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MECHANISMS OF RESISTANCE

Plasmodium falciparum Isolates Carrying *pfk*13 Polymorphisms Harbor the SVMNT Allele of *pfcrt* in Northwestern Indonesia

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ABSTRACT Artemisinin-based combination therapy (ACT) is the first-line antimalarial regimen in Indonesia. Susceptibility of *Plasmodium falciparum* to artemisinin is falling in the Greater Mekong subregion, 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 to 76) was the most prevalent and displayed significant linkage disequilibrium with the *pfmdr1* haplotype YY (codons 86 and 184) (odds ratio [OR] 26.7; 95% confidence interval [CI], 5.96 to 239.4; $P < 0.001$). This contrasts with Mekong countries, where the CVIET haplotype of *pfcrt* predominates. Among 231 evaluable isolates, only 9 (3.9%) showed any evidence of nonsynonymous gene variants in the propeller domain of *pfk13*. The Thr474Ala variant was seen in six individuals, and Cys580Tyr was 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, the treatment outcome was not associated with pretreatment genotype. However, submicroscopic persistent parasites at day 28 or day 42 of follow-up were significantly more likely to harbor the *pfmdr1* haplotype NF (codons 86 and 184) than were pretreatment isolates ($P < 0.001$ for both treatment groups). Current ACT regimens appear to be effective in Sumatera, but evidence of persistent submicroscopic infection in some patients suggests further detailed studies of drug susceptibility should be undertaken.

KEYWORDS Indonesia, antimalarial agents, drug resistance mechanisms

Successful strategies for the elimination of malaria require effective first-line chemotherapies. Failure of the antimalarials chloroquine and sulfadoxine-pyrimethamine compromised malaria control strategies in many 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 2 decades (3). Nevertheless, decreased susceptibility of *P. falciparum* parasites to artemisinin and partner drugs has emerged in the Greater Mekong subregion (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.

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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 to 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 has been used in Indonesia since 2004 after efficacy of chloroquine was severely reduced by the spread of parasites harboring 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 countrywide 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 a lack of information on parasite polymorphisms in this study. Also of great concern is that artemisinin-resistant parasites harboring *pf*k13 mutants have now spread across Southeast Asia, and so, with their proximity to the Mekong and a 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 ACTs, artemether-lumefantrine (AL) and DP (27), and tested for evidence of an association between variants of these three loci and treatment outcomes.

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 submicroscopic, low-density parasitemia (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%) (Fig. 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%).

