ORIGINAL ARTICLE

Increasing Prevalence of Artemisinin-Resistant HRP2-Negative Malaria in Eritrea

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ABSTRACT

BACKGROUND

Although the clinical efficacy of antimalarial artemisinin-based combination therapies in Africa remains high, the recent emergence of partial resistance to artemisinin in *Plasmodium falciparum* on the continent is troubling, given the lack of alternative treatments.

METHODS

In this study, we used data from drug-efficacy studies conducted between 2016 and 2019 that evaluated 3-day courses of artemisinin-based combination therapy (artesunate–amodiaquine or artemether–lumefantrine) for uncomplicated malaria in Eritrea to estimate the percentage of patients with day-3 positivity (i.e., persistent *P. falciparum* parasitemia 3 days after the initiation of therapy). We also assayed parasites for mutations in *Pfkelch13* as predictive markers of partial resistance to artemisinin and screened for deletions in *hrp2* and *hrp3* that result in variable performance of histidine rich protein 2 (HRP2)–based rapid diagnostic tests for malaria.

RESULTS

We noted an increase in the percentage of patients with day-3 positivity from 0.4% (1 of 273) in 2016 to 1.9% (4 of 209) in 2017 and 4.2% (15 of 359) in 2019. An increase was also noted in the prevalence of the *Pfkelch13* R622I mutation, which was detected in 109 of 818 isolates before treatment, from 8.6% (24 of 278) in 2016 to 21.0% (69 of 329) in 2019. The odds of day-3 positivity increased by a factor of 6.2 (95% confidence interval, 2.5 to 15.5) among the patients with *Pfkelch13* 622I variant parasites. Partial resistance to artemisinin, as defined by the World Health Organization, was observed in Eritrea. More than 5% of the patients younger than 15 years of age with day-3 positivity also had parasites that carried *Pfkelch13* R622I. In vitro, the R622I mutation conferred a low level of resistance to artemisinin when edited into NF54 and Dd2 parasite lines. Deletions in both *hrp2* and *hrp3* were identified in 16.9% of the parasites that carried the *Pfkelch13* R622I mutation, which made them potentially undetectable by HRP2-based rapid diagnostic tests.

CONCLUSIONS

The emergence and spread of *P. falciparum* lineages with both *Pfkelch13*-mediated partial resistance to artemisinin and deletions in *hrp2* and *hrp3* in Eritrea threaten to compromise regional malaria control and elimination campaigns. (Funded by the Bill and Melinda Gates Foundation and others; Australian New Zealand Clinical Trials Registry numbers, ACTRN12618001223224, ACTRN12618000353291, and ACTRN12619000859189.)

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This is the *New England Journal of Medicine* version of record, which includes all *Journal* editing and enhancements. The Author Accepted Manuscript, which is the author's version after external peer review and before publication in the *Journal*, is available at PubMed Central.

N Engl J Med 2023;389:1191-202.
DOI: 10.1056/NEJMoa2210956
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Artemisinin-based combination therapies, which combine fast-acting and potent artemisinin derivatives with longer-acting partner drugs, are essential first-line treatments for uncomplicated *Plasmodium falciparum* malaria.[1] Over the past 15 years, *P. falciparum* parasites in the Greater Mekong Subregion in Southeast Asia have developed partial resistance to artemisinin, which manifests as delayed parasite clearance or persistence of parasites 3 days after the initiation of artemisinin-based combination therapy. Resistance in vitro results from a decreased susceptibility to artemisinin in intraerythrocytic ring-stage parasites.[1-6] Artemisinin-based combination therapy has had an increasing incidence of failure in this subregion, where parasites have also acquired resistance to the partner drug piperaquine and previously to the partner drug mefloquine.[7]

Although the clinical efficacy of artemisinin-based combination therapies in African regions is currently high, the recent emergence of partial resistance to artemisinin in Rwanda and Uganda is a major concern.[1,8-13] Molecular studies have confirmed the presence of nonsynonymous mutations in *Pfkelch13* (PF3D7_1343700), the primary determinant of partial resistance to artemisinin.[14,15] These mutations include R561H in Rwanda and C469Y and A675V in Uganda. All three are associated with delayed parasite clearance, persistent parasitemia on day 3, or both and have displayed increasing prevalence over time (7.8% in 2015 to 12.8% in 2018 in Rwanda and 3.9% in 2015 to 19.8% in 2019 in Uganda).[10,12] Results of ex vivo and in vitro assays measuring survival of *Pfkelch13* R561H and C469Y parasites (either gene-edited lines or field isolates) support these mutations as markers of in vitro partial resistance to artemisinin in a manner dependent on the genetic background of the parasite.[10,11,16] Genomic analyses have shown the independent emergence and local expansion of these *Pfkelch13*-variant parasites.[10-12]

In Eritrea, artesunate–amodiaquine, first introduced in 2007 as the first-line treatment for uncomplicated falciparum malaria, is now available free of charge at health facilities and at the community level. In 2015, a single dose of primaquine was added to artesunate–amodiaquine as a transmission-blocking agent.[13] Artemether–lumefantrine, which has been recommended as second-line treatment, was implemented in 2019 at health facilities as an alternative first-line treatment for uncomplicated malaria.

