Plasmodium faciparum carrying *pf*k13 polymorphisms harbour the SVMNT allele of *pfcrt* 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 *pfcrt, 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 *pfcrt* 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 *pfcrt* 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 artemisinin-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 artemisinin and partner drugs has emerged in 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 dihydroartemisinin-piperaquine (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 artemisinins was first observed in the GMS over a decade ago (6). This is caused by mutations in the *P. falciparum* gene *pf*k13 which affect the propeller domain of the kelch-13 protein (7, 8). Amplification of *plasmepsin II* gene copy number is linked to piperaquine resistance in the same region (9). Resistance to aminoquinolines is known to be mediated by the putative transporter *pfcrt* (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 artemisinin 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 artemisinin 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 *pfcrt* (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 *pf*k13 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 *pf*k13*, pfcrt* and *pfmdr1* 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 *pfcrt*, *pfmdr1* and the *pf*kelch13 propeller domain using established methods with minor modifications. Polymorphisms at codons 72-76 in *pfcrt* were determined using multiplex qPCR (28). Polymorphisms at codons 86, 184, 1034, 1042 and 1246 in *pfmdr1* were identified by direct sequencing (Humphreys *et al.*, 2007). *Pf*k13 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 *pfmdr1* 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 *Pfcrt*

Pfcrt genotyping 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 *Pf*k13

*Kelch*13 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 *pf*k13 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 C580Y 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 *Pfcrt, Pfmdr1* and *Pf*k13 polymorphisms in the *P. falciparum* population

We investigated any evidence of linkage disequilibrium between the *pfcrt* and *pfmdr1* polymorphisms among isolates in our cross-sectional survey. Isolates carrying the SVMNT *pfcrt* 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 *pfcrt* SVMNT also carried the *pfmdr1* haplotype NF (7.0%), compared to 11 of 26 harbouring other *pfcrt* genotypes (OR 0.101, 95% CI 0.031 - 0.333; *P*<0.001). We observed that *pf*k13 propeller domain variant alleles were present in a background of *pfcrt* 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.

***Pfcrt, Pfmdr1* and *Pf*k13 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 *pfcrt*, *pfmdr1* and *pf*k13 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 *pfcrt* (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 *pfcrt* 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 *pf*k13*, w*ild-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 *pf*k13 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*=0.0003; 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 *pf*k13 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 *pf*k13 variants, although rare, were present in parasites harbouring the SVMNT genotype at codons 72-76 of *pfcrt,* which is the predominant haplotype in our three study sites. This contrasts with *P. falciparum* in the GMS, where *pfk13* variant parasites carry the CVIET *pfcrt* allele at codons 72-76 (30), together with additional acquired mutations associated with piperaquine resistance at other *pfcrt* codons (31). Decreased piperaquine susceptibility is associated with the C350R *pfcrt* polymorphism in French Guiana, where it occurrs 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 *pf*k13 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 *pfcrt* 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 *pfcrt* 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 piperaquine resistance, and studies of polymorphisms known to be associated with piperaquine 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 *pfcrt*. Our findings are consistent with these data, as *Pfmdr1* 86Y is at moderate prevalence, but accompanied by high prevalence of mutant *Pfcrt* 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 *pfcrt* 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).

*Pf*k13 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 C580Y, 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 *pf*k13 allele G497V (29), and no *pf*k13 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 *in vivo* and *in vitro*. We have also confirmed that selective impact of ACT favouring the *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 *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 *pfcrt* and *pfmdr1*, and evidence of more recent counter-selection by current regimens on the *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 *Pf*k13 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 *P. falciparum* infection in North Sumatera, but further efficacy studies are needed.

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453 **Table 1. Non-synonymous single-nucleotide polymorphisms in the *Pf*k13 propeller domain of**
454 **nine isolates in the community sample among 231 sequenced.**

Regency	ID	Codon	Coverage	Evidence*
Batubara	BB02030	mixed** T474A	both strands	high confidence
	BB02033	mixed T474A	both strands	moderate confidence
	BB13019	unmixed T535A, C542R	one strand	low confidence
	BB22036	unmixed N523S, T535A, T593A	one strand	low confidence
Langkat	LK01061	mixed T474A, mutant peak low	both strands	moderate confidence
	LK06042	mixed T474A	both strands	moderate confidence
	LK10083	mixed T474A	both strands	moderate confidence
South Nias	NS23031	mixed T474A	both strands	moderate confidence
	NS27031	mixed E461G, C580Y mixed synon a->g codon 521	one strand	low confidence

455

456 * Only polymorphisms confirmed on all available DNA strand sequence reads are presented. Equivocal sequences, or
457 polynorphisms observed on only one of two strands, were not considered to have been verified and were scored as
458 wild-type. For isolates BB13019, BB22036 and NS 27031 only a single strand was available and so the results are
459 presented as of low confidence.

