Directional Selection at the *pfmdr1*, *pfcrt*, *pfubp1*, and *pfap2mu* Loci of *Plasmodium falciparum* in Kenyan Children Treated With ACT

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Background. The efficacy of artemisinin-based combination therapy (ACT) for *Plasmodium falciparum* malaria may be threatened by parasites with reduced responsiveness to artemisinins. Among 298 ACT-treated children from Mbita, Kenya, submicroscopic persistence of *P. falciparum* on day 3 posttreatment was associated with subsequent microscopically detected parasitemia at days 28 or 42.

Methods. DNA sequences of resistance-associated parasite loci *pfcrt*, *pfmdr1*, *pfubp1*, and *pfap2mu* were determined in the Mbita cohort before treatment, on days 2 and 3 after initiation of treatment, and on the day of treatment failure.

Results. Parasites surviving ACT on day 2 or day 3 posttreatment were significantly more likely than the baseline population to carry the wild-type haplotypes of *pfcrt* (CVMNK at codons 72–76; $P < .001$) and *pfmdr1* (NFD at codons 86, 184, 1246; $P < .001$). In contrast, variant alleles of the novel candidate resistance genes *pfap2mu* (S160N/T; $P = .006$) and *pfubp-1* (E1528D; $P < .001$) were significantly more prevalent posttreatment. No genetic similarities were found to artemisinin-tolerant parasites recently described in Cambodia.

Conclusions. Among treated children in western Kenya, certain *P. falciparum* genotypes defined at *pfcrt*, *pfmdr1*, *pfap2mu*, and *pfubp1* more often survive ACT at the submicroscopic level, and contribute to onward transmission and subsequent patent recrudescence.

Keywords. artemisinin; drug resistance; genetics; malaria.

Over the last decade, the widespread deployment of artemisinin-based combination therapy (ACT), with improved vector control and other measures, has contributed to a reduction of malaria-related morbidity and mortality across sub–Saharan Africa [1, 2]. These public health gains may be lost if the efficacy of ACT against *Plasmodium falciparum* is not sustained. Recent evidence suggests that parasite genotypes have arisen in Cambodia and surrounding countries that are less rapidly cleared by artesunate monotherapy [3–6]. This has been interpreted by some as bona fide emerging artemisinin resistance [4, 6], and by others as "treatment failure," with full-blown resistance to ACT not yet present [7]. The efficacy of ACT remains high in Africa, with no reports to date of slow clearance as in Cambodia. However, evidence based on classical microscopic parasite detection suggests that a proportion of ACT-treated children in Kenya do not completely clear *P. falciparum* parasitemia [8]. More recently, quantitative polymerase chain reaction (qPCR)–detectable submicroscopic

residual parasitemia on day 2 or 3 post-ACT in Kenyan children was shown to be a significant risk factor for parasite transmission to mosquitoes, and for microscopically detected parasitemia recurring on day 28 or 42 [9]. Thus, residual asexual parasitemia, if genetically determined, may provide an enhanced likelihood of infecting the vector population, and thus further human hosts. Such evolutionary success, where parasites harboring genetic markers of drug resistance exhibit a transmission advantage, has previously been implicated in the development of resistance to chloroquine and antifolate malaria drugs [10–12].

The possibility that *P. falciparum* populations in Kenya may be less responsive to ACT therapy than previously reported provides a strong rationale for further investigation, for 2 reasons. First, identification of any heritable genetic signature for this phenotype would enable monitoring of its spread by tracking relevant molecular markers. Second, it is important to determine whether the submicroscopic persisting parasite phenotype described in Kenya is related to the more pronounced microscopically detectable slow clearance described from western Cambodia [3, 4], given important differences in transmission intensity, population prevalence of acquired immunity, and treatment policy. Genetic characterization of the persisting Kenyan parasites at candidate loci known or suspected to be under selection by ACT would address both objectives. Candidate loci include, first, markers for resistance to the quinoline drugs chloroquine and amodiaquine, known to be selected by ACT toward quinoline-sensitive alleles [13–16]. Second, new candidates *pcubp1* (encoding ubiquitin carboxyl-terminal hydrolase 1) and *pcap2mu* (encoding clathrin vesicle-associated adaptor 2, μ subunit), both implicated in artemisinin resistance in the rodent parasite *P. chabaudi*, have been shown to have polymorphic homologues in *P. falciparum* [17, 18]. These polymorphisms have yet to be fully validated as markers in parasites with reduced sensitivity to artemisinin derivatives in vivo. However, genome-wide association studies of *P. falciparum* isolates from coastal Kenya in which the *pfubp1* variant E1528D was associated with reduced susceptibility to artemisinin in vitro were recently reported [19].