Here, we describe the results of therapeutic efficacy studies conducted between 2016 and 2019 at five sites in Eritrea evaluating 3-day courses of artesunate–amodiaquine or artemether–lumefantrine for uncomplicated falciparum malaria. We assessed the percentage of patients with day-3 positivity (i.e., persistent *P. falciparum* parasitemia 3 days after the initiation of therapy) and assayed parasites for molecular signatures of partial resistance to artemisinin. We also screened for deletions in *hrp2* and *hrp3* that result in variable performance of HRP2-based rapid diagnostic tests for malaria.

Methods

Study Design and Population

We conducted an analysis of three previously unpublished, open-label, single-group, multisite, clinical drug-efficacy studies that were designed to assess clinical partial resistance to artemisinin in Eritrea, as determined by the percentage of patients with day-3 positivity after artesunate–amodiaquine or artemether–lumefantrine treatment. The studies were conducted in 2016, 2017, and 2019 at health centers or hospitals at five sites in western Eritrea, and the three study protocols are available with the full text of this article at NEJM.org. The studies were approved by the Eritrean ethical committee and the World Health Organization (WHO) ethical review committee. Patients were at least 6 months of age, and eligibility was determined according to WHO inclusion and exclusion criteria. Informed written consent was obtained from the adult patients and from the parents or caretakers of children enrolled in the studies.

Treatment, Follow-up Procedure, and Outcomes

Patients were assigned a supervised standard 3-day course of artesunate–amodiaquine (2016 and 2019) or artemether–lumefantrine (2017) and were monitored clinically. Thick and thin blood smears were obtained by finger prick on the day of recruitment (day 0) and during follow-up visits on days 1, 2, 3, 7, 14, 21, and 28 to screen for *P. falciparum* and estimate parasite

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density. Additional follow-up visits were scheduled if further symptoms occurred. Dried blood-spot filter papers were used for molecular studies. The primary outcome was day-3 positivity, as assessed by microscopic examination of thick blood smears on day 3 after a 3-day course of artemisinin-based combination therapy (days 0, 1, and 2).[1] A secondary outcome was the polymerase-chain-reaction (PCR)–adjusted clinical response to the designated treatment on day 28.

MOLECULAR ANALYSIS

We used the QIAamp DNA Blood Mini Kit (Qiagen) to extract parasite DNA from dried blood spots before and after (in cases of recurrence) treatment. Genotyping of the polymorphic genetic markers *msp1*, *msp2*, and *polyα* was carried out by PCR testing, and post-treatment infections were classified as either recrudescent (same genotype as on day 0) or new (different genotype) infections.[17]

Paired DNA samples (obtained on day 0 and on the day of recurrence) were analyzed for mutations in the propeller domain of *Pfkelch13* (codons 430 to 720) and in *pfcrt*, *pfmdr1*, *dhfr*, and *dhps*, which are associated with decreased parasite susceptibility to artemisinin derivatives, 4-aminoquinolines (piperaquine and chloroquine), amino alcohols (mefloquine and lumefantrine), pyrimethamine, and sulfadoxine, respectively.[18] We also screened for *hrp2* and *hrp3* deletions that can cause false negative results with HRP2-based rapid diagnostic tests.[19]

Whole-genome sequencing was performed with the use of Illumina paired-end sequencing after selective amplification of parasite DNA.[20] Read alignments against the 3D7 genome (PlasmoDB, Release 45) were used to infer a phylogenetic tree. The Genome Analysis Toolkit[21] was used to identify single-nucleotide polymorphisms, genotype isolates, and assess the genetic identity of *Pfkelch13* variants from Eritrea. Principal coordinate analysis, hierarchical clustering, and an analysis of molecular variance were performed on the basis of pairwise Euclidean genetic distances between samples.

GENE-EDITED LINES AND IN VITRO SUSCEPTIBILITY

The *Pfkelch13* R622I mutation was introduced into African (NF54) and Asian (Dd2) parasite lines by CRISPR-Cas9 (clustered regularly interspaced short palindromic repeats and associated Cas9 homing endonucleases)–mediated gene editing. Susceptibility to artemisinin in the edited parasites and wild-type controls was assessed in vitro with the use of the ring-stage survival assay, performed on ring-stage parasites synchronized to a 0-to-3-hour window after merozoite invasion of red cells ($RSA_{0\text{-}3hr}$) (see the Supplementary Appendix, available at NEJM.org).[22]

STATISTICAL ANALYSIS

Data were analyzed with the use of GraphPad Prism, version 9.3.1 (GraphPad Software). Because the analyses presented here were not originally specified in the protocols for the three component studies, all analyses are descriptive. All estimates include 95% confidence intervals, but the widths of the confidence intervals have not been adjusted for multiple comparisons and should not be used in place of hypothesis testing. The primary analysis was conducted on the basis of complete cases only and excluded data from patients with missing outcomes. The Kaplan–Meier analyses were conducted as an alternative to the complete-case analysis.

RESULTS

PATIENTS AND STUDY DESIGN

A total of 852 patients with uncomplicated *P. falciparum* malaria were enrolled (Table 1). Of these, 841 patients (98.7%) were assessed for the day-3 positivity outcome and 825 (96.8%) for the clinical efficacy outcome (PCR-adjusted clinical response to the designated treatment on day 28). The remaining patients either withdrew consent (10 patients) or were lost to follow-up (17 patients) (Fig. S1 in the Supplementary Appendix).

