

We performed a survey of antimalarial drug resistance markers in north-western Indonesia to identify genetic polymorphisms present in the *P. falciparum* parasite population. We found that *pfk*13 variants, although rare, were present in parasites harbouring the SVMNT genotype at codons 72-76 of *pfcr*, which is the predominant haplotype in our three study sites. This contrasts with *P. falciparum* in the GMS, where *pfk*13 variant parasites carry the CVIET *pfcr* allele at codons 72-76 (30), together with additional acquired mutations associated with piperazquinine resistance at other *pfcr* codons (31). Decreased piperazquinine susceptibility is associated with the C350R *pfcr* polymorphism in French Guiana, where it occurs with the SVMNT haplotype at codons 72-76, although this is not linked to artemisinin resistance (32). Among a subset of symptomatic participants randomized to receive the ACT regimens DP or AL, we found strong evidence of directional selection on *pfmdr1*. In both drug arms the NF haplotype at codons 86 and 184 was much more abundant in persistent sub-patent parasites identified at day 28 or day 42 of follow-up than in the pre-treatment population. We identified only nine *pfk*13 propeller domain variant alleles with moderate to high confidence in the cross-sectional survey, six of which encoded the Thr to Ala change at codon 474.

We observed a high proportion of parasite genotypes associated with amodiaquine and chloroquine resistance in our samples with 76.5% carrying the *pfcr* haplotype SVMNT and 20.2% the CVIET haplotype. Despite the discontinuation of chloroquine in 2004 and subsequent introduction of ACT, the proportion of mutant 76T in this region remains above 90%, similar to pre-2004 data (19, 20), likely due to the use of ASAQ. This contrasts with data from East Africa where wild-type *pfcr* has recovered to high prevalence following the widespread deployment of AL (17). Evidence of treatment failure with ASAQ triggered a recent change in recommendations for treating *P. falciparum* infection in Indonesia (22 - 25). DP is now the approved first-line regimen, with AL licensed and widely available in the private sector. Recently, evidence has accumulated of decreased DP efficacy in western Cambodia, and the phenotype has been associated with increased copy number of the *plasmepsin II* gene and other emerging gene variants (9, 31, 33). This leads to concern that Indonesian parasites may also develop piperazquinine resistance, and studies of polymorphisms known to be associated with piperazquinine susceptibility are now needed. We found PCR-based evidence of sub-microscopic parasite persistence at D28 and/or D42 in both drug arms (30% of evaluable patients in the AL arm, 40% in the DP arm) (27), as has previously been observed in imported *P. falciparum* malaria cases in France (34).

The *pfmdr1* 86Y allele was formerly common in Southeast Asian region, but significantly decreased in frequency consistent with the abandonment of chloroquine and amodiaquine (35). A similar dramatic fall in the prevalence of 86Y was also observed in Nias, from 100% in 2003 (20) to 31.4% in 2005 (36). Nevertheless, this was not concomitant with an increase in abundance of wild-type *pfcr*. Our findings are consistent with these data, as *Pfmdr1* 86Y is at moderate prevalence, but accompanied by high prevalence of mutant *Pfcr* 76T (Fig. 2). 184F has also slowly disappeared in mainland Southeast Asia,
possibly driven by pressure from mefloquine, except in western Cambodia and eastern Thailand (37), but as mefloquine is not available in Indonesia, this cannot explain the relatively low prevalence of 184F in Sumatera. It is important to also recognize compelling evidence in the literature that artemisinins themselves directly select for the NF haplotype of pfmdr1, both in vivo (16) and in genome editing experiments in vitro (15).

We show a strong association between the pfcr t SVMNT and the pfmdr1 YY haplotypes among our parasite populations. Both alleles have been associated with amodiaquine resistance (12, 13, 35). The SVMNT haplotype is distributed across Indonesia, Papua New Guinea, East Timor, south Asia and, as an allele with an independent origin, in South America (18, 38, 39). However, these high grade amodiaquine-resistant parasites remain uncommon in most parts of mainland Southeast Asia and are absent from Africa, where CVIET predominates and amodiaquine may still be effective (37). The ongoing presence of these gene mutations in our study sites is likely the result of extended drug pressure from amodiaquine, as the partner drug in the previously recommended ASAQ regimen, and the continuing access to chloroquine in the private sector. This occurrence of SVMNT alleles may therefore explain the low clinical efficacy of ASAQ for treatment of P. falciparum infection observed in Indonesian efficacy studies (22 - 25).

Pfk13 propeller domain polymorphisms have been linked to reduced sensitivity to artemisinin in Southeast Asia and are thought to have emerged independently in Cambodia and Myanmar. The mutants CS80Y, R539T and M446I associated with slow clearance of P. falciparum after artesunate monotherapy or ACT are the most frequent and geographically specific in mainland Southeast Asia. In Eastern Indonesia, this trend has not been seen as only 0.9% of 106 samples from Sumba harboured the pfk13 allele G497V (29), and no pfk13 mutation was detected among 65 samples from southern Papua (40). In our study sites 6 of 9 variant isolates harbored the T474A propeller domain polymorphism, which is not prevalent in the GMS, although a T474I variant has been described (29). Codon 474 variants have not been associated with reduced susceptibility to artemisinin to date. We were unable to evaluate the impact of this genotype on parasite clearance, and phenotypic studies of these mutants are now needed to assess their significance.