To characterize any genetic signature of slow clearing submicroscopic infections in ACT-treated children from Mbita, Kenya [9], *pfcrt*, *pfmdr1*, *pfubp1*, and *pfap2mu* genotypes were determined on days 2 and 3, and on day of recrudescence in the case of later treatment failures. These were compared with pretreatment parasite genotypes isolated from children at baseline, and evaluated for evidence of posttreatment selection in vivo.

METHODS

Trial Design and Participants
As previously described in detail [20], 298 children aged 6 months to 10 years were randomly allocated to receive a 6-dose regimen of artemether-lumefantrine (AL) or a 3-dose regimen of dihydro-artemisinin-piperaquine (DP), fully observed, and followed for 42 days, according to a protocol approved by the Kenya Medical Research Institute Scientific Steering Committee (#1556, 2009) and the London School of Hygiene and Tropical Medicine Ethics Committee (#5455, 2009). Detailed parasite clearance dynamics were assessed by qPCR as described elsewhere [9].

Detection of Genetic Polymorphisms in Loci of Interest
Parasite DNA was extracted from approximately 10 μL blood spots collected on Whatman filter paper prior to treatment (day 0) and on days 2, 3, 7, 14, 28, and 42 after treatment as previously described [9]. Polymorphisms in the *pfcrt* gene (PF3D7_0709000), encoding the *P. falciparum* chloroquine resistance transporter, were determined in parasite DNA by multiplex qPCR as described [21]. Polymorphisms at codons 86, 184, and 1246 in the *pfmdr1* gene (PF3D7_0523000), encoding P-glycoprotein H1, were identified using direct sequencing of PCR products as previously described with minor modifications [16].

Polymorphisms in the *pfap2mu* gene (PF3D7_1218300), encoding the μ-subunit of clathrin-associated AP2 adaptor protein [18], were determined by PCR amplification and direct sequencing of 3 fragments encompassing codons 1–174, 121–399, and 377–621, as described [22].

Polymorphisms in *pfubp-1* (PF3D7_0104300) in the 300 base-pair region encompassing codons 1463–1563 were determined using a PCR strategy designed and optimized for this study. Nested PCR products were generated using the primer sets:

Nest 1 Forward Primer (pfubp1_1452_1F) CGCCCGTACTAT GAAGAAGATC
Nest 1 Reverse Primer (pfubp1_1612_1R) GGCTTTTACCT GAACTGTTCAGG
Nest 2 Forward Primer (pfubp1_1463_2F) CGTAAACAGAA TATTCAGGATTGC
Nest 2 Reverse Primer (pfubp1_1563_2R) CTAGCCCTTT ATTATCATTATCG

Amplification was performed with 2 nested reactions of 40 cycles of 3-step PCR, with 30 seconds annealing at 53°C (nest 1) or 57°C (nest 2) and elongation at 72°C for 45 seconds. Products were characterized by direct sequencing as described [22]. Ambiguous mixed haplotypes of *pfmdr1* were excluded from the analysis as previously described [14]. Mixed genotypes were included in the analysis at the *pfcrt* locus, where only 2 haplotypes were found at codons 72–76, and at both the *pfap2-mu* and *pfubp1* loci, for which only a single variable codon was evaluated.

Analysis of Locus Polymorphism in Global *P. falciparum* Genome Data
Single-nucleotide polymorphic residues in the *pfap2mu* and *pfubp1* loci were examined in Plasmoview (http://pathogenseq.

in an ordered set of 631 *P. falciparum* genomes stratified by geographical region.

Statistical Analysis

Binary variables were compared across pairs of categories, and odds ratios (ORs) with 95% confidence intervals (CIs) estimated. Significance was determined using χ^2 distribution. Directional selection in paired samples from the same patient at different time points was tested for statistical significance using McNemar's symmetry test. All analyses were performed in STATA 11 (Timberlake Associates, College Station, TX).

