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Evidence of Artemisinin-Resistant Malaria in Africa

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ABSTRACT

BACKGROUND

In the six Southeast Asian countries that make up the Greater Mekong Subregion, *Plasmodium falciparum* has developed resistance to derivatives of artemisinin, the main component of first-line treatments for malaria. Clinical resistance to artemisinin monotherapy in other global regions, including Africa, would be problematic.

METHODS

In this longitudinal study conducted in Northern Uganda, we treated patients who had *P. falciparum* infection with intravenous artesunate (a water-soluble artemisinin derivative) and estimated the parasite clearance half-life. We evaluated ex vivo susceptibility of the parasite using a ring-stage survival assay and genotyped resistance-related genes.

RESULTS

From 2017 through 2019, a total of 14 of 240 patients who received intravenous artesunate had evidence of in vivo artemisinin resistance (parasite clearance half-life, >5 hours). Of these 14 patients, 13 were infected with *P. falciparum* parasites with mutations in the A675V or C469Y allele in the *kelch13* gene. Such mutations were associated with prolonged parasite clearance half-lives (geometric mean, 3.95 hours for A675V and 3.30 hours for C469Y, vs. 1.78 hours for wild-type allele; P<0.001 and P=0.05, respectively). The ring-stage survival assay showed a higher frequency of parasite survival among organisms with the A675V allele than among those with the wild-type allele. The prevalence of parasites with *kelch13* mutations increased significantly, from 3.9% in 2015 to 19.8% in 2019, due primarily to the increased frequency of the A675V and C469Y alleles (P<0.001 and P=0.004, respectively). Single-nucleotide polymorphisms flanking the A675V mutation in Uganda were substantially different from those in Southeast Asia.

CONCLUSIONS

The independent emergence and local spread of clinically artemisinin-resistant *P. falciparum* has been identified in Africa. The two *kelch13* mutations may be markers for detection of these resistant parasites. (Funded by the Japan Society for the Promotion of Science and others.)

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In 2019, an estimated 229 million cases of malaria resulting in 409,000 deaths were reported worldwide.[1] Artemisinin-based combination therapies, the recommended first-line treatment in all countries in which malaria is endemic, have contributed considerably toward reducing the malaria burden.[2] However, artemisinin-resistant *Plasmodium falciparum* parasites have spread in the Greater Mekong Subregion of Southeast Asia during the past 10 years.[3-7] Because 90% of malaria cases and deaths are currently reported in Africa, the emergence of artemisinin resistance poses a serious threat to malaria control worldwide.

Artemisinin resistance has been assessed primarily by means of the gene encoding the kelch protein (*kelch13*; PF3D7_1343700). To date, 10 mutations in *kelch13* have been validated as molecular markers.[8] In Africa, several mutations (M476I, P553L, R561H, and P574L) have been sporadically detected.[8,9] The prevalence of three of these mutations was low, but in 2018, the R561H mutation was found in 12.8% of *P. falciparum* cases in Rwanda and was associated with positive parasitemia on day 3 of testing.[10] Because no alternative to artemisinin derivatives is currently available, the spread of artemisinin-resistant parasites in Africa is problematic.

Artemether–lumefantrine was officially introduced in 2006 in Northern Uganda. Between 2014 and 2016, we identified in vitro artemisinin-resistant isolates in the region using a ring-stage survival assay.[11] Two *kelch13* mutations, A675V and C469Y, were particularly noted as candidates for artemisinin resistance in recent studies.[12,13] In vivo artemisinin resistance has been best defined by a prolonged parasite clearance half-life.[14,15] Although such resistance has been confirmed by this method in Southeast Asia, it has not been confirmed in Africa. Here, we describe the results of a longitudinal study to determine the phenotype of clinical artemisinin resistance in Northern Uganda, as determined quantitatively by the half-life of parasite clearance after monotherapy.

Methods

Study Location and Patient Recruitment

This study was conducted from 2015 through 2019 at St. Mary's Hospital Lacor in Gulu, Northern Uganda.[11,16] In this region, malaria transmission is high and perennial,[17,18] although some decline was reported between 2017 and 2018.[19] Patients with *P. falciparum* infection who were hospitalized for intravenous artesunate treatment (under the hospital's standard-of-care guidelines) were recruited. Patients were eligible for enrollment if they were at least 6 months of age and had *P. falciparum* monoinfection with parasitemia of more than 10,000 organisms per microliter. Patients were excluded if they had received an antimalarial drug within 2 weeks before enrollment or if they had a coexisting chronic medical condition.