We observed diversity in the P. falciparum genetic signature among the three study sites, which is in line with differences in transmission intensity, treatment-seeking behavior, access to health care and antimalarial use in these communities. However, our study was not designed to scrutinize the factors contributing to these differences in genetic profiles, and so their importance remains unclear. A limitation of our study was the difficulty of obtaining high quality genotypes from multiple loci in these parasite isolates, the majority of which were low density asymptomatic infections. Even among patients with clinical malaria enrolled in our prospective study, post-treatment isolates were difficult to analyse at all the loci of interest, even when evidence of persisting P. falciparum was obtained from at least one gene amplification. Another limitation of our study is the use of a convenience sampling approach (26), and this may have introduced bias in the proportion of drug resistance markers presented. Nevertheless, new evidence of mutations in the Pf k13 propeller domain in western Indonesia was found. The lack of information on the
associated phenotypic profiles warrants future studies to measure artemisinin susceptibility of these parasites \textit{in vivo} and \textit{in vitro}. We have also confirmed that selective impact of ACT favouring the \textit{pfmdr1} haplotype NF (codons 86, 184), originally described in African studies, is also clearly evident in Sumatera.

In summary, our study provides new information on the genetic profiles of \textit{P. falciparum} parasites in western Indonesia. We provide evidence of selective pressure from ASAQ in the recent past, including linkage disequilibrium between certain alleles of \textit{pfcr} and \textit{pfmdr1}, and evidence of more recent counter-selection by current regimens on the \textit{pfmdr1} locus in particular. This can guide antimalarial policy for ACT use in the country. We found no evidence that artemisinin-resistant parasites had spread from the nearby GMS. The presence of some \textit{Pfk13} mutations among the sampled parasite population is of potential concern and demonstrates the need to further evaluate artemisinin susceptibility of parasites from western Indonesia. DP and AL currently appear to be effective treatment options for \textit{P. falciparum} infection in North Sumatera, but further efficacy studies are needed.

\textbf{Word Count: 3581}
REFERENCES


Table 1. Non-synonymous single-nucleotide polymorphisms in the Pfk13 propeller domain of nine isolates in the community sample among 231 sequenced.

<table>
<thead>
<tr>
<th>Regency</th>
<th>ID</th>
<th>Codon</th>
<th>Coverage</th>
<th>Evidence*</th>
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<tbody>
<tr>
<td>Batubara</td>
<td>BB02030</td>
<td>mixed** T474A</td>
<td>both strands</td>
<td>high confidence</td>
</tr>
<tr>
<td></td>
<td>BB02033</td>
<td>mixed T474A</td>
<td>both strands</td>
<td>moderate confidence</td>
</tr>
<tr>
<td>BB13019</td>
<td>unmixed T535A, C542R</td>
<td>one strand</td>
<td>low confidence</td>
<td></td>
</tr>
<tr>
<td>BB22036</td>
<td>unmixed N523S, T535A, T593A</td>
<td>one strand</td>
<td>low confidence</td>
<td></td>
</tr>
<tr>
<td>Langkat</td>
<td>LK01061</td>
<td>mixed T474A, mutant peak low</td>
<td>both strands</td>
<td>moderate confidence</td>
</tr>
<tr>
<td></td>
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<td>mixed T474A</td>
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<td>South Nias</td>
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<td>both strands</td>
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<td></td>
<td>NS27031</td>
<td>mixed E461G, C580Y</td>
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<td>mixed synon a-&gt;g codon 521</td>
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* Only polymorphisms confirmed on all available DNA strand sequence reads are presented. Equivocal sequences, or polymorphisms observed on only one of two strands, were not considered to have been verified and were scored as wild-type. For isolates BB13019, BB22036 and NS 27031 only a single strand was available and so the results are presented as of low confidence.

** “mixed” denotes the presence of two different DNA sequences at the codon named in the isolate, indicative of a multi-clonal infection
Figure Legends

Figure 1. Prevalence of genotypes of interest in pfcr, pfmdr1 and pfk13 in a cross-sectional community sample in 3 Regencies

Genotypes are shown for (A) pfcr at codons 72-76 (B) codons 86/184 of pfmdr1 gene and (C) the pfk13 propeller domain in three study sites in North Sumatera province. Pfcr haplotypes were identified by multiplex qPCR, pfmdr1 and pfk13 genotypes were established by direct sequencing of PCR products (see Materials and Methods).

Denominators are:

(A) n=183 (n=49 for Batubara, n=39 for Langkat, n=95 for South Nias)

(B) n=261 (n=59 for Batubara, n=57 for Langkat, n=145 for South Nias)

(C) n=232 (n=66 for Batubara, n=60 for Langkat, n=106 for South Nias)

Figure 2. Pre-treatment prevalence of variants in codons of interest in the pfcr, pfmdr1, and pfkelch13 genes, by Regency.

Allele-specific qPCR (pfcr only) or direct sequencing of nested PCR products was used to enumerate P. falciparum alleles of interest present among pre-treatment samples from prospective trial participants (N=117). These alleles were at the following codons:

pfcr 72-76

pfmdr1 86/184

pfkelch13 474 (propeller domain)

Figure 3. Prevalence of pfmdr1 alleles in 15 and 13 individuals randomized to the DP and AL treatment groups, respectively, with PCR-detectable P. falciparum at days 28 or 42 during follow-up.

“Baseline” denotes the pre-treatment isolates in the same individuals evaluated at days 28 and 42. Pale blue colour denotes the wild-type allele, red the mutant allele associated with aminoquinoline resistance, and orange a mixture of both alleles present simultaneously.
Figure 2
Figure 3

**Pfmdr1 86**

- **DP**
  - Day 0: n=19
  - Day 28: n=12
  - Day 42: n=14

- **AL**
  - Day 0: n=14
  - Day 28: n=14
  - Day 42: n=13

**Pfmdr1 184**

- **DP**
  - Day 0: n=18
  - Day 28: n=12
  - Day 42: n=14

- **AL**
  - Day 0: n=14
  - Day 28: n=14
  - Day 42: n=12

Legend:
- N86
- Y86Y
- B6Y
- 86S
- Y184
- Y184F
- 184F