RESULTS

Codons 72–76 of *pfcrt*

The wild-type *pfcrt* allele, encoding CVMNK at codons 72–76 of the CRT protein, was confirmed as present in 143 of the 278 (51.4%) evaluable parasite isolates prior to treatment (day 0), of which 89 also harbored the CVIET allele. The remaining 135 isolates (48.6%) were CVIET alone. The CVMNK allele was significantly more common among submicroscopic infections at day 3, being detected in 66 of the 73 successfully evaluated (90.4%; OR, 8.90; 95% CI, 3.88–23.7; $P < .001$). A significant proportion on day 3 (68%) were mixed infections, comprising both the CVMNK and CVIET genotypes. Thirty-five of the 65 children with detectable CVMNK parasites at day 3 did not carry this genotype at day 0, compared to only 3 children presenting with CVMNK but having only CVIET detected at day 3, demonstrating significant asymmetry, interpretable as strong directional within-host selection for the CVMNK allele ($\chi^2 = 26.95$, 1 degree of freedom; $P < .001$). This effect remained significant after stratification for treatment group (Table 1). These findings suggest that in those children with both *pfcrt* genotypes present prior to treatment, CVMNK significantly increased in relative abundance from day 0 to day 3. There was

evidence that carriage of CVMNK prior to treatment was associated with risk of parasite recurrence at day 28 or day 42 for AL (OR, 2.461, 95% CI, 1.004–6.23; $P = .030$) but not for DP (OR, 1.405, 95% CI, .154–17.3; $P = .714$).

Codons 86, 184 and 1246 of *pfmdr1*

Previous studies have proposed that the haplotype NFD at codons 86, 184, and 1246 is associated with parasite recurrence after AL. We collated information at these 3 positions into haplotypes, using strict criteria to eliminate ambiguity due to mixed infections by excluding all isolates with mixed alleles called at 2 or more of the codons of interest [14]. The proportion of children carrying the haplotype NFD prior to treatment was 38.4%, but this rose to 69.1% on day 3 (N = 42; OR, 3.59; 95% CI, 1.68–7.95; $P < .001$). However, this strict haplotype definition may have led to biased loss of data from pretreatment isolates because of higher parasite density, and thus greater clonal multiplicity. The analysis was repeated using less stringent criteria for assigning *pfmdr1* haplotypes (ie, assigning the NFD haplotype to all ambiguous isolates where this may have been present). This also showed significantly higher prevalence of the NFD haplotype (sensu lato) on day 3 than in the baseline population (N = 48 at day 3; OR, 4.31, 95% CI, 2.09–9.30; $P < .001$). Paired analysis on both days 0 and 3 at all 3 codon positions was available for 38 children, and evidence of significant within-host directional selection was found for the NFD haplotype sensu stricto ($P = .0016$). Despite loss of statistical power, stratification by treatment group suggested that this effect occurred in both the AL (N = 22) and DP arms (N = 15) ($P = .011, .059$, respectively; Table 1). Presence of the NFD haplotype at day 0 was not associated with risk of recrudescence in either arm (data not shown).

Polymorphisms in *pfap2mu*

As this is the first study to examine the role of *pfap2mu* polymorphisms in a prospective study of ACT sensitivity, sequence

Table 1. Prevalence of *pfcrt* and *pfmdr1* Haplotypes Before and After Treatment With Artemether-Lumefantrine or Dihydroartemisinin-Piperaquine

Gene	Odds Ratio: Occurrence on Day 3 vs Baseline (95% CI)	Directional Selection Within-Host[a]		
		LOSS (Frequency)	GAIN (Frequency)	*P* Value
pfcrt codons 72-76 CVMNK				
AL (N = 43 143)	10.32 (3.42–41.4) **P < .001**	1	15	**P < .001** (N = 43)
DP (N = 30 135)	7.64 (2.17–40.8) **P < .001**	1	9	**P = .0114** (N = 30)
Combined ACT (N = 73 278)	8.90 (3.88–23.69) **P < .001**	2	24	**P < .001** (N = 73)
pfmdr1 codons 86, 184, 1246 NFD				
AL (N = 26 101)	3.15 (1.19–8.60) **P = .001**	1	9	**P = .0114** (N = 22)
DP (N = 15 104)	8.52 (1.76–80.3) **P = .002**	1	6	P = .0588 (N = 15)
Combined ACT (N = 42 206)	3.59 (1.68–7.95) **P < .001**	2	15	**P = .0016** (N = 38)

Abbreviations: ACT, artemisinin-based combination therapy; AL, artemether-lumefantrine; DP, dihydro-artemisinin-piperaquine.