Written informed consent was obtained from adults (≥18 years of age) or from children's parents or guardians. In addition, assent was sought from children between the ages of 7 and 17 years. In the presence of an impartial witness, oral informed consent and fingerprints were obtained from patients who were unable to read or write. This study was approved by the institutional research board at Lacor Hospital, the Uganda National Council for Science and Technology, and the ethics committee at Juntendo University.

Artemisinin Monotherapy and Follow-up Study

As part of the overall study, we assessed the efficacy of artesunate monotherapy from 2017 through 2019. A form of artesunate (Guilin Pharmaceutical) that had been prequalified by the World Health Organization (WHO) was administered intravenously at a dose of 3 mg per kilogram of body weight (in patients weighing <20 kg) or 2.4 mg per kilogram (in those weighing ≥20 kg) at 0, 12, and 24 hours, followed by a standard 3-day course of artemether–lumefantrine.[20]

Thin and thick blood smears were collected in duplicate at 0, 4, and 6 hours and then at 6-hour intervals until two consecutive negative blood smears had been obtained. If parasites were still observed after the initial three doses, additional doses of intravenous artesunate were administered every 24 hours until parasite clearance was observed. Parasite density was independently determined by two examiners by counting 1000 erythrocytes on a thin smear or 200 leukocytes on a thick smear (parasitemia, <0.1%). We determined the parasite clearance half-life using the Parasite Clearance Estimator developed by the Worldwide Antimalarial Resistance Network.[21] If the difference in clearance half-life that was determined by two examiners was more than

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1 hour, a third examiner also provided a measure; the half-life reported by the third examiner was considered in the calculation of the average value. An average clearance half-life of more than 5 hours was judged to indicate in vivo artemisinin resistance.[8]

EX VIVO RING-STAGE SURVIVAL ASSAYS

Ex vivo ring-stage survival assays were performed in 2017 and 2018, as reported previously.[11,22] The erythrocyte fraction of a sample was suspended in RPMI-1640 culture medium containing 10% O+ serum and 50 mg per liter of gentamicin (Thermo Fisher Scientific) and then stored at 4°C overnight before the assay was performed. Patients with a non-O blood type were excluded from the analysis. Two examiners independently evaluated the proportion of erythrocytes infected with viable parasites by counting 10,000 erythrocytes on a thin smear. If the proportion of infected erythrocytes in the control wells was less than 20% of the initial parasitemia, the results were excluded. The survival rate was determined from the ratio of viable parasites in the drug-exposed and control wells. The cutoff value for ex vivo resistance was set at more than 1%.[8]

GENOTYPING

Genotyping was performed from 2015 to 2019. After the removal of human leukocytes with the use of Acrodisc filters (Pall), blood was spotted onto filter paper. Parasite DNA was extracted by means of a QIAamp DNA Mini Kit (Qiagen). Direct sequencing was performed to determine polymorphisms in the propeller domain of *kelch13* (Tables S1 and S2 in the Supplementary Appendix, available with the full text of this article at NEJM.org).[16,23] For samples with *kelch13* mutations associated with resistance, six background mutations were also assessed: V127M in apicoplast ribosomal protein S10 (*arps10*), I356T in chloroquine resistance transporter (*crt*), D193Y in ferredoxin (*fd*), T484I in multidrug resistance protein 2 (*mdr2*), V1157L in protein phosphatase (*nif4*, also known as *pph*), and C1484F in phosphoinositide-binding protein (*pibp*).[16,24] A mixed genotype was recognized when the minor peak was higher than a third of the major peak. The mixed genotypes with wild-type and mutant alleles were classified as mutants.

Polymorphisms in microsatellite loci located −33.9, −8.1, −1.9, 4.1, 9.3, and 43.9 kb from *kelch13* were genotyped by means of polymerase-chain-reaction (PCR)–based fragment analysis.[25,26] To determine single-nucleotide polymorphism (SNP) haplotypes flanking *kelch13*, whole-genome sequencing was performed on A675V and randomly selected wild-type isolates, as reported previously.[11] Sequencing data were compared with isolates obtained along the Thai–Myanmar border that contained both A675V and wild-type alleles, which were determined from the genome data available in the Short Read Archive at the National Center for Biotechnology Information.