DAY-3 POSITIVITY

Among 841 patients, 20 (2.4%) from the towns of Guluj, Shambuko, and Tokombia, Eritrea, remained parasitemic 3 days after the initiation of therapy (Fig. 1A and Table S1). The percentage of patients with day-3 positivity increased from 0.4% (95% confidence interval [CI], 0.0 to 2.0 [1 of 273 patients]) in 2016 to 1.9% (95% CI, 0.5 to 4.9 [4 of 209]) in 2017 and 4.2% (95% CI, 2.3 to 6.9 [15 of 359]) in 2019. The towns with the highest percentages of patients with day-3 positivity in 2019 were Tokombia (10.2%) and Sham-

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Table 1. Characteristics of the Three Eritrean Studies and of the Patients at Baseline, Day-3 Positivity, and *Pfkelch13* Genotypes in Blood Samples Collected before Artemisinin-Based Combination Therapy.*

Variable	Study Year			Total
	2016	2017	2019	
Study characteristic				
Period	Jan.–Dec.	Sept.–Dec.	Aug.–Nov.	
Antimalarial treatment	Artesunate–amodiaquine	Artemether–lumefantrine	Artesunate–amodiaquine	
Patients — no.				
Total†	280	211	361	852
Distribution according to site				
Akordat	58	19	88	165
Ghindae	NA	15	NA	15
Guluj	73	25	97	195
Shambuko	73	64	88	225
Tokombia	76	88	88	252
Patient characteristic at baseline				
Median age (IQR) — yr	13.0 (8.0–19.0)	13.0 (9.0–22.7)	17.5 (12.0–29.0)	15.0 (10.0–25.0)
Female/male — no.	120/160	82/129	119/242	321/531
Median body temperature (IQR) — °C	38.0 (38.0–39.0)	38.0 (38.0–38.3)	38.0 (38.0–38.5)	38.0 (38.0–39.0)
Median parasite density (IQR) — μl	7530 (2736–18,036)	7900 (2677–22,335)	9312 (2720–23,933)	8280 (2715–21,902)
Day-3 positivity				
Patients with day-3 positivity — no./total no. (%)				
Total	1/273 (0.4)	4/209 (1.9)	15/359 (4.2)	20/841 (2.4)
Distribution according to site				
Akordat	0/54	0/19	0/88	0/161
Ghindae	NA	0/15	NA	0/15
Guluj	0/71	0/23	1/95 (1.1)	1/189 (0.5)
Shambuko	1/73 (1.4)	4/64 (6.2)	5/88 (5.7)	10/225 (4.4)
Tokombia	0/75	0/88	9/88 (10.2)	9/251 (3.6)
***Pfkelch13* genotype detected in pre-treatment blood sample**				
Missing samples — no./total no. (%)	0/280	0/211	23/352 (6.5)	23/852 (2.7)
Missing data — no./total no. (%)	2/280 (0.7)	0/211	9/352 (2.5)	11/852 (1.3)
Pfkelch13 wild type	251/278 (90.3)	193/211 (91.4)	254/329 (77.2)	698/818 (85.3)
Pfkelch13 position: amino acid substitution				
503: K→W		1/211 (0.5)		1/818 (0.1)
515: R→G		1/211 (0.5)		1/818 (0.1)
520: V→A	1/278 (0.3)			1/818 (0.1)
532: C→W			1/329 (0.3)	1/818 (0.1)
533: G→N			1/329 (0.3)	1/818 (0.1)
543: I→V			1/329 (0.3)	1/818 (0.1)
548: G→C			1/329 (0.3)	1/818 (0.1)

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Table 1. (Continued.)

Variable	Study Year			Total
	2016	2017	2019	
556: E→K			1/329 (0.3)	1/818 (0.1)
561: R→H			1/329 (0.3)	1/818 (0.1)
591: G→N	1/278 (0.3)			1/818 (0.1)
622: R→I	24/278 (8.6)	16/211 (7.6)	69/329 (21.0)	109/818 (13.3)
658: K→E	1/278 (0.3)			1/818 (0.1)

* Day-3 positivity was defined as persistent *P. falciparum* parasitemia on 3 days after the initiation of therapy. IQR denotes interquartile range, and NA data not available.

† The target number of patients to be enrolled at each study site was 73, as estimated on the basis of power calculations. Lower-than-expected numbers of patients were enrolled in Akordat (2016 and 2017), Ghindae (2017), and Guluj (2017), mainly because of the low number of malaria cases seen at health centers in this low malaria-transmission region during the study period.

buko (5.7%). A total of 825 of 852 (96.8%) patients were evaluated at day 28 (Table S2). Of the 27 recurrent infections, 17 were classified as recrudescent. The PCR-corrected complete-case and Kaplan–Meier estimates of efficacy with artesunate–amodiaquine and with artemether–lumefantrine were greater than 94%, which is above the 90% threshold recommended by the WHO for a treatment policy change (Tables S3, S4, and S5).

PFKELCH13 GENOTYPING

Of the 828 available pretreatment samples, 818 (98.8%) were successfully genotyped. Twelve *Pfkelch13* nonsynonymous mutations were detected in 120 samples (Table 1). The *Pfkelch13* R561H mutation, a validated marker for partial resistance to artemisinin, was observed in one isolate (Shambuko, 2019).[11,12] A novel *Pfkelch13* R622I mutation was detected in 109 of 818 samples (13.3%). The prevalence of this variant increased from 8.6% (95% CI, 5.5 to 12.8 [24 of 278 patients]) in 2016 and 7.6% (95% CI, 4.3 to 12.3 [16 of 211]) in 2017 to 21.0% (95% CI, 16.3 to 26.5 [69 of 329]) in 2019 (Fig. 1B and 1C and Table S6). In 2019, the prevalence was 8.1% in Shambuko, 21.9% in Guluj, 26.2% in Akordat, and 29.1% in Tokombia.

A NOVEL MARKER OF PARTIAL RESISTANCE TO ARTEMISININ

The percentage of patients with parasites carrying the *Pfkelch13* R622I mutation before treatment was higher among those with day-3 positivity (45%; 95% CI, 20.6 to 85.4 [9 of 20 patients]) than among those who were negative on day 3 (12.3%; 95% CI, 10.0 to 15.0 [97 of 787]) (risk ratio, 5.4; 95% CI, 2.3 to 12.7). In vivo partial resistance to artemisinin was confirmed in Eritrea. Among the patients with day-3 positivity who were younger than 15 years of age, parasites carrying *Pfkelch13* R622I were detected on day 0 in more than 5% in Tokombia (7.0% in 2019) and Shambuko (5.4% in 2017) (Table S7).[23] The odds of day-3 positivity were higher by a factor of 6.2 (95% CI, 2.5 to 15.5) among the patients with the *Pfkelch13* 622I variant in isolates collected before artemisinin-based combination therapy than among those with *Pfkelch13* wild-type parasites (Table 2). Although the failure rates of artesunate–amodiaquine treatment were similar among patients with parasites carrying *Pfkelch13* R622I and those with wild-type parasites on day 0 (2.3% [2 of 87] vs. 3.0% [15 of 495]), *Pfkelch13* genotyping of paired isolates obtained on day 0 and on the day of recrudescence indicated selective pressure for the 622I variant after artesunate–amodiaquine administration (an increase by a factor of 4.4, from 12% on day 0 to 53% on the day of recrudescence). Using amplicon deep sequencing, we detected the presence of *Pfkelch13* 622I genotypes in minor percentages (1.4 to 3.2% in 7 of 9 samples obtained on day 0 that had been previously classified as wild type), findings that provide evidence of intrahost selection after administration of artesunate–amodiaquine (Table S8).

IN VITRO SURVIVAL OF *PFKELCH13* 622I VARIANT PARASITES

The *Pfkelch13* R622I mutation was edited into NF54 (African) and Dd2 (Asian) parasites. Re-

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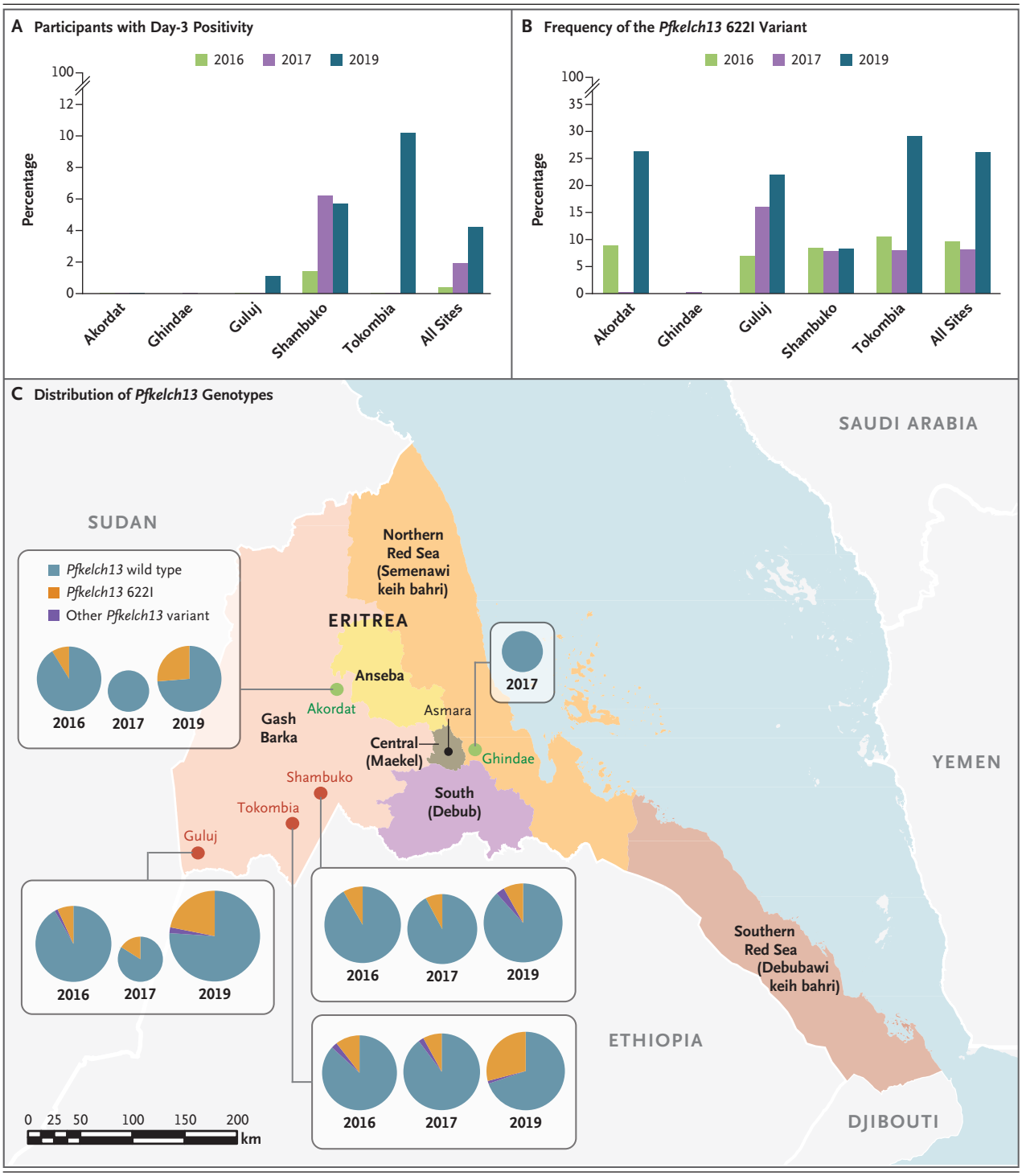

combinant clones were tested with the $RSA_{0\text{-}3hr}$, which measures the survival of early ring-stage parasites exposed to 700nM dihydroartemisinin for 6 hours. Survival (calculated relative to dimethylsulfoxide mock-treated parasites) greater than 1% indicates in vitro resistance to artemisinin. Results showed that the *Pfkelch13* R622I mutation conferred low-level partial resistance

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Figure 1 (facing page). Evidence of Delayed Parasite Clearance Associated with the Expansion of *Pfkelch13* 622I Variants in Eritrea.

Panel A shows the percentages of patients with day-3 positivity (i.e., persistent *P. falciparum* parasitemia 3 days after the initiation of artemisinin-based combination therapy). Day-3 positivity was not observed in Akordat in 2016, 2017, and 2019; Ghindae in 2017 (no data were available in 2016 and 2019); Guluj in 2016 and 2017; or Tokombia in 2016 and 2017. Panel B shows the frequency of the *Pfkelch13* 622I variant according to site and year. Only *Pfkelch13* wild-type parasites were observed in Akordat and Ghindae in 2017 (no data were available in 2016 and 2019). Panel C shows the distribution of *Pfkelch13* genotypes according to site and year. The proportions of each *Pfkelch13* allele are shown per year in pie charts (except for Ghindae, where only 2017 data were available). In the pie charts, the *Pfkelch13* wild-type allele is shown in light blue, the *Pfkelch13* 622I allele in orange, and other *Pfkelch13* variants in purple. The size of the pie chart is proportional to the sample size. The study sites in green correspond to areas where no cases of day-3 positivity were observed, and those in red correspond to areas where day-3 positivity was detected (Guluj, Tokombia, and Shambuko). Additional information is provided in Tables S1, S6, and S7 in the Supplementary Appendix.

to artemisinin in NF54 622I parasites as compared with the isogenic wild-type control line (survival, 3.3% vs. 0.6%). In vitro resistance was borderline in the Dd2 R622I line (survival, 1.5% in the variant vs. 0.7% in the isogenic control). In NF54 parasites, the R622I mutation conferred somewhat lower levels of resistance than the C580Y mutation that predominates across Southeast Asia ($RSA_{0\text{-}3hr}$ survival, 4.3%) (Fig. 2).

ORIGINS OF THE *PFKELCH13* 622I GENOTYPE

We compared whole-genome sequences of 291 samples, including 128 Eritrean *P. falciparum* sequences generated for this study, 162 publicly available sequences, and the 3D7 reference genome from Africa (Table S9). A maximum-likelihood phylogenetic tree showed that the *Pfkelch13* 622I variants were scattered among the East African wild-type isolates (Fig. 3).

We then explored haplotype diversity in the genomic regions flanking the R622I mutation. A principal coordinate analysis based on a pairwise genetic distance matrix indicated a shared genetic background between Eritrean 622I variants and wild-type isolates (Figs. S2 and S3). Haplotype similarity in a region of approximately 300 kb around the mutation pointed to a shared ancestry among variants found across different sites (Fig. S4). In the absence of accurate estimates of recombination rates in populations of *P. falciparum*, the age of the R622I mutation could not be properly assessed. Nevertheless, the limited segment of haplotype homozygosity, as well as the lack of space–time structure in the distribution of haplotypes, does not suggest a recent clonal expansion of the *Pfkelch13* R622I mutation (Figs. S5 and S6 and Table S10).

GENETIC BACKGROUND OF ERITREAN *PFKELCH13* 622I VARIANTS

We investigated the genetic background of Eritrean *Pfkelch13* 622I variants by profiling both variant and wild-type parasites at known antimalarial drug-resistance loci and by measuring the frequency of *hrp2* and *hrp3* deletions, a genomic feature previously observed in *P. falciparum* in Eritrea (Table S11).[24] We assessed 67 *Pfkelch13* 622I and 311 wild-type parasite samples for mutations in four genes. Differences in the mutation frequencies were observed in *pfcrt* and *dhfr*, which mediate resistance to chloroquine and piperaquine and to pyrimethamine, respectively.[25] Most of the parasites carrying *Pfkelch13* R622I had PfCRT M74I/N75E/K76T mutations (present in 92.5% of the 622I variants vs. 66.9% of *Pfkelch13* wild-type parasites), and N51I/S108N *dhfr* mutations (74.6% of the variant parasites vs. 49.5% of the wild-type parasites). We evaluated 29 *Pfkelch13* 622I and 139 wild-type parasites for amplification of *plasmepsin II* or *pfmdr1*, as these are considered to be markers of reduced susceptibility to piperaquine and to lumefantrine and mefloquine, respectively.[25] No parasites had *pfmdr1* amplification, and the percentage of isolates harboring at least two copies of *plasmepsin2* was similar in both variant (31.0%) and wild-type (32.4%) parasites.

We also tested for *hrp2* and *hrp3* deletions in 65 *Pfkelch13* 622I variant and 280 wild-type parasites. The majority (69.2% [45 of 65]) of *Pfkelch13* 622I parasites had an *hrp3* deletion, as compared with 22.5% (63 of 280) of wild-type parasites. More worrisome is that we detected both *hrp2* and *hrp3* deletions in a substantial percentage of 622I variant parasites (16.9% [9 of 65]), as compared with 21.8% (61 of 280) in wild-type parasites, a finding that potentially threatens the

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Table 2. Multiple Regression Analysis of Day-3 Positivity among 841 Patients with Uncomplicated *P. falciparum* Malaria in Eritrea (2016–2019).*

Covariate	Coefficient	Standard Error	Odds Ratio (95% CI)
Age	–0.0063	0.016	0.99 (0.96–1.02)
Sex	–0.0081	0.487	0.98 (0.38–2.57)
Initial parasitemia	0.0000087	0.0000058	1.00 (1.00–1.00)
Pfkelch13 622I	1.824	0.466	6.2 (2.5–15.5)

* Multiple regression was used to analyze the relationship between day-3 positivity and age (which is related to host immunity and the capacity of the immune system to clear parasites independent of treatment), sex, initial parasitemia (an initially high degree of parasitemia can lead to parasite persistence on day 3), and *Pfkelch13* 622I variant status. We found that when all other covariates were kept constant, the odds of day-3 positivity were higher among patients carrying the *Pfkelch13* R622I mutation in isolates before artemisinin-based combination therapy than among patients carrying *Pfkelch13* wild-type parasites by a factor of 6.2 (95% CI, 2.5 to 15.5). The goodness-of-fit of our multiple regression model was evaluated with the use of the Hosmer–Lemeshow test (P>0.05) and by receiver-operating-characteristic (ROC) curve analysis (mean [±SD] area under the ROC curve estimate, 0.726±0.07; 95% CI, 0.69 to 0.75).

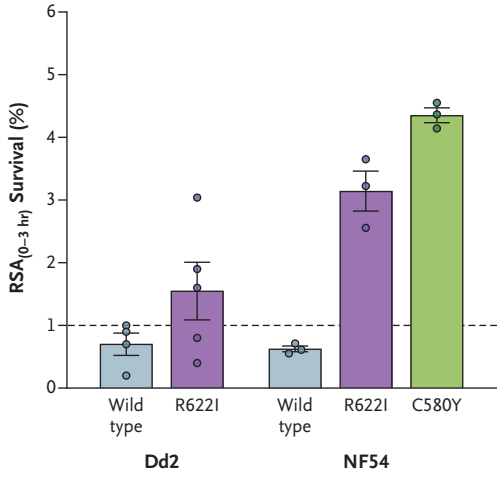

Figure 2. *Pfkelch13* R622I and Low-Level In Vitro Artemisinin Resistance in *P. falciparum* Parasites.

The *Pfkelch13* R622I mutation was introduced into African (NF54) and Asian (Dd2) parasite lines by CRISPR-Cas9 (clustered regularly interspaced short palindromic repeats and associated Cas9 homing endonucleases)–mediated gene editing. The ring-stage survival assay, performed on ring-stage parasites synchronized to a 0-to-3-hour window after merozoite invasion of red cells ($RSA_{0\text{-}3hr}$), was used to measure the survival of early ring-stage parasites exposed to 700nM dihydroartemisinin for 6 hours, relative to dimethylsulfoxide mock-treated parasites that were assayed in parallel. Percent survival values are shown as means; I bars indicate standard errors. The dashed horizontal line indicates the 1% threshold for survival, above which parasites are considered to exhibit in vitro resistance to dihydroartemisinin. Results were obtained from three to five independent experiments (circles), each performed in duplicate or triplicate.

efficacy of HRP2-based rapid diagnostic tests. No parasites carrying the *Pfkelch13* R622I mutation had only an *hrp2* deletion, as compared with 5.7% (16 of 280) of wild-type parasites.

DISCUSSION

Partial resistance to artemisinin in *P. falciparum* is now firmly established in Africa. Although partial resistance has been confirmed only in Central (Rwanda) and East (Uganda) Africa to date,[10-12] here we provide evidence of an additional hotspot of partial resistance to artemisinin in the Horn of Africa. More worrisome was the finding that the emergence and spread of a novel *Pfkelch13* 622I variant lineage was accompanied by deletions in both *hrp2* and *hrp3* in a

Figure 3 (facing page). Genomewide Phylogenetic Tree.

This maximum-likelihood tree is based on 128 *P. falciparum* Eritrean isolates, together with 162 isolates collected worldwide (Africa, Asia, and South America) and the 3D7 reference genome from Africa. Labels on Eritrean isolates include the year of collection. The non-Eritrean isolates were sourced from the MalariaGEN *P. falciparum* Community Project (https://www.malariagen.net/data/open-dataset-plasmodium-falciparum-v70) and are labeled with their accession identifier. Each leaf represents one sample and is colored according to the country of collection. Eritrean parasites carrying the *Pfkelch13* R622I mutation are identified by red stars at the tip. Eritrean parasites carrying *Pfkelch13* 622I are closely related to *Pfkelch13* wild-type parasites originating from East African countries. The scale bar corresponds to 0.00005 nucleotide substitutions per character.

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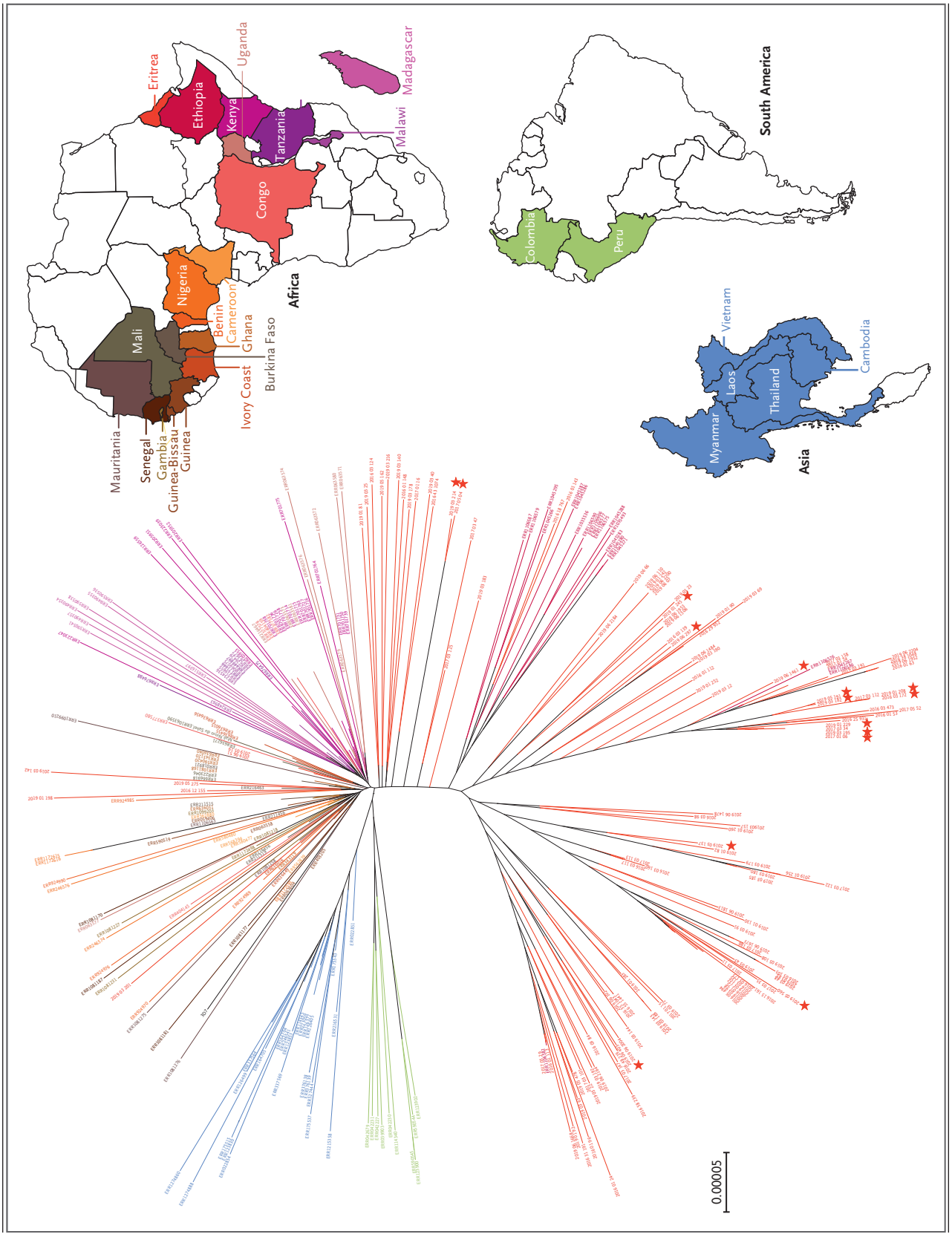

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substantial percentage of parasites (16.9%), thereby rendering these parasites more likely to be undetected by HRP2-based rapid diagnostic tests.

In vivo partial resistance to artemisinin in Eritrea was evidenced by a substantial increase over time in the percentage of patients with day-3 positivity after artemisinin-based combination therapy (from 0.4% in 2016 to 4.2% in 2019). We also witnessed a substantial rise in the percentage of parasites carrying the *Pfkelch13* R622I mutation (from 8.6% in 2016 to 21.0% in 2019). We determined that this mutation, which has not been observed previously in Southeast Asia, confers in vitro resistance to dihydroartemisinin in the African parasite strain NF54, at slightly lower levels than the C580Y mutation that predominates in Southeast Asia (mean $RSA_{0\text{-}3hr}$ survival, 3.3% vs. 4.3%). The R622I mutation conferred only low-level survival (1.5%) in Dd2 parasites (an Asian reference strain), a finding consistent with previous evidence that *Pfkelch13* mutations do not afford resistance across all strains and that in vitro resistance to artemisinin levels can be substantially modulated by the parasite genetic background.[16] We also documented substantial intrahost selection of the *Pfkelch13* R622I mutation in recrudescent infections after artesunate–amodiaquine treatment (from 12% on day 0 to 53% on day 28, an increase by a factor of 4.4). These findings suggest that the *Pfkelch13* R622I mutation is a molecular marker of partial resistance to artemisinin.

Eritrean *Pfkelch13* 622I variants were phylogenetically closely related to other African parasites, clustering with both Eritrean and Ethiopian wild-type isolates. Hallmarks of the spread of a newly arisen mutation would be expected to include extended haplotype homozygosity in the genomic region flanking the *Pfkelch13* R622I mutation. By contrast, we observed a limited identity of core haplotypes among the variants. This finding may reflect the spread of a preexisting *Pfkelch13* resistance allele in *P. falciparum* populations from the Horn of Africa, as supported by previous reports of low-frequency detection of the *Pfkelch13* 622I variant in Eritrea and neighboring countries (0.8% in Eritrea [2013 and 2014], 2.4% in Ethiopia [2013–2014], 0.7% in Somalia [2016–2017], and 0.3% in Zambia [2012]),[26-29] and in Chinese travelers returning from Mozambique or Somalia (2016–2018).[30]

Although recent data on treatment efficacy at sites with a high prevalence of *Pfkelch13* variants are limited in Eritrea,[31-33] our data showed that more than 94% of cases of uncomplicated falciparum malaria were cured with artesunate–amodiaquine or artemether–lumefantrine, a result that is presumably attributable to the continued efficacy of the partner drugs amodiaquine and lumefantrine. These data are concordant with those from a recent study of artemether–lumefantrine efficacy conducted in Rwanda.[12] The lower efficacy of artemether–lumefantrine (<90%) that was recently reported in Angola,[34] the Democratic Republic of Congo,[35] and east-central Uganda[36] remains questionable, because the methodologic deviation from the WHO standard genotyping protocol might have underestimated the efficacy.

Our study also showed that a substantial percentage (16.9%) of parasites carrying the *Pfkelch13* R622I mutation had deletions in both *hrp2* and *hrp3* (a similar percentage to that among *Pfkelch13* wild-type parasites), a characteristic that may result in false negative results on HRP2-based rapid diagnostic tests.[19] This genomic trait, frequently observed in Eritrea[37] with a prevalence ranging from 7% in Shambuko to 81% in Ghindae,[24] resulted in a policy switch to the use of lactate dehydrogenase–based rapid diagnostic tests in 2016. This finding emphasizes the need to conduct further research to evaluate the performance of lactate dehydrogenase–based tests for detecting the *Pfkelch13* 622I variant with *hrp2* and *hrp3* deletions.

Over the past two decades, Eritrea has achieved substantial reductions in malaria-related morbidity and mortality through active government engagement and effective implementation of insecticide-treated nets, indoor residual spraying, larvicidal activities, and malaria case management.[38,39] However, decreased malaria prevalence may in turn have favored the emergence and spread of partial resistance to artemisinin by reducing parasite genetic diversity and naturally acquired immunity and increasing per-patient drug pressure as fewer infections are cleared naturally and therefore require treatment.[40] These data suggest that in locations where strategies to reduce malaria transmission are efficiently implemented, surveillance of the emergence and spread of drug resistance should be prioritized.

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The results of our study show the presence of partial resistance to artemisinin in *P. falciparum*, along with deletions in both *hrp2* and *hrp3* in parasite populations in Eritrea. Strategies to contain the spread of these lineages across the Horn of Africa are needed because the potential occurrence of partner-drug resistance could lead to an increased incidence of treatment failure and uncontrolled expansion of *P. falciparum* parasites beyond this region.

Drs. Zehaie Kassahun and Ringwald are staff members of the World Health Organization (WHO) and note that the views expressed in this publication do not necessarily represent the decisions, policy, or views of the WHO.

Supported by the Bill and Melinda Gates Foundation through the WHO (grant OPP1209843); the Global Fund to Fight AIDS, Tuberculosis, and Malaria through the Ministry of Health, Eritrea; the Institut Pasteur; the French Government (Agence Nationale de la Recherche), Laboratoire d'Excellence "French Parasitology Alliance for Health Care" (ANR-11-15 LABX-0024-PARAFRAP); the University of Strasbourg through the Programme IdEX 2022 (to Dr. Ménard); and National Institutes of Health (grant R01 AI109023, to Dr. Fidock).

Disclosure forms provided by the authors are available with the full text of this article at NEJM.org.

A data sharing statement provided by the authors is available with the full text of this article at NEJM.org.

We thank all the patients who contributed samples, their guardians in the communities of Akordat, Ghindae, Guluj, Shambuko, and Tokombia, and all team members in the health centers; the members of the national Malaria Control Program in Eritrea for their support; the personnel at the High Performance Computing Core Facility, the Biomics Platform, and the Center for Technological Resources and Research at the Institut Pasteur, Paris, France, for their help with this work; the Integrated Collections for Adaptive Research in Biomedicine team of the Center for Translational Science and the BioResource Center of Institut Pasteur for providing blood samples from healthy volunteers; Hélène Laude, Laurence Arowas, Ayla Zayoud, and Marie Noelle Ungeheuer for managing the patients' visits; the healthy volunteers for their participation in the study; Emmanuel Roux, Alain Li, Dorian Cheval, Sophie Vacant, Sophie Chaouche, and Elsa Lievin for preparing the blood samples from the donors; and Aurélie Claës (Biology of Host–Parasite Interactions Unit, INSERM U1201, Institut Pasteur) for providing advice on *P. falciparum* genome editing.

APPENDIX

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