[a] Change in genotype in the same patient between day 0 and day 3 is scored as loss or gain; significance tested using McNemar's test of asymmetry.

Table 2. Synonymous and Nonsynonymous Single-Nucleotide Polymorphisms in the *pfap2-mu* Gene, and Prevalence at Day 0, Day 2/3, and Day of Failure After ACT Treatment

Codon	Reference Sequence	AA	Nucleotide Substitution	AA Changes	Prevalence Day 0 (N = 244)[a]	Prevalence Day 2/3 (N = 32)	Prevalence Day Failure (N = 28)
5	G	Leu	15A	. . .	8.4%	4.2%	No data
146	G	Arg	437A	Lys	1.2%	0%	3.6%
160	G	Ser	479A	Asn	18.0%	37.5%	25.0%
			479C	Thr	1.2%	3.1%	0%
161	G	Val	483A	. . .	0.4%	0%	0%
162	T	Ile	486C	. . .	9.8%	3.1%	0%
163	A	Glu	489G	. . .	16.8%	18.8%	17.9%
188	A	Arg	564G	. . .	0.8%	3.1%	0%
199	A	Lys	596C	Thr	3.3%	3.1%	7.1%
200	A	Asn	598T	Tyr	0.4%	0%	0%
233	T	Asn	699G	Lys	0.4%	0%	0%
337	C	Ala	1010G	Gly	1.2%	0%	No data
324	T	Cys	1026C	. . .	0.6%	0%	No data
437	C	Phe	1311A	Leu	4.2%	9.4%	No data
476	T	Ser	1428G	. . .	4.2%	3.1%	No data
478	A	Val	1434T	. . .	2.4%	0%	No data

Abbreviations: AA, amino acid; ACT, artemisinin-based combination therapy.

[a] The middle sequencing fragment (codons 121–399) was successfully analyzed in all isolates. Codon 5 was not determined in 2 isolates without fragment 1 data; codons 5, 337, 324, 437, 476, and 478 were not determined in 74 isolates without fragment 1 or 3 data, and codons 337, 324, 437, 476, and 478 were not determined in 1 isolate without fragment 3 data.

data was obtained at all 621 codons for 166 pretreatment isolates, and for codons 121–399 for a further 78 isolates. A variety of mutations, including synonymous and nonsynonymous single nucleotide polymorphisms, deletions, and insertions, were identified within this gene in the study population, and these are summarized in Tables 2 and 3. We were able to assemble 57 distinct haplotypes from the 166 full-length *pfap2mu* sequences, 38 of which only occurred once. The most common haplotype, found in 32 pretreatment isolates, was identical to the reference sequence from 3D7.

Pilot analysis of the first 20 isolates found to have persisting parasites on day 2 or day 3 identified the nonsynonymous polymorphism at codon 160, Ser to either Asn or Thr (Table 2), as differing in prevalence between pre- and posttreatment infections. We therefore focused on this mutation for detailed analysis. Among 244 evaluable pretreatment samples, 18.3% and

1.2% carried the Asn or Thr alleles, respectively, at codon 160 of *pfap2mu*. In contrast, combining 32 evaluable day 2 and day 3 isolates, the prevalence of parasites with either the S160N or S160T variant rose immediately following ACT treatment to 40.6% (OR, 2.87; 95% CI, 1.20–6.61; $P = .006$). Among these 32 individuals, evidence of directional selection for either variant was found between day 0 and day 2/3 ($P = .046$). This selection was not seen at the day of failure, when the overall prevalence of codon 160 mutations was 25% (Table 2). There was no evidence that carriage of the S160N or S160T variant prior to treatment was associated with risk of recurrent infection (data not shown).

Polymorphisms in *pfubp1*

An amplification strategy was designed for a region of *pfubp1* from codons 1463–1563, encompassing the polymorphic

Table 3. Indels Identified in the *pfap2-mu* Gene, Consequent Amino Acid Replacements, and Prevalence at Day 0, Day 2/3, and Day Fail After Treatment

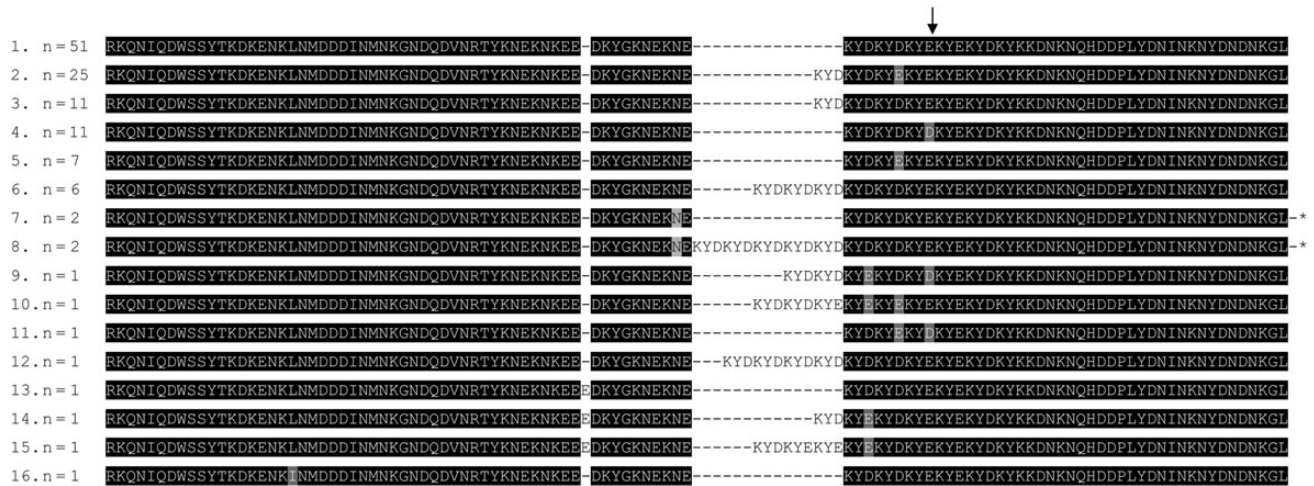| Codon	Reference Sequence	Deletions/ Insertions	Prevalence Day 0 (N = 244)	Prevalence Day 2/3 (N = 32)	Prevalence Day Failure (N = 28)
226	7xAsn	5xAsn/6xAsn	0.4%/0.4%	0%/0%	0%/0%
		8xAsn/9xAsn	17.6%/1.2%	9.4%/3.1%	25.0%/0%
233	1xLys	2xLys	2.0%	3.1%	7.1%
319	5xAsn	6xAsn	12.7%	6.3%	42.9%
326	4xAsn	5xAsn	13.1%	9.4%	3.6%

Figure 1. Haplotype frequencies at codons 1463–1563 of *pfubp1* in 123 pretreatment isolates. A 303-nucleotide fragment was amplified from genomic DNA using primers described in the text, and sequenced directly. Sequences were ordered from the most frequent to the least, and aligned in Clustal W and formatted in Boxshade, with additional hand-editing for clarity. Codon 1258, as identified by Borrman et al [19] is arrowed. Haplotype 1 is identical to the sequence found in the 3D7 reference genome. *Haplotypes 7 and 8 carry a synonymous mutation at codon 1518, encoding Asn.

codon 1528 identified by Borrmann et al [19] as being associated with in vitro parasite responses to artemisinin in Kenya. Direct sequencing of this region was successful in 123 pretreatment isolates, and identified 16 distinct haplotypes defined by synonymous and nonsynonymous point mutations, indels of 1 to 15 amino acids and repeat number variations in low-complexity nucleotide tracts (Figure 1). We focused on the codon E1528D substitution, which occurred in 12 of 123 evaluable individuals (9.8%) prior to treatment. Day 3 finger-prick samples had been largely exhausted by investigations of the other 3 loci, and by previous qPCR analysis [9], but sequencing of *pfubp1* was possible for 35 samples from day 3, of which 6 (17.1%) encoded Asp at codon 1528. To increase the sample, we amplified this region from 33 parasitological failures identified by microscopy on day 28 or day 42 [20], of which 14 (42.4%) carried 1528D. Thus evidence was found that the E1528D mutation was significantly more common among parasite isolates after treatment (days 3, 28, or 42) than among the pretreatment population (OR, 3.85, 95% CI, 1.63–9.32; $P < .001$). Paired analysis in the 36 evaluable individuals (i.e. those with *pfubp1* sequence data on day 0 and at least 1 of day 3, day 28, or day 42) showed strong evidence of directional selection ($P = .002$). Carriage of the 1528D variant at day 0 was not associated with risk of recurrent infections (data not shown).

Polymorphism of *pfap2mu* and *pfubp1* in the Global Genome Sequence Database

Having identified 2 novel candidate loci contributing to the genetic signature of persisting submicroscopic *P. falciparum* in our treated Kenyan children, we sought to examine diversity in these 2 loci across 631 genomes using PlasmoView software

[23]. For *pfap2mu*, the S160N/T polymorphism occurred across both West African (Burkina Faso, Gambia, Ghana, Mali, Senegal) and East African (Kenya, Malawi) parasite populations. This polymorphism did not occur in SE Asian populations (Thailand, Cambodia, Vietnam) nor in Papua New Guinea (Figure 2, top). For *pfubp1*, diversity across the locus was less geographically distinct, and mutations around codons 1520–1540 occurred in all populations (Figure 2, bottom). By contrast, the recently described polymorphisms at codons 493, 539, 543, and 580 of the kelch gene (PF3D7_1343700), associated with artemisinin sensitivity in Cambodia [6], were restricted to SE Asia, and do not occur in available African *P. falciparum* genomes. In contrast, the kelch polymorphism K189T was very common among African isolates but absent from Asian parasites included in the publicly available sample (data not shown).

DISCUSSION

We have genetically characterized submicroscopic *P. falciparum* infections in ACT-treated children from Mbita, Kenya, and described a novel genetic signature at the *pfcrt*, *pfmdr1*, *pfap2mu*, and *pfubp1* candidate drug-resistance marker loci. For 3 of these loci, the signature was already present 3 days after treatment with either AL or DP. For *pfubp1*, selection was seen on day of recrudescence in the case of later treatment failures. By microscopy, all children were parasite-free at day 3 and afebrile, and thus these were persisting, submicroscopic and asymptomatic infections which, in some cases, contributed to mosquito transmission potential on day 7 and patent recrudescent parasitemia on day 28 or day 42 [9, 20]. The evidence that these variant genotypes were overrepresented on day 3, immediately
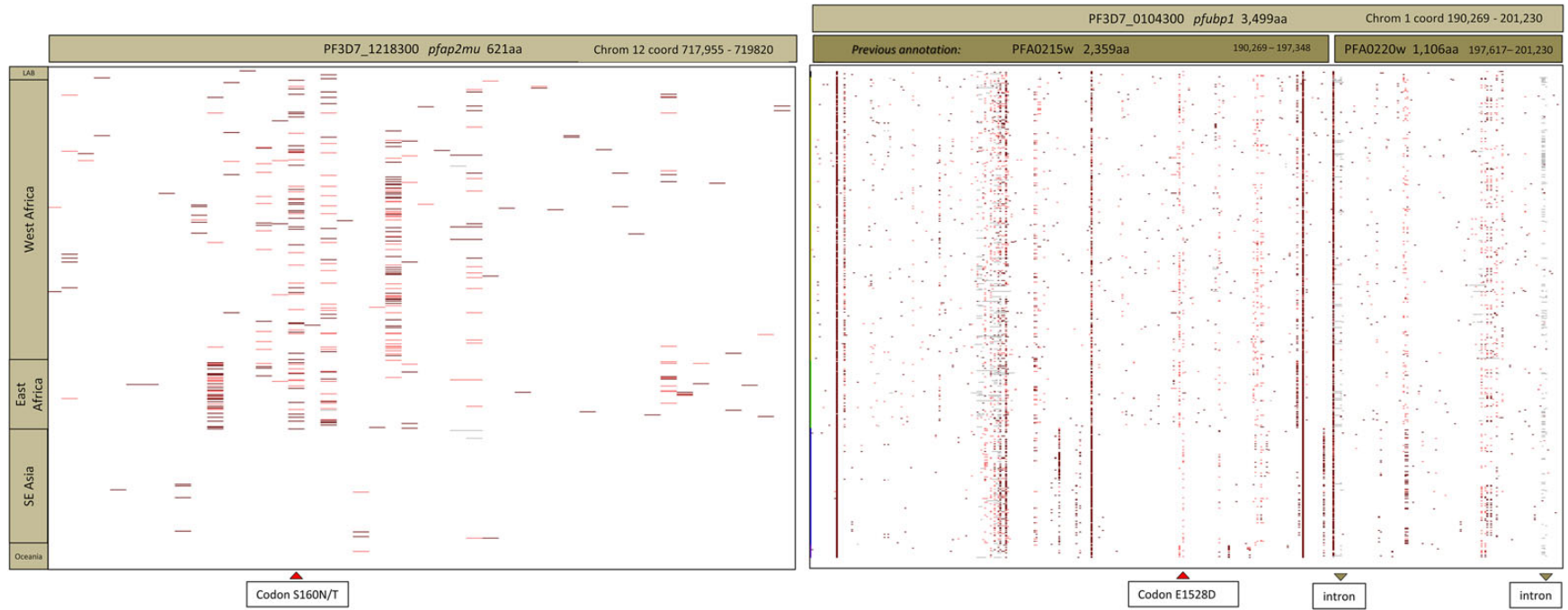
Figure 2. Global sequence diversity in *pfap2mu* (left) and *pfubp1* (right) across genome sequences from 631 *P. falciparum* isolates mapped to the 3D7 reference genome [23]. Colored bars at any chromosome position (horizontal axis) denote a nonreference substitution at that residue in the relevant isolate (vertical axis). At top is the regional origin of isolates in the analysis, comprising 8 laboratory isolates (L), 367 isolates from West Africa, 88 from East Africa, 151 from SE Asia, and 17 from Oceania (Oc). Codon S160N/T of *pfap2mu* and codon 1528 of *pfupb1* are highlighted by red arrows at the bottom of the figure. For *pfubp1*, the position of 2 carboxy-terminal introns is also marked. Previous annotation of this region of chromosome 1 as 2 distinct genes (PFA0215w and PFA0220w) is shown above the right-hand figure.

following completion of the full (observed) course of either AL or DP, suggests that selection is exerted partly or primarily by the artemisinin component of combination therapy, and that *P. falciparum* response to artemisinin in vivo is modulated by variation at these loci. Although not directly shown by our data, this interpretation is supported by a recent study in Uganda [24], which found that lumefantrine and piperaquine did not select for the same haplotypes of *pfmdr1* in recurrent infections, implying that early selection for the NFD haplotype is exerted by the artemisinin component in both treatment arms.

Previous studies have documented selection at the *pfcrt* and *pfmdr1* loci in late recurrent infections in ACT-treated African children [13–16], but this is the first demonstration of selection on day 3. The favored CQ-sensitive haplotypes *pfcrt* CVMNK and *pfmdr1* NFD were supplanted in the late 20th century by the spread of CQ resistance. Thus, it is difficult to distinguish between a general disadvantage in vivo caused by the relative lack of fitness of CQ-resistant *P. falciparum* in the absence of CQ [25, 26], and a specific interaction between these loci and artemisinin. Our study was not powered, nor designed, to explore interlocus interactions, but we observed both *pfmdr1*-NFD and *pfcrt*-CVMNK in 43 of 204 evaluable patients on day 0, and in 21 of 34 evaluable submicroscopic infections on day 3 (OR, 6.05, 95% CI, 2.62–14.2; $P < .001$). This may suggest the benefit of the 2 wild-type genes was enhanced by the presence of artemisinin. Another weakness of our study was that some isolates of interest were not sequenced at all loci, mainly due to exhaustion of small, finite blood samples. Although the limited number of observations in each of the treatment arms precluded comparisons of effect estimates due to lack of power, an exploratory stratification was carried out, generating odds ratios with very wide confidence intervals (data not shown). The directional selection for *pfap2mu* 160 (AL: $P = .015$; DP: $P = .063$) and *pfubp1* (AL: $P = .0002$; DP: $P = .029$) remained evident in both arms. Future studies with adequate power can now be designed using these estimates of prevalence and effect size. Moreover, age and multiplicity of infection were associated with parasite persistence in the Mbita trial [9], and thus are also potential confounders. These parameters should be investigated by multivariate regression in studies with sufficient power to further dissect the interplay among parasite genotypes, host factors, and different treatment regimens.

This study provides the first preliminary evidence that *P. falciparum* response to artemisinin in vivo may be modulated by polymorphisms in the loci *pfap2mu* and *pfubp1*, both homologues of genes identified in studies of experimentally induced artemisinin resistance in the rodent parasite *P. chabaudi* [17, 18]. Clathrin-mediated endocytosis is a mechanism common to eukaryotic cells by which membrane-associated proteins and other cargo are transported from the plasma membrane, *trans*-Golgi network, or endosomes to acceptor compartments; the adaptor complex 2 is associated with cell surface endocytosis,

recruiting to the vesicle both cargo and structural components including clathrin, and may have a role in hemoglobin trafficking in malaria parasites. The ubiquitination of proteins modulates endocytosis, and proteosomal recycling, with known substrates including P-glycoproteins such as Pgh1 (encoded in malaria parasites by *pfmdr1*) [27]. Thus, these 2 loci may both contribute to endosomal digestion of hemoglobin in malaria parasites [18].

The persistence of microscopically undetectable parasites following treatment of clinical malaria does not constitute in vivo antimalarial resistance as normally defined, and may have been equally common in the pre-ACT era. The application of a novel endpoint, qPCR positivity within 72 hours of treatment, has merely permitted this to be observed for the first time. In settings where a significant proportion of malaria patients are semi-immune, newly acquired super-infecting parasites may flourish and cause symptomatic malaria, despite the presence of preexisting "symbiotic" genotypes hitherto stably suppressed by the immune response, but adapted to continuing survival. As we have previously hypothesized [28], it may be these preexisting parasites that remain in some individuals after drug clearance of the symptomatic infection. This hypothesis is only plausible if current ACT regimens are ineffective against low density parasitemia, but recent data do suggest this is the case [28, 29]. Evaluation of current and alternative regimens against asymptomatic parasitemia, using appropriate study protocols specifically designed for this purpose, is required to test this possibility.

Our interpretation is that the phenomenon studied here is not antimalarial resistance per se. Consistent with this, the genetic signature described does not resemble that of the "artemisinin-resistant" parasites associated with the slow-clearance phenotype in Cambodia. First, the latter do not carry the CVMNK haplotype of *pfcrt* and, second, *pfmdr1* polymorphisms are not associated with the phenotype [30]. Finally, we found no evidence of codon 160 polymorphism in *pfap2mu* occurring in SE Asian isolates in the available genomic sequence database, nor evidence of kelch propeller domain polymorphism in any African isolates [6]. We thus conclude that the persisting submicroscopic parasites observed in our cohort of ACT-treated Kenyan children are not related to slow-clearing parasites as seen in Cambodia and nearby countries. Only polymorphisms in *pfubp1* may be shared determinants in Cambodia and Kenya. PF3D7_0104300 encompasses 2 loci, PFA0215w and PFA0220w, from earlier genome annotations (Figure 2B), and variant alleles of the former were identified in subpopulations of *P. falciparum* in Cambodia associated with slow clearance after artesunate monotherapy [31].

We have described a novel genetic signature at 4 loci in *P. falciparum*, and implicate these genes in modulating in vivo parasite response to artemisinin. Further evaluation in purpose-designed, adequately powered studies of asymptomatic malaria in vivo, and in genetically modified *P. falciparum* in

vitro, are needed to determine the potential public health implications of these observations, and the utility of these loci as markers of artemisinin sensitivity in natural populations of *P. falciparum* worldwide.

Notes

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