MAJOR ARTICLE

Comparative Impacts Over 5 Years of Artemisinin-Based Combination Therapies on *Plasmodium falciparum* Polymorphisms That Modulate Drug Sensitivity in Ugandan Children

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(See the editorial commentary by Taylor and Juliano on pages 335–7.)

Background. Artemisinin-based combination therapies, including artemether-lumefantrine (AL) and dihydroartemisinin-piperaquine (DP), are recommended to treat uncomplicated falciparum malaria. Sensitivities to components of AL and DP are impacted by polymorphisms in *pfmdr1* and *pfcrt*. We monitored changes in prevalences of polymorphisms in Tororo, Uganda, from 2008 to 2012.

Methods. Polymorphic loci in *pfmdr1* and *pfcrt* were characterized in samples from 312 children randomized to AL or DP for each episode of uncomplicated malaria (50 samples per arm for each 3-month interval) utilizing a fluorescent microsphere assay. Treatment outcomes and impacts of prior therapies were also characterized.

Results. Prevalence increased significantly over time for *pfmdr1* N86 (AL: odds ratio [OR], 2.08 [95% confidence interval {CI}, 1.83–2.38]; DP: 1.41 [95% CI, 1.25–1.57]), *pfmdr1* D1246 (AL: 1.46 [95% CI, 1.29–1.64]; DP: 1.36 [95% CI, 1.23–1.50]), and *pfcrt* K76 (AL: 3.37 [95% CI, 1.85–6.16]; DP: 5.84 [95% CI, 1.94–17.53], and decreased for *pfmdr1* Y184 (AL: 0.78 [95% CI, .70–.86]; DP: 0.84 [95% CI, .76–1.50]); changes were consistently greater in the AL arm. Recent AL treatment selected for *pfmdr1* N86, D1246, and 184F in subsequent episodes; DP selected for the opposite alleles.

Conclusions. Genotypes with decreased sensitivity to AL components increased over time. This increase was greater in children receiving AL, suggesting that the choice of treatment regimen can profoundly influence parasite genetics and drug sensitivity.

Clinical Trials Registration. NCT00527800.

Keywords. *Plasmodium falciparum*; artemether-lumefantrine; dihydroartemisinin-piperaquine; *pfcrt*; *pfmdr1*.

Artemisinin-based combination therapies (ACTs) have shown excellent efficacy and are now recommended to treat falciparum malaria in nearly all countries [1].

Received 19 December 2013; accepted 30 January 2014; electronically published 8 March 2014.

Presented in part: 62nd Annual Meeting of the American Society of Tropical Medicine and Hygiene, Washington, D.C., 13–17 November 2013. Abstract 504.

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The Journal of Infectious Diseases 2014;210:344–53

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DOI: 10.1093/infdis/jiu141

ACTs include potent, short-acting artemisinins that rapidly reduce parasite biomass and alleviate malaria symptoms and longer-acting partner drugs that improve antimalarial efficacy and reduce the risk of selection for artemisinin resistance [2]. However, as partner drugs circulate well after artemisinins have been cleared, there is concern that subsequent infections will be exposed to subtherapeutic concentrations, facilitating the selection of parasites with reduced sensitivity to the partner drugs.

Artemether-lumefantrine (AL) is the most widely recommended ACT in Africa and the national malaria treatment regimen in Uganda [1, 3]. It has shown outstanding efficacy [4, 5], but treatment selects in recurrent *Plasmodium falciparum* infections for polymorphisms

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in *pfcrt* and *pfmdr1* [6–11]—genes encoding 2 putative drug transporters—that reduce sensitivity to artemether, lumefantrine, and other antimalarial drugs [12–15]. AL exerts an opposite selective pressure compared to that of the aminoquinolines chloroquine and amodiaquine. Specifically, the aminoquinolines select for the mutant *pfcrt* 76T, *pfmdr1* 86Y, and *pfmdr1* 1246Y alleles, which decrease sensitivity to these drugs, whereas AL selects for the wild-type alleles [6–11, 16]. AL also selected for the I876 polymorphism in *pfmrp1*, which encodes another putative drug transporter, in Tanzania [17]. Three additional *pfmdr1* polymorphisms (S1034C, N1042D, and increased gene copy number) are associated with altered sensitivity to some drugs, but are primarily seen outside of Africa [12, 15, 18–20]. Ex vivo sensitivities of field isolates to lumefantrine have varied widely, but clinically relevant resistance does not appear to be a problem [14, 21, 22]. Analysis of parasites selected in vitro for high-level lumefantrine resistance demonstrated multiple differentially expressed genes, including *pfmdr1*, but the phenotype was unstable [23].

Dihydroartemisinin-piperaquine (DP) has shown excellent efficacy in clinical trials in Africa [16, 24–27], but has only been adopted as a first-line therapy in Southeast Asia [1]. Particularly in areas with high malaria transmission intensity, DP benefits from the pharmacokinetics of piperaquine, which has a much longer half-life (3–4 weeks) than that of lumefantrine (3–5 days) and other ACT partner drugs [28], yielding a long posttreatment prophylactic effect [5, 24, 27]. Piperaquine was used extensively to prevent and treat malaria decades ago in China [29, 30], but reported resistance led to reduced use by the 1980s. More recently, with implementation of DP as a standard therapy, piperaquine resistance does not appear to be a major problem, although ex vivo sensitivities of field isolates to piperaquine have varied [14, 22, 31, 32]. Mechanisms of resistance to piperaquine are poorly understood. Parasites selected in vitro for resistance acquired a number of genetic changes, including a novel mutation in *pfcrt* and deamplification of *pfmdr1*, but the phenotype was unstable [33].