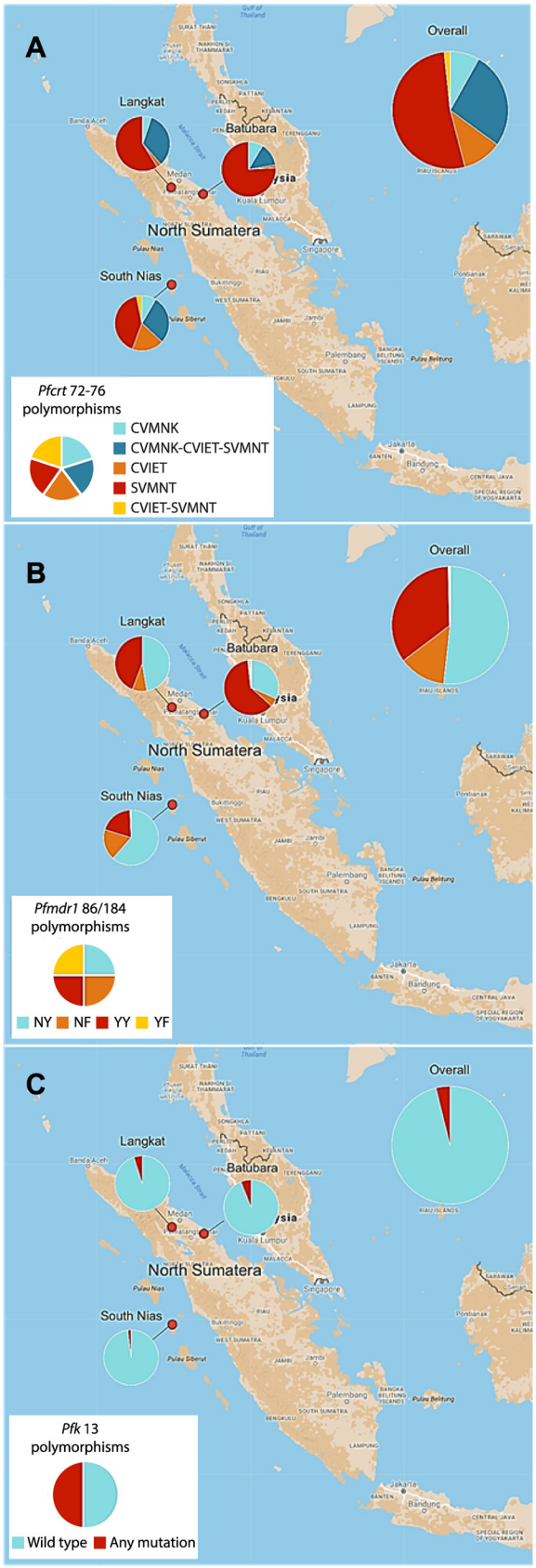

FIG 1 Prevalence of genotypes of interest in *pfcrt*, *pfmdr1*, and *pfk13* in a cross-sectional community sample in 3 regencies. Genotypes are shown for *pfcrt* at codons 72 to 76 (A), codons 86/184 of *pfmdr1*

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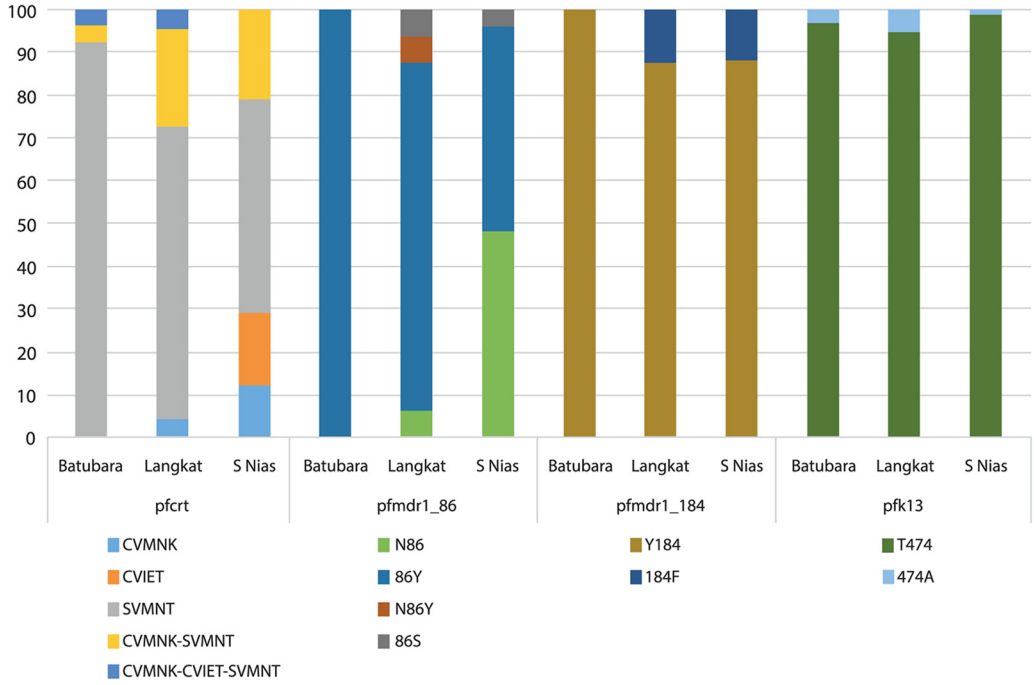

FIG 2 Pretreatment prevalence of variants in codons of interest in the *pfcrt*, *pfmdr1*, and *pfkelch13* genes by regency. Allele-specific qPCR (*pfcrt* only) or direct sequencing of nested PCR products was used to enumerate *P. falciparum* alleles of interest present among pretreatment samples from prospective trial participants ($n = 117$). These alleles were at the following codons: 72 to 76 (*pfcrt*), 86 and 184 (*pfmdr1*), and 474 (*pfkelch13*) (propeller domain).

Polymorphisms in *Pfmdr1*. Codons 25 to 201 of *pfmdr1* were successfully amplified and sequenced for 267 isolates (66.1%). The prevalence of the *Pfmdr1* N86 (Asn) wild-type allele was predominant overall (174/267, 65.2%) but varied 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 (Fig. 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 (Fig. 1B).

Population prevalence of polymorphisms in *Pf*k13. The *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

FIG 1 Legend (Continued)
gene (B), and the *pfk13* propeller domain (C) in three study sites in North Sumatera province. *Pfcrt* haplotypes were identified by multiplex qPCR; *pfmdr1* and *pfk13* genotypes were established by direct sequencing of PCR products (see Materials and Methods). Denominators are $n = 183$ ($n = 49$ for Batubara, $n = 39$ for Langkat, and $n = 95$ for South Nias) in panel A, $n = 261$ ($n = 59$ for Batubara, $n = 57$ for Langkat, and $n = 145$ for South Nias) in panel B, and $n = 232$ ($n = 66$ for Batubara, $n = 60$ for Langkat, and $n = 106$ for South Nias) in panel C.

TABLE 1 Nonsynonymous single-nucleotide polymorphisms in the *Pf*k13 propeller domain of nine isolates in the community sample among 231 sequenced

Regency	Identifier	Codon	Coverage	Evidence[a]
Batubara	BB02030	Mixed[b] 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[c]	One strand	Low confidence

[a]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 low confidence.
[b]"Mixed" denotes the presence of two different DNA sequences at the codon named in the isolate, indicative of a multiclonal infection.
[c]Nucleotide change at codon 521 did not alter the amino acid encoded.

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 nonsynonymous 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 (28). Although K13 polymorphisms occurred in all 3 sites, the prevalence was uniformly low, with 4 of 66 in Batubara, 3 of 60 in Langkat, and 2 of 106 in South Nias (Fig. 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 (odds ratio [OR], 26.7; 95% confidence interval [CI], 5.96 to 239.4; $P < 0.001$). Conversely, only 8 of 116 isolates harboring *pfcrt* SVMNT also carried the *pfmdr1* haplotype NF (7.0%), compared to 11 of 26 harboring other *pfcrt* genotypes (OR, 0.101; 95% CI, 0.031 to 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 unexpectedly high proportion of ACT-treated patients with persisting subpatent *P. falciparum* parasites, and so, we explored whether *pfcrt*, *pfmdr1*, and *pf*k13 genotypes in the pretreatment 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 to 76) dominated in both treatment groups (28/34 in the DP group [82.4%]; 35/37 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 pretreatment 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 pretreatment population for both ACT groups (32/49, 65.3% for DP; 36/47, 76.6% for AL), reflecting the high prevalence of this haplotype observed in the cross-sectional population survey (Fig. S1). The rare 86S allele was also identified in two individuals (Fig. 2). The 86N allele was common only in South Nias and rare in pretreatment isolates from the other 2 regencies. For *pf*k13, wild-type genotypes (96%, 72/75) dominated in the propeller domain. The T474A polymorphism was detected in 3 (4.0%) pretreatment isolates in the AL group, in each case, mixed with the wild-type sequence. All parasite isolates harboring *pf*k13 mutations also carried the SVMNT haplotype of *pfcrt*. We found no evidence of slow clearance by quantitative PCR (qPCR) during the first 72h following treatment with either ACT, except in a single DP-treated patient who 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 them 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 days 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 the presence of the NF haplotype before treatment was associated with persistent parasitemia 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 to 143.8; $P < 0.001$ and OR, 21.0; 95% CI, 2.9 to 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 to 72.5; $P = 0.0003$ and OR, 24; 95% CI, 3.5 to 254.7; $P < 0.001$, respectively). Paired analysis of pre- and posttreatment *pfmdr1* genotypes by McNemar's test of asymmetry confirmed directional selection favoring 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 subpatent 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 northwestern Indonesia to identify genetic polymorphisms present in the *P. falciparum* parasite population. We found that *pf*k13 variants, although rare, were present in parasites harboring the SVMNT genotype at codons 72 to 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 to 76 (29), together with additional acquired mutations associated with piperaquine resistance at other *pfcrt* codons (30). Decreased piperaquine susceptibility is associated with the C350R *pfcrt* polymorphism in French Guiana, where it occurs with the SVMNT haplotype at codons 72 to 76, although this is not linked to artemisinin resistance (31). 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 subpatent parasites identified at day 28 or day 42 of follow-up than in the pretreatment 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

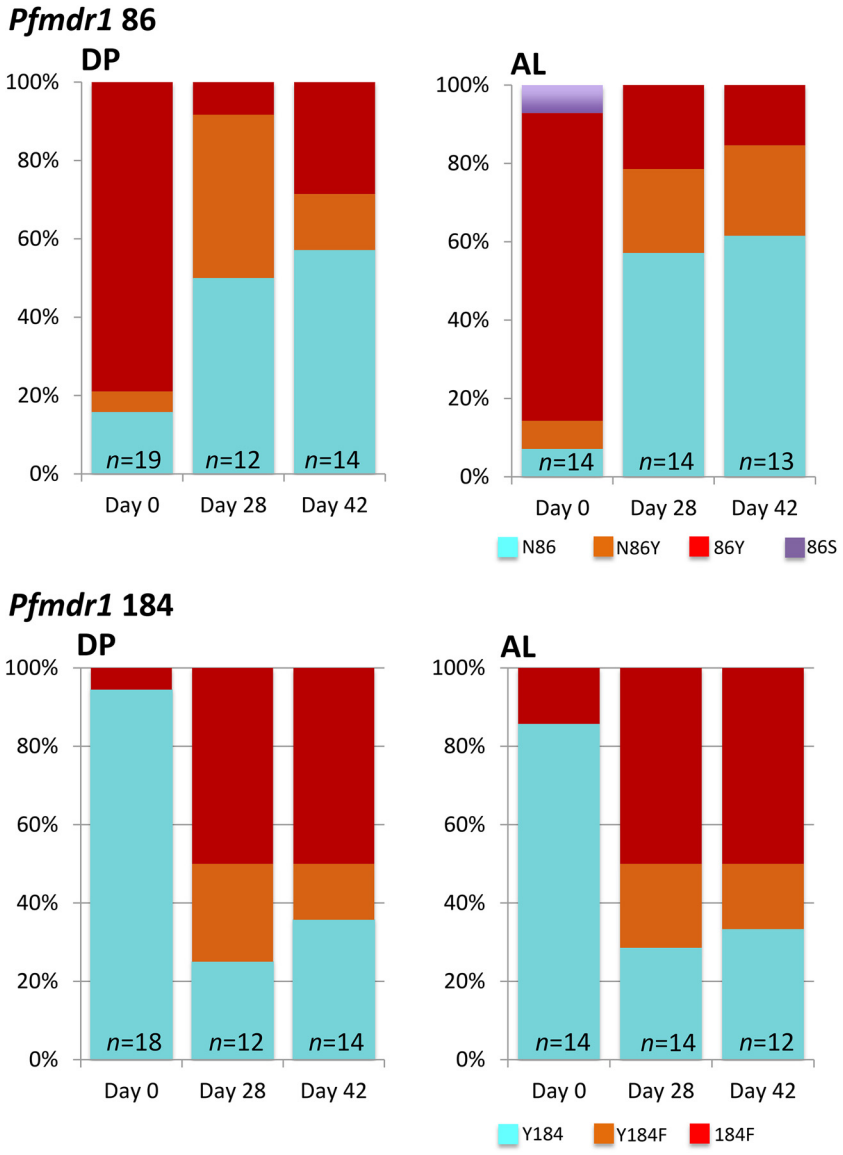

FIG 3 Prevalence of *pfmdr1* alleles in 15 and 13 individuals randomized to the DP and AL treatment groups, respectively, with PCR-detectable *P. falciparum* at days 28 or 42 during follow-up. "Baseline" denotes the pretreatment isolates in the same individuals evaluated at days 28 and 42. Pale blue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

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 an increased copy number of the *plasmepsin II* gene and other emerging gene variants (9, 30, 32). 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 submicroscopic 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 (33).

The *pfmdr1* 86Y allele was formerly common in the Southeast Asian region, but it significantly decreased in frequency, consistent with the abandonment of chloroquine and amodiaquine (34). A similar dramatic fall in the prevalence of 86Y was also observed in Nias, from 100% in 2003 (20) to 31.4% in 2005 (35). 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 a high prevalence of mutant *Pfcrt* 76T (Fig. 2). The 184F allele has also slowly disappeared in mainland Southeast Asia, possibly driven by pressure from mefloquine, except in western Cambodia and eastern Thailand (36), 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, 34). 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, 37, 38). 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 (36). 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 harbored the *pf*k13 allele G497V (28), and no *pf*k13 mutation was detected among 65 samples from southern Papua (39). 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 (28). 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, posttreatment isolates were difficult to analyze 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 favoring the *pfmdr1* haplotype NF (codons 86 and184), 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 counterselection 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.

MATERIALS AND 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 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 versus 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 (401/KOMET/FK USU/2014) and the London School of Hygiene and Tropical Medicine, United Kingdom (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 to 76 in *pfcrt* were determined using multiplex qPCR (40). Polymorphisms at codons 86, 184, 1034, 1042, and 1246 in *pfmdr1* were identified by direct sequencing (13). *Pf*k13 polymorphisms were identified by nested amplification and direct sequencing of PCR products (7, 28). 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 (ORs) with 95% confidence intervals (CIs), and significance was determined using the X^2 distribution. Linkage disequilibrium between loci was examined in 2 by 2 contingency tables.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

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