STATISTICAL ANALYSIS

We assessed chronologic changes in parasite prevalence using the Cochran–Armitage test. We used Spearman's correlation coefficient to evaluate the correlation between in vivo and ex vivo efficacy. To study the association between drug efficacy and genotypes, we used the Wilcoxon rank-sum test, Fisher's exact test, and multiple regression analysis. A P value of less than 0.05 was considered to indicate statistical significance. Multiple comparisons were adjusted with the use of the Bonferroni method. All reported P values are two-sided. All analyses was performed with the use of R software, version 4.0.2.[27]

RESULTS

PATIENTS

A total of 274 patients were enrolled from 2017 through 2019. Of these patients, 27 were excluded because of coinfection with plasmodia species other than falciparum, a low parasite density, the receipt of antimalarial treatment within 2 weeks before enrollment, the presence of human immunodeficiency virus infection, or withdrawal of consent. Consequently, 247 patients were assessed for in vivo parasite clearance after artemisinin monotherapy (Fig. 1).

IN VIVO AND EX VIVO DRUG EFFICACY

The parasite clearance half-life was successfully determined in 240 patients. The median age of the patients was 2.6 years (Table 1 and Table S3). Parasites were rapidly cleared in most patients, with a median half-life of 1.9 hours (interquartile range, 1.5 to 2.3). However, 14 patients (5.8%) had evidence of slow parasite clearance (defined as a half-life of >5 hours) and were considered to have in vivo artemisinin resistance (Fig. 2A and Fig. S1). The prevalence of in vivo resistance increased during the study years —

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Table 1. Characteristics of the Patients with Measured Parasite Clearance Half-Life, According to Study Year.*

Characteristic	2017 (N=87)	2018 (N=60)	2019 (N=93)	Total (N=240)
Median age (IQR) — yr	2.5 (1.6–4.0)	2.8 (1.8–4.4)	2.7 (1.5–4.0)	2.6 (1.6–4.0)
Female sex — no. (%)	42 (48)	29 (48)	36 (39)	107 (45)
Median temperature (IQR) — °C	38.6 (37.7–39.4)	38.5 (37.9–39.2)	38.4 (37.8–39.5)	38.6 (37.8–39.3)
Laboratory findings				
Median hemoglobin (IQR) — g/dl	12.8 (10.9–14.3)	10.5 (9.3–11.5)	9.4 (8.4–10.7)	10.6 (9.2–12.3)
Median parasite density (IQR) — mm^3	138,000 (73,000–228,000)	91,800 (67,000–127,000)	128,700 (84,600–186,000)	117,000 (70,000–189,000)
Gametocytemia — no. (%)	2 (2)	1 (2)	4 (4)	7 (3)
Median parasite clearance half-life (IQR) — hr	2.00 (1.62–2.42)	1.71 (1.53–2.14)	1.77 (1.48–2.22)	1.85 (1.52–2.32)
Parasite clearance half-life of >5 hr — no. (%)	4 (5)	4 (7)	6 (6)	14 (6)
kelch13 genotype — no. (%)†				
C469Y	0	1 (2)	4 (4)	5 (2)
A675V	8 (9)	8 (13)	11 (12)	27 (11)
Other mutation	0	2 (3)	4 (4)	6 (2)
Wild type	79 (91)	49 (82)	74 (80)	202 (84)

* The parasite clearance half-life was successfully determined in 240 of the study patients. The prevalence of in vivo resistance (defined as a parasite clearance half-life of >5 hours) increased slightly during the study period, but the difference with the prevalence at baseline was not significant (P=0.60). IQR denotes interquartile range.

† During the study period, mixed alleles were present in 1 patient with the C469Y allele, in 10 patients with the A675V allele, and in 3 patients with other mutations.