AL replaced chloroquine plus sulfadoxine-pyrimethamine as the first-line regimen for uncomplicated malaria in Uganda in 2004, although implementation did not begin until 2006 and was initially slow [34, 35]. With improved utilization of AL in recent years, it was of interest to determine the prevalence over time of parasite polymorphisms that alter sensitivity to ACT components and to determine how use of AL impacts upon these polymorphisms. Therefore, we analyzed the prevalence of polymorphisms of interest in samples from a 5-year longitudinal trial comparing the antimalarial efficacies of AL and DP in Ugandan children. Polymorphisms associated with reduced sensitivity to AL increased markedly in prevalence over the course of the study, and this increase was greater in children treated with AL compared to those treated with DP, consistent with our demonstration of opposite selective pressures of the 2 regimens.

MATERIALS AND METHODS

Clinical Trial

Samples were from a longitudinal trial conducted in Tororo, Uganda, from 2007 to 2012 [27, 36]. In brief, a cohort 6 weeks to 12 months of age was enrolled and followed for all medical problems. Subjects with fever and a positive thick blood smear were diagnosed with malaria. At the time of their first episode of uncomplicated malaria, participants ≥4 months of age and ≥5 kg in weight were randomly assigned to AL or DP, administered according to weight-based guidelines, and participants received the same assigned treatment for each subsequent episode of uncomplicated malaria [27, 36]. This trial was approved by the institutional review boards of Makerere University and the University of California, San Francisco, and is registered at ClinicalTrials.gov (NCT00527800).

Selection of Samples for Testing of Parasite Polymorphisms

To establish baseline prevalences, samples from the first malaria episode after enrollment for 50 study participants were randomly selected for assessment of polymorphisms of interest. To follow changes in prevalences over time, samples from 50 malaria episodes from each treatment arm were randomly selected for each 3-month interval from January 2008 through June 2012. Fewer samples were selected for July–December 2013 (50 randomly selected samples from the AL arm and all 39 from the DP arm) due to a reduced number available. A total of 1889 longitudinal samples, each of which had been preceded by at least 1 prior episode treated with study drugs, was studied. For *pfmdr1* copy number assessment and multiplicity of infection (MOI) quantification, 10 recurrent malaria episodes from each 3-month interval were randomly selected from each treatment arm.

Characterization of Parasite Polymorphisms

DNA was extracted from filter paper blood spots into 100 µL of water using Chelex-100 [37]. Gene fragments spanning all loci of interest were amplified [38], and failed reactions were repeated using nested polymerase chain reaction (PCR) (Supplementary Table 1). To detect polymorphisms, multiplex ligase detection reaction–fluorescent microsphere assays were performed as previously described [38]. *Pfmdr1* copy number was quantified using TaqMan real-time PCR as previously described, with 3D7 and Dd2 strain standards [8]. Reactions were performed in quadruplicate, and repeated if results did not conform to exponential kinetics, if the standard deviation of the cycle threshold was >0.30, or if copy number was >1.3 or <0.7.

Multiplicity of Infection and Allele Frequency

MOI was estimated by characterizing complexity of the *msp1* and *msp2* genes using capillary electrophoresis, as previously described (Supplementary Table 2) [39–41]. Fluorescently labeled amplicons were multiplexed in Hi-Di formamide. MOI was

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defined as the highest number of alleles found at either locus. Allele frequencies were estimated using *MalHaploFreq* [42] (available at: http://pcwww.liv.ac.uk/hastings/MalHaploFreq), which utilizes allele prevalences and MOI data to estimate allele frequencies using maximum likelihood methodology.

Treatment Outcomes

We compared genotypes between paired samples from 50 randomly selected consecutive episodes of malaria from each year from 2009 to 2012 with recurrence within 63 days. Comparisons were performed using 6 previously described genetic markers and capillary electrophoresis (Supplementary Tables 2 and 3) [43]. Paired samples that shared alleles at all successfully genotyped loci were categorized as recrudescences; those that did not were categorized as new infections.

Statistical Methods

Data analysis was done using Stata version 12 (StataCorp). Outcomes of interest were the prevalence of wild-type alleles (excluding mixed infections) for each locus of interest. Exposure variables of interest were calendar time (date of treatment) and duration since prior malaria treatment for each episode of malaria. Calendar time was evaluated as a continuous variable for each locus except *pfcrt* 76, due to a lack of linearity for this locus. Duration since last treatment was evaluated as a categorical variable, with the selection of categories driven by the distribution of recurrent malaria episodes over time (Table 1); this differed between the AL and DP arms, as expected due to the different pharmacokinetics of lumefantrine and piperaquine. Independent associations between outcomes and exposure variables were measured using multivariate generalized estimating equations with exchangeable correlations and robust standard errors to account for repeated measures in the same child. To compare arms, treatment with AL or DP was added as an exposure variable, and duration since treatment was categorized using the delineations utilized for the AL arm. Differences in rates of change in allele prevalence were tested by assessing the significance of an additional variable representing the interaction between calendar time and treatment arm. All other analyses used Fisher exact or Kruskal–Wallis tests. In all analyses, a 2-tailed P value <.05 was considered significant.

Table 1. Characteristics of Children and Malaria Episodes

	Treatment Arm	
Characteristic/Episode	AL	DP
Characteristic	n = 134	n = 140
Median age at randomization, mo (range)	10.6 (3.8–35.9)	10.2 (4.3–45.3)
Median duration of observation, y (range)	4.5 (0.32–4.87)	4.6 (0.48–4.87)
Female sex, No.	73 (54.5%)	58 (41.4%)
Living in a rural area, No.	117 (87.3%)	117 (83.6%)
Malaria episodes	n = 950	n = 939
Recrudescences[a]	1/200 (0.5%)	2/200 (1.0%)
Mean multiplicity of infection[b] (range)	2.81 (1–7)	3.09 (1–7)
Median time since last malaria episode, d (IQR)	40 (28–63)	54 (44–74)
Duration since prior treatment for each recurrent episode		
≤28 d	286 (30%)	33 (4%)
29–42 d	235 (25%)	182 (19%)
43–56 d	150 (16%)	308 (33%)
57–70 d	79 (8%)	163 (17%)
>70 d	200 (21%)	253 (27%)

Abbreviations: AL, artemether-lumefantrine; DP, dihydroartemisinin-piperaquine; IQR, interquartile range.

[a] Ten episodes assessed by 6-allele genotyping for each 3-month interval for each treatment arm.

[b] Ten episodes assayed by *msp1* and *msp2* genotyping for each 3-month interval for each treatment arm.

RESULTS

Treatment Outcomes and Sample Selection

We enrolled 351 children and randomized 312 upon their first malaria episode following enrollment to treatment with AL or DP for every episode of uncomplicated malaria during the course of the study (August 2007 to December 2012) (Figure 1) [36]. The incidence of malaria was very high (treated episodes per person-year: 4.53 in the DP arm and 5.31 in the AL arm). Study children received 5564 treatments for uncomplicated malaria over 1260 person-years of follow-up. Recurrent malaria 14–63 days after therapy was common [36], but characterization of treatment outcomes for 50 pairs of successive isolates per treatment arm from each year during 2009–2012 indicated that treatment efficacies were outstanding. Only 3 outcomes were classified as recrudescence, one from the AL arm in 2012 and 2 from the DP arm in 2010. A similarly low incidence of recrudescence was reported for outcomes from 2007 to 2008 [27]. Thus, we can assume that nearly all samples evaluated for this study were from independent episodes of malaria.

Samples from a subset of malaria episodes were selected for molecular analyses. These totaled 1889 episodes from 274 individuals, 140 randomized to DP and 134 to AL. There were no significant differences in characteristics of treatment arm subjects, except that the DP arm had a lower proportion of females (41.4% vs 54.5%, $P = .031$; Table 1). The median age at randomization was 10.5 months, and children were followed for a median of 4.5 years following randomization. We detected no difference in the MOI for episodes that occurred in the 2 treatment arms (mean, 2.95 [95% confidence interval [CI], 2.81–3.08]). Children in the DP arm had a longer duration between episodes of malaria ($P = .0012$).

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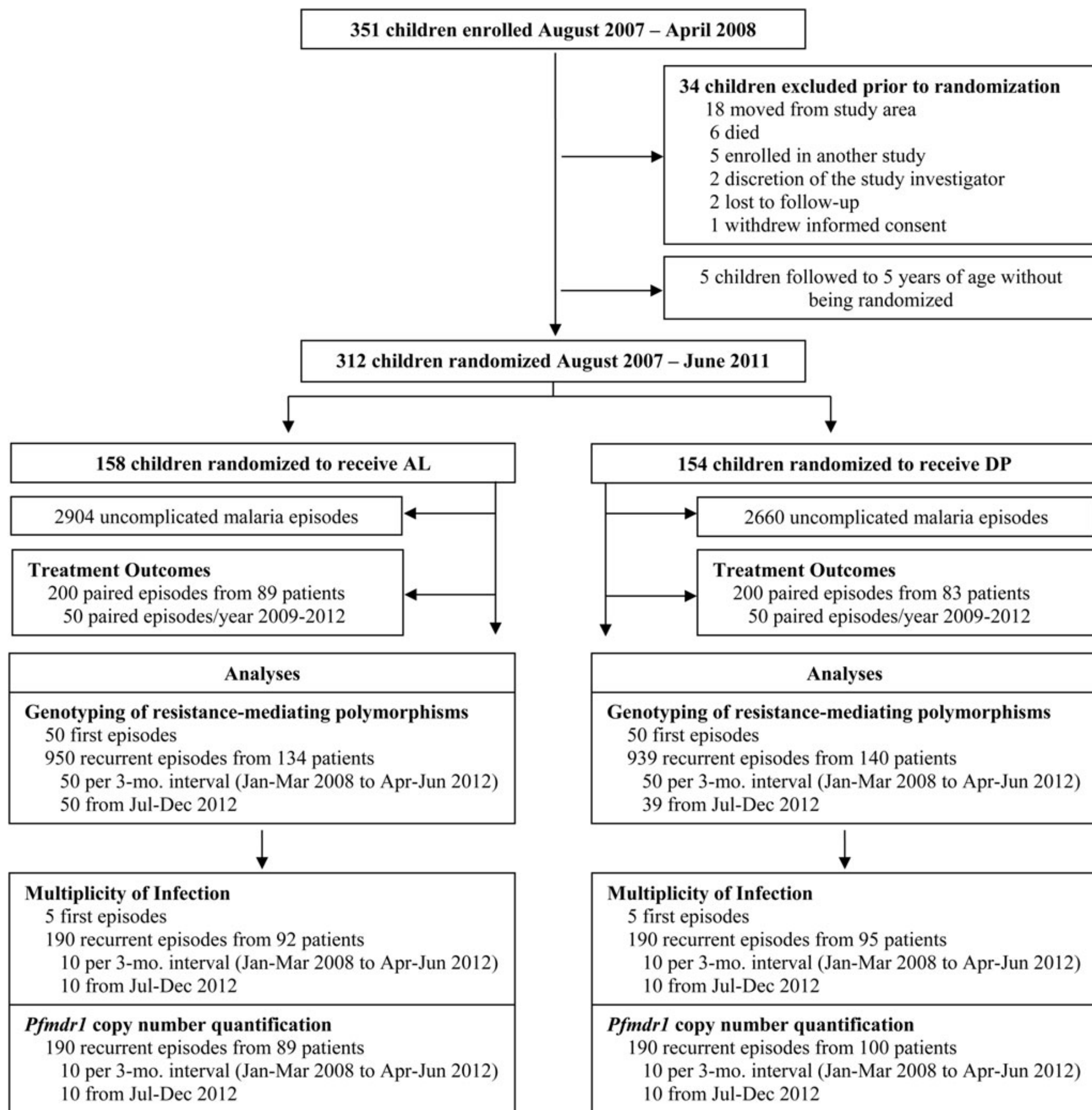

Figure 1. Trial and experimental profile. Abbreviations: AL, artemether-lumefantrine; DP, dihydroartemisinin-piperaquine.

Detection of Polymorphisms in First and Recurrent Malaria Episodes

To establish baseline prevalences of polymorphisms of interest independent of study drugs, we assessed 50 samples for each treatment arm from first episodes after enrollment. These samples were assessed at 8 loci (N86Y, Y184F, S1034C, N1042D, and D1246Y in *pfmdr1*; K76T in *pfcrt*; and I876V and K1466R in *pfmrp1*), and, as expected, there were no significant differences between the baseline allele prevalences in the 2 treatment groups (Table 2).

Samples from the 1889 recurrent malaria episodes selected as described above were assessed at the same 8 loci. More than 99% of the loci were successfully genotyped (Supplementary Table 4). All but 2 of the loci were highly polymorphic; only wild-type alleles were detected for *pfmdr1* 1034 and 1042, consistent with prior reports from Uganda [11] (Figure 1; Supplementary Table 4).

Changes in Allele Prevalence Over Time

We observed changes in the prevalence of *pfmdr1* 86, 184, and 1246 wild-type alleles across the 5 years surveyed for both treatment arms (Figure 2; Table 3). Multivariate analyses incorporating calendar time and the duration of time since the subject's last malaria treatment showed that in both treatment arms the pure wild-type genotype increased over time for *pfmdr1* N86 (AL: odds ratio [OR], 2.08/year, $P < .001$; DP: OR, 1.41/year, $P < .001$) and *pfmdr1* D1246 (AL: OR, 1.46/year, $P < .001$; DP: OR, 1.36/year, $P < .001$), and decreased over time for *pfmdr1* Y184 (AL: OR, 0.78/year, $P < .001$; DP: OR, 0.84/year,

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Table 2. Baseline Allele Prevalences in the 2 Treatment Arms

Locus	AL (n = 50)			DP (n = 50)			
	Wild-type	Mutant	Mixed	Wild-type	Mutant	Mixed	*P* Value[a]
Pfmdr1 N86Y	20%	46%	34%	26%	56%	18%	.207
Pfmdr1 Y184F	34%	6%	60%	32%	12%	56%	.684
Pfmdr1 D1246Y	41%	39%	20%	56%	35%	8%	.151
Pfcrt K76T	4%	92%	4%	0%	94%	6%	.678
Pfmrp1 I876V	34%	42%	24%	48%	32%	20%	.436
Pfmrp1 K1466R	39%	37%	24%	29%	41%	31%	.622

Abbreviations: AL, artemether-lumefantrine; DP, dihydroartemisinin-piperaquine.

[a] Fisher exact test, comparing categorical composition of wild-type, mutant, and mixed alleles.

P < .001). We also detected an increased prevalence of wild-type *pfcrt* K76 in 2012 compared with the previous 4 years (AL: OR, 3.37, *P* < .001; DP: OR, 5.84, *P* = .002) and a decreased prevalence of *pfmrp1* I876 limited to the AL arm (OR, 0.90/year, *P* = .034). No changes were detected over time for *pfmrp1* 1466.

Of particular interest were differences in prevalences of polymorphisms between treatment arms. Importantly, across the 5-year course of the study, the AL treatment arm was associated with greater prevalences, compared to the DP arm, for the wild-type alleles *pfmdr1* N86 (OR, 3.74 [95% CI, 2.90–4.81], *P* < .001), *pfmdr1* D1246 (OR, 2.06 [95% CI, 1.65–2.57], *P* < .001) and *pfcrt* K76 (OR, 2.38 [95% CI, 1.31–4.30], *P* = .004), and lower prevalences for wild-type *pfmdr1* Y184 (OR, 0.82 [95% CI, .68–1.00], *P* = .052). For *pfmdr1* 86, the rate of increase for the wild-type allele was significantly more rapid for the AL treatment arm compared with the DP arm (*P* < .001); for the other *pfmdr1* alleles rates of change were consistently higher for the AL arm, but the differences were not significant. At the end of the study, differences between treatment arms were large: during the last 6 months of 2012, prevalences of the pure wild-type alleles for AL and DP, respectively, were 94% vs 51% for *pfmdr1* N86 (*P* < .001), 14% vs 28% for *pfmdr1* Y184 (*P* = .10), 78% vs 56% for *pfmdr1* D1246 (*P* = .030), and 14% vs 5% for *pfcrt* K76 (*P* = .172) (Supplementary Table 4).

Impacts of Prior Therapies on Allele Prevalence

Malaria treatments may have an impact on subsequent malaria episodes by selecting for polymorphisms in parasites that emerge soon after prior therapy. Therefore, we examined associations between duration since prior treatment and allele prevalence. Recent treatment with AL was associated with higher prevalences of wild-type alleles at *pfmdr1* N86, *pfmdr1* D1246, and *pfcrt* K76, and with a lower prevalence of *pfmdr1* Y184, with decreasing influence of prior therapy as the duration since the therapy increased (Figure 3). Allele prevalences were significantly different than baseline (prevalence in parasites that emerged >56 days after prior AL treatment) in samples that emerged up to 56 days after prior therapy for *pfmdr1* N86 (*P* ≤ .004), 42 days for *pfmdr1* Y184 (*P* ≤ .025) and D1246 (*P* ≤ .013), and 21 days for *pfcrt* K76 (*P* = .009). Similar associations were seen with multivariate analyses adjusting for calendar time (Table 3). In contrast, recent treatment with DP was associated with changes in prevalence in the opposite direction for each studied allele, although the extent of selection was generally lower. Genotype prevalences were significantly different than baseline (prevalence in parasites that emerged >70 days since prior DP treatment) in samples that emerged up to 56 days after prior DP treatment for *pfmdr1* N86 (*P* ≤ .002) and 49 days for *pfmdr1* D1246 (*P* < .001) (Figure 3). As with AL, similar associations were seen with multivariate analyses adjusting for calendar time (Table 3), and the influence of prior therapy decreased as the duration since the therapy increased. We saw no selection by either regimen for the studied *pfmrp1* alleles.

Comparative Allele Frequencies

In areas with high MOI, malaria infections often contain several genetically distinct parasite clones. As a result, genotyping effectively detects allele prevalence but may not reflect allele frequency, as different clones typically comprise different proportions of an infection [42]. To determine if variation in MOI between treatment arms and over time might bias our prevalence estimates, we genotyped samples from 380 malaria episodes evenly distributed over time and treatment arm. We estimated a mean MOI of 2.94 among these samples, with little variation between years, treatment arms, or duration since last treatment (Table 1). In addition, we modeled allele frequency over time using the *MalHaploFreq* program [42]. The dynamics of estimated allele frequencies were consistent with the dynamics of calculated allele prevalences (Figure 2).

Pfmdr1 Copy Number Variation

We assessed *pfmdr1* copy number in 10 isolates from each treatment arm selected randomly for every 3-month interval during 2008–2012. Among the 380 isolates, copy number was ≥1.5 for

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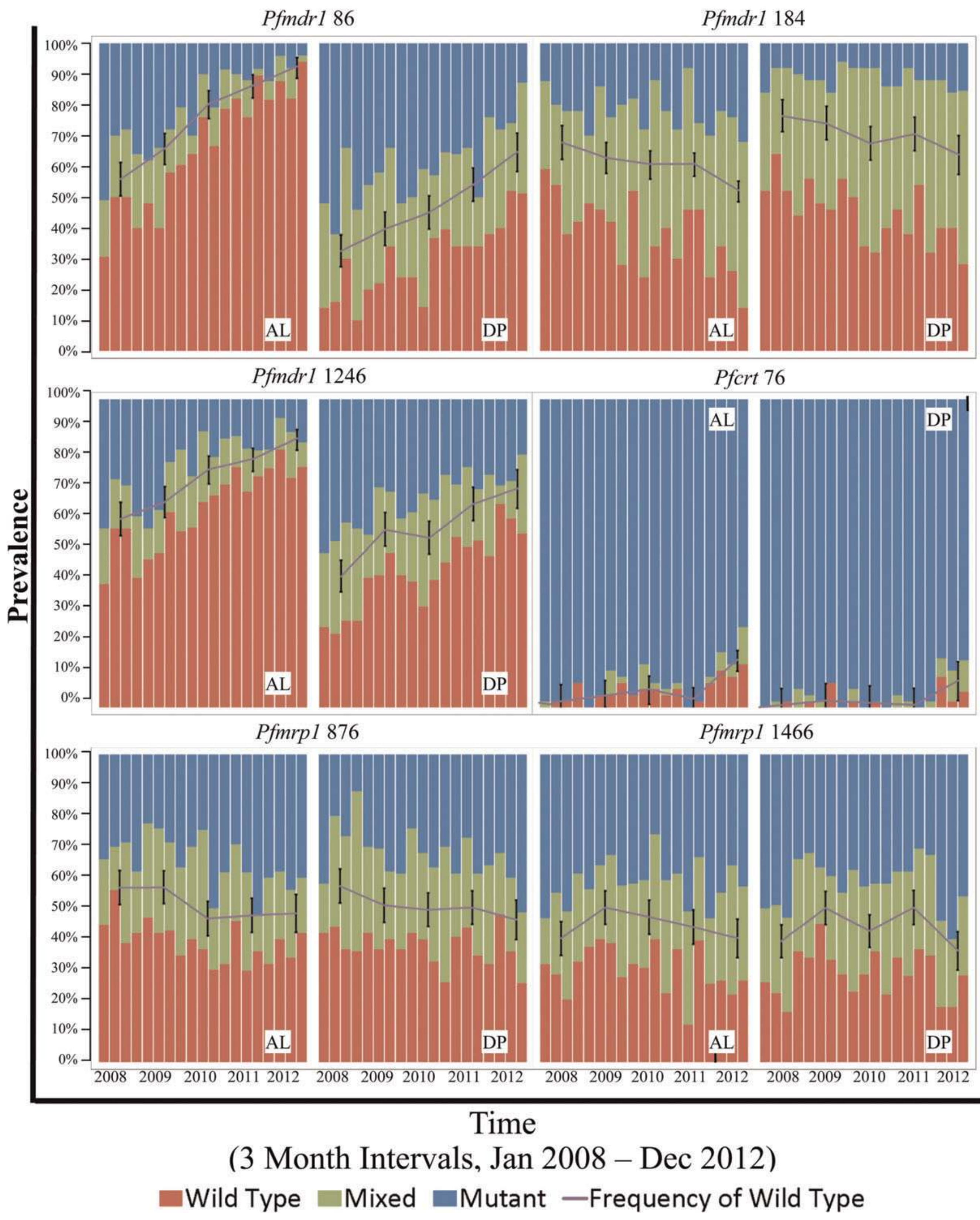

Figure 2. Allele prevalences over time. Prevalences, each based on analysis of 50 samples per treatment arm, are shown over 3-month intervals for wild-type, mixed, and mutant alleles of *pfmdr1* 86, 184, and 1246; *pfcrt* 76; and *pfmrp1* 876 and 1466. Frequency curves, based on *MalHaploFreq* frequency estimations for each year, are superimposed; error bars represent the 95% confidence intervals for the estimates. Abbreviations: AL, artemether-lumefantrine; DP, dihydroartemisinin-piperaquine.

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Table 3. Changes in *pfmdr1* 86, 184, and 1246; *pfcrt* 76; and *pfmrp1* 876 and 1466 Wild-type Allele Prevalences by Treatment Arm

Treatment	Risk Factor	Category	*Pfmdr1* 86 AOR (95% CI)	*P* Value	*Pfmdr1* 184 AOR (95% CI)	*P* Value	*Pfmdr1* 1246 AOR (95% CI)	*P* Value	*Pfcrt* 76	AOR (95% CI)	*P* Value	*Pfmrp1* 876 AOR (95% CI)	*P* Value	*Pfmrp1* 1466 AOR (95% CI)	*P* Value
AL	Time	Continuous[a]	**2.08 (1.83–2.38)**	**<.001**	**0.78 (.70–.86)**	**<.001**	**1.46 (1.29–1.64)**	**<.001**	2008–2011	1.0 (ref)	. . .	**0.90 (.82–.99)**	**.034**	0.93 (.85–1.02)	.148
									2012	**3.37 (1.85–6.16)**	**<.001**				
	Time since treatment	>56 d	1.0 (reference)	. . .	1.0 (reference)	. . .	1.0 (reference)	. . .		1.0 (reference)	. . .	1.0 (reference)	. . .	1.0 (reference)	. . .
		43–56 d	**1.89 (1.16–3.09)**	**.011**	0.87 (.59–1.29)	.499	1.43 (.97–2.11)	.075		0.46 (.13–1.65)	.233	0.75 (.46–1.20)	.226	0.74 (.47–1.16)	.194
		29–42 d	**3.21 (2.10–4.91)**	**<.001**	**0.65 (.44–.94)**	**.022**	**1.57 (1.11–2.23)**	**.011**		1.78 (.78–4.09)	.170	1.28 (.86–1.89)	.218	1.28 (.91–1.79)	.155
		22–28 d	**6.52 (3.83–11.10)**	**<.001**	**0.64 (.41–.99)**	**.044**	**1.81 (1.23–2.67)**	**.003**		1.85 (.85–4.04)	.120	1.14 (.77–1.68)	.528	0.68 (.46–1.01)	.056
		≤21 d	**13.38 (5.76–31.05)**	**<.001**	**0.58 (.35–.96)**	**.035**	**2.63 (1.36–5.07)**	**.004**		**3.65 (1.48–9.01)**	**.005**	1.42 (.81–2.48)	.221	1.17 (.67–2.05)	.573
DP	Time	Continuous	**1.41 (1.25–1.59)**	**<.001**	**0.84 (.76–.92)**	**<.001**	**1.36 (1.23–1.50)**	**<.001**	2008–2011	1.0 (ref)	. . .	0.94 (.86–1.03)	.166	0.98 (.89–1.09)	.767
									2012	**5.84 (1.94–17.53)**	**.002**				
	Time since treatment	>70 d	1.0 (reference)	. . .	1.0 (reference)	. . .	1.0 (reference)	. . .		1.0 (reference)	. . .	1.0 (reference)	. . .	1.0 (reference)	. . .
		57–70 d	0.58 (.41–.81)	.057	1.06 (.71–1.57)	.777	0.94 (.62–1.41)	.759		1.71 (.57–5.13)	.340	1.22 (.83–1.78)	.309	0.88 (.54–1.45)	.616
		50–56 d	**0.53 (.36–.78)**	**.001**	1.37 (.99–1.88)	.054	**0.69 (.49–.97)**	**.035**		0.65 (.12–3.58)	.620	0.86 (.62–1.20)	.376	0.83 (.57–1.21)	.329
		≤49 d	**0.49 (.32–.75)**	**.001**	1.27 (.86–1.88)	.235	**0.61 (.41–.91)**	**.015**		0.38 (.08–1.78)	.218	0.89 (.60–1.32)	.564	0.92 (.61–1.37)	.669

Abbreviations: AL, artemether-lumefantrine; AOR, adjusted odds ratio; CI, confidence interval; DP, dihydroartemisinin-piperaquine; OR, odds ratio. Bold face indicates statistical significance.

[a] Odds ratios are reported per year. Because the change in the prevalence of *pfcrt* K76 over time did not follow a linear pattern, time was evaluated as a categorical variable.

14 (3.7%) and ≥2.6 for 3 (0.8%). Ten of these isolates were from AL-treated patients (7 from 2008 and 3 from 2009); 4 were from DP-treated patients (all from 2008). We found no association between increased copy number and treatment arm and did not see a trend toward increased *pfmdr1* copy number over time.

DISCUSSION

We examined changes in the prevalence of *P. falciparum* polymorphisms associated with altered drug sensitivity in a cohort of Ugandan children randomly assigned to receive either AL or DP for each episode of uncomplicated malaria from 2007 to 2012, a period during which AL was increasingly utilized as the national treatment regimen. Both treatments were highly efficacious, consistent with prior reports [24, 27]. However, the prevalences of 4 polymorphisms associated with reduced sensitivity to AL components—*pfmdr1* N86, *pfmdr1* 184F, *pfmdr1* D1246, and *pfcrt* K76—all increased over time. Comparing results for samples from the 2 treatment arms, the prevalences of all of these alleles were greater in the AL treatment arm. Thus, over a 5-year span during which AL was increasingly utilized to treat malaria, parasites increasingly contained polymorphisms associated with decreased sensitivity to lumefantrine. These changes were seen in children treated with either AL or DP, indicative of the selective pressure of widespread use of AL in Uganda. However, the changes were greater in children treated for each episode of malaria with AL, compared to DP, consistent with the opposite selective pressures of AL and DP that we demonstrated. Thus, the choice of national antimalarial regimen can have a profound impact on parasite genetics, and specifically on the selection of parasites with altered sensitivity to ACT components.

Emerging resistance to artemisinins is of great concern [44, 45], although at present the problem appears to be limited to Southeast Asia [46]. Of more urgent concern in Africa is resistance to artemisinin partner drugs, which may be readily selected. Evaluation of the ex vivo sensitivities of Ugandan [22] and other African [14, 21, 47] parasites to lumefantrine and piperaquine have shown a range of sensitivities, but it is unclear if clinically relevant resistance is yet occurring. Mediators of high-level resistance are uncertain [23, 33], but sensitivity to many antimalarials is affected by polymorphisms in *pfcrt* and *pfmdr1*. Relevant to our study, lumefantrine selects for the wild-type *pfcrt* K76, *pfmdr1* N86, and *pfmdr1* D1246 alleles, which are associated with decreased lumefantrine sensitivity [12–14]. In the only available study to consider this question, DP did not exert selective pressure on these alleles in Burkina Faso [9].

With the deployment of AL as the standard treatment for malaria in Uganda and the threat of emerging drug resistance, understanding the dynamics of changes in key parasite alleles, and how these alleles are influenced by the use of different ACTs, is of great importance. Parasites causing uncomplicated malaria in

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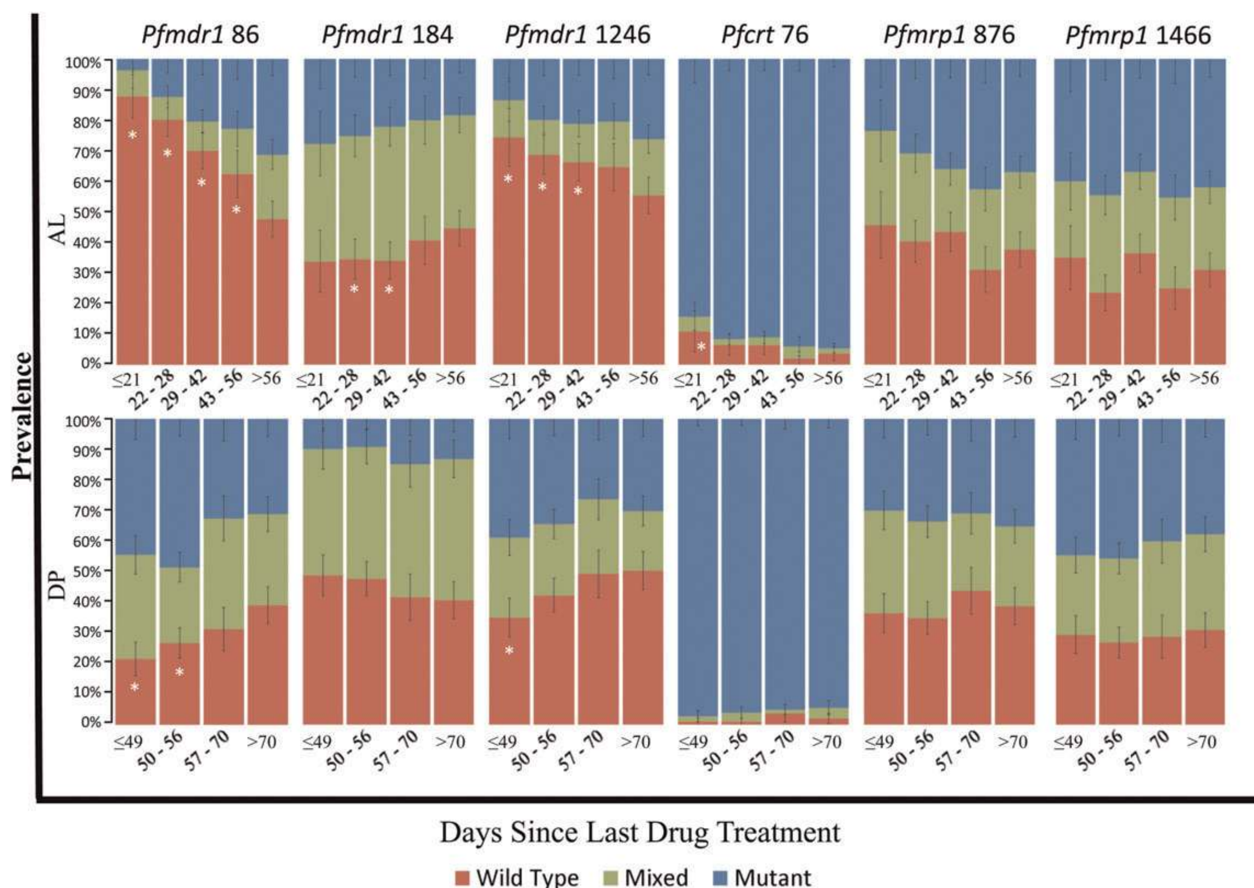

Figure 3. Impact of prior therapy on allele prevalences. The prevalences of wild-type, mixed, and mutant sequences at the indicated alleles are shown for samples from episodes that emerged within the indicated time after a prior treatment with artemether-lumefantrine (AL) or dihydroartemisinin-piperaquine (DP). Based on consideration of the distribution of recurrent malaria episodes over time, samples from episodes that emerged more than 56 days and 70 days after treatment are considered not to be under selection for AL and DP, respectively. Asterisks indicate wild-type allele prevalences that are significantly different from those for samples not under selection ($P < .05$, χ^2 test). Abbreviations: AL, artemether-lumefantrine; DP, dihydroartemisinin-piperaquine.

Tororo changed markedly, with changes likely facilitated both by decreasing selective pressure from chloroquine and increasing pressure from AL, as supported by the demonstration of increased selection toward relevant genotypes in the AL treatment arm of our study. Differing selection between treatment arms was also apparent upon examination of parasites that emerged after recent treatment, as, consistent with prior studies [6–11, 16], treatment with AL selected for the same wild-type alleles in parasites that emerged after therapy. Importantly, recent DP treatment selected for the opposite variants, consistent with selection seen for amodiaquine [7, 16, 48], but differing from results for DP from Burkina Faso [9]. The difference between sites might be explained by different genetic backgrounds of parasites in West Africa (eg, low prevalence of the *pfmdr1* 1246Y mutation), differences in experimental designs, or other factors.

Our results highlight the profound influence that the choice of national malaria treatment regimen can have on parasite genetics and specifically on potential mediators of drug resistance. In Uganda, replacement of a chloroquine-containing regimen with AL has been accompanied by marked changes in parasite genotypes, with selection of alleles that mediate diminished sensitivity to AL components. In children who were treated for each episode of malaria with AL, following national treatment guidelines, selection was enhanced compared with that in children treated with DP, another highly efficacious regimen. This observation is consistent with our finding that DP selects in the opposite direction as AL, as has been observed with the related 4-aminoquinolines chloroquine and amodiaquine [7, 16, 48]. Importantly, the parasite polymorphisms described here do not appear to mediate high-level drug resistance. Nonetheless, they will likely facilitate continued selection toward clinically relevant resistance, which is commonly stepwise in *P. falciparum* [49]. Thus, consideration should be given to changes in malaria treatment regimens, possibly with sequential administration of different ACTs, to limit the selection of parasites with decreasing drug sensitivity.

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Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online (http://jid.oxfordjournals.org/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

Acknowledgments. We thank the clinical study team and our administrative staff. We are grateful to the children who participated in this study and to their parents and guardians.

Disclaimer. The contents of this manuscript are solely the responsibility of the authors and do not necessarily represent the official views of Centers for Disease Control and Prevention or the Doris Duke Charitable Foundation.

Financial support. This work was supported by the US President's Emergency Plan for AIDS Relief and the Department of Health and Human Services/Centers for Disease Control and Prevention; National Center for HIV, Viral Hepatitis, STD, and TB Prevention; Global AIDS Program (U62P024421); National Institutes of Health (AI089674 and AI075045); and Doris Duke Charitable Foundation.

Potential conflicts of interest. All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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