460 ** "mixed" denotes the presence of two different DNA sequences at the codon named in the isolate, indicative of a
461 multi-clonal infection

462 **Figure Legends**

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

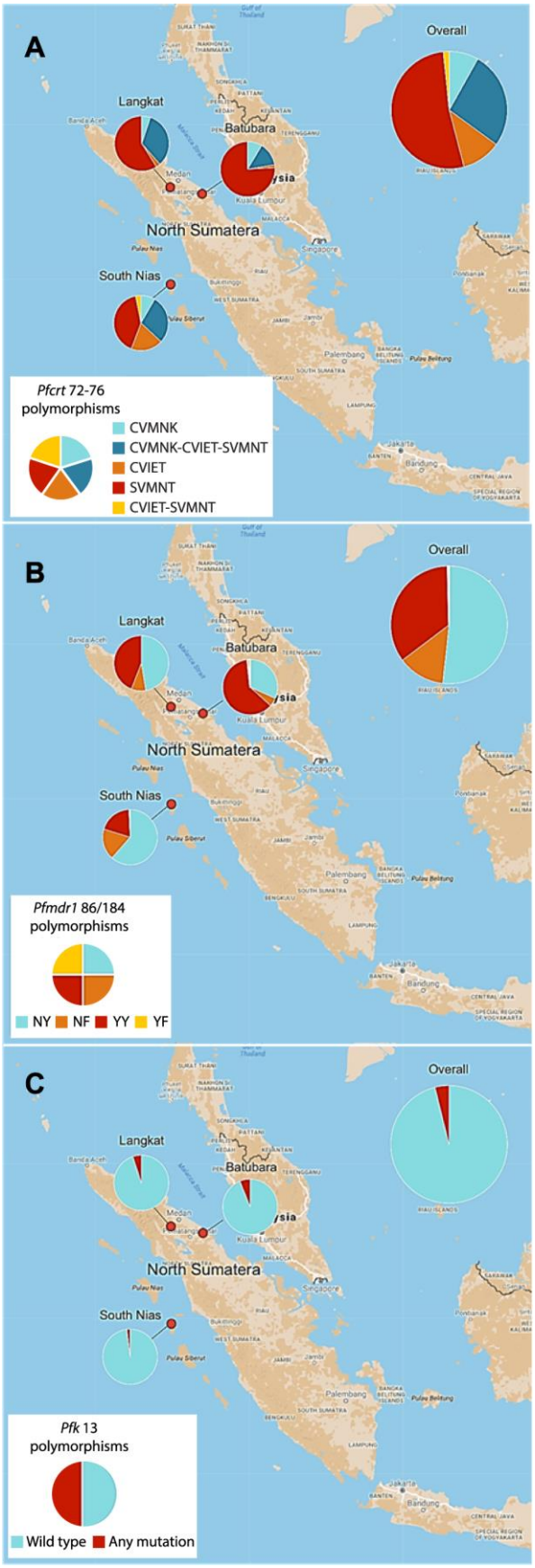
465 Genotypes are shown for (A) *pfcrt* at codons 72-76 (B) codons 86/184 of *pfmdr1* gene and (C)
466 the *pfk13* propeller domain in three study sites in North Sumatera province. *Pfcrt* haplotypes
467 were identified by multiplex qPCR, *pfmdr1* and *pfk13* genotypes were estabished by direct
468 sequencing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

469 Denominators are:
470 (A) *n*=183 (*n*=49 for Batubara, *n*=39 for Langkat, *n*=95 for South Nias)
471 (B) *n*=261 (*n*=59 for Batubara, *n*=57 for Langkat, *n*=145 for South Nias)
472 (C) *n*=232 (*n*=66 for Batubara, *n*=60 for Langkat, *n*=106 for South Nias)
473

474 **Figure 2. Pre-treatment prevalence of variants in codons of interest in the *pfcrt*, *pfmdr1*, and**
475 ***pfkelch13* genes, by Regency.**
476 Allele-specific qPCR (*pfcrt* only) or direct sequencing of nested PCR products was used to
477 enumerate *P. falciparum* alleles of interest present among pre-treatment samples from
478 prospective trial participants (N=117). These alleles were at the following codons:
479 *pfcrt* 72-76
480 *pfmdr1* 86/184
481 *pfkelch13* 474 (propeller domain)
482

483 **Figure 3. Prevalence of *pfmdr1* alleles in 15 and 13 individuals randomized to the DP and AL treatment**
484 **groups, respectively, with PCR-detectable *P. falciparum* at days 28 or 42 during follow-up.**
485 "Baseline" denotes the pre-treatment isolates in the same individuals evaluated at days 28 and
486 42. Pale blue colour denotes the wild-type allele, red the mutant allele associated with
487 aminoquinoline resistance, and orange a mixture of both alleles present simultaneously.

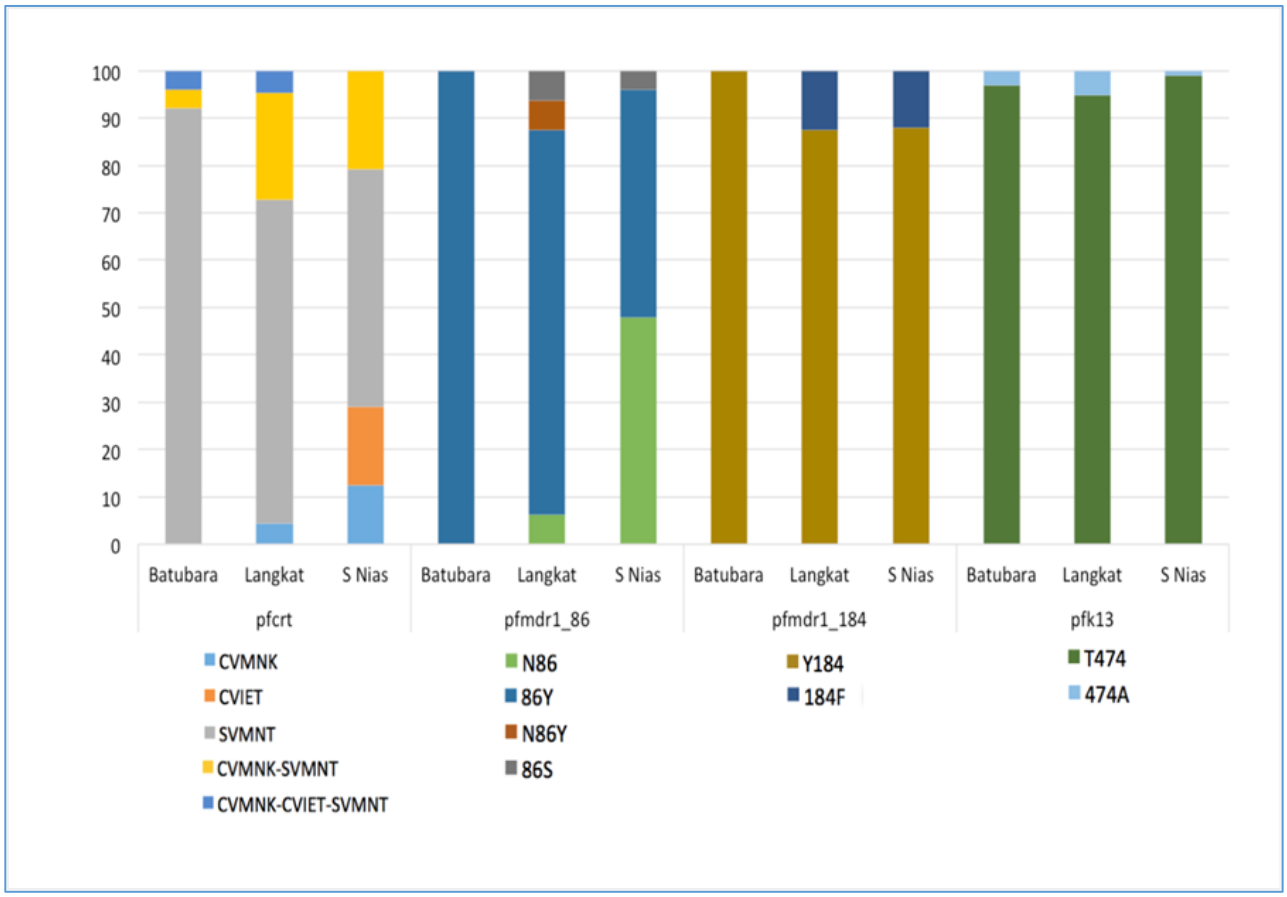
488 Figure 1


A
Overall
Langkat
Batubara
North Sumatera
South Nias
Pfcrt 72-76
polymorphisms
CVMNK
CVMNK-CVIET-SVMNT
CVIET
SVMNT
CVIET-SVMNT
B
Overall
Langkat
Batubara
North Sumatera
South Nias
Pfmdr1 86/184
polymorphisms
NY NF YY YF
C
Overall
Langkat
Batubara
North Sumatera
South Nias
Pfk 13
polymorphisms
Wild type
Any mutation

489 Figure 2

490

491



492 Figure 3

493