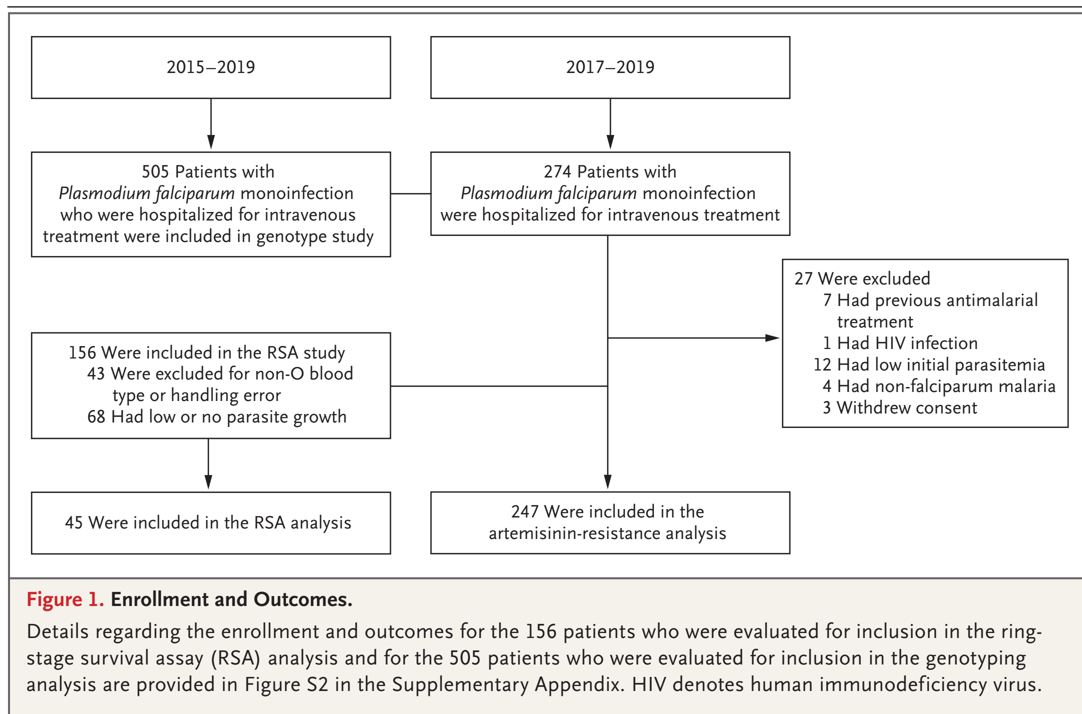

Figure 1. Enrollment and Outcomes.

Details regarding the enrollment and outcomes for the 156 patients who were evaluated for inclusion in the ring-stage survival assay (RSA) analysis and for the 505 patients who were evaluated for inclusion in the genotyping analysis are provided in Figure S2 in the Supplementary Appendix. HIV denotes human immunodeficiency virus.

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4.6% in 2017, 6.7% in 2018, and 6.5% in 2019 — but the difference was not significant (P=0.60). None of the cases fulfilled the criteria for early treatment failure. Gametocytes were detected in 7 patients.

Successful results on ex vivo ring-stage survival assays were obtained for 45 of 113 patients (Fig. S2); of these patients, 13 (28.9%) had a parasite survival of more than 1% (Fig. 2B). The ex vivo survival of parasites correlated with the in vivo clearance half-life (correlation coefficient, 0.29; P=0.05). Of the 14 patients with in vivo artemisinin resistance, ring-stage survival assays were performed on samples obtained from 7 patients. Among these patients, 4 had successful results on ring-stage survival assays, including 3 who had parasite survival of more than 1% (Fig. S3).

ASSOCIATION BETWEEN *KELCH13* MUTATIONS AND ARTEMISININ EFFICACY

Seven *kelch13* mutations were detected in 38 of 240 patients (15.8%) whose parasite clearance half-life had been determined (Table 1). Most of the mutations were either A675V (in 27 patients) or C469Y (in 5), which are the candidate mutations for artemisinin resistance currently listed by the WHO.[6] In the *kelch13* wild-type population, the geometric mean and upper 95th percentile of clearance half-life were 1.78 and 2.76 hours, respectively (Table S4). The half-lives of organisms with A675V and C469Y alleles (geometric mean, 3.95 and 3.30 hours, respectively) were significantly longer than those of organisms with wild-type alleles (P<0.001 and P=0.05, respectively, by the Bonferroni-corrected Wilcoxon rank-sum test) (Fig. 3A and Fig. S4). Furthermore, of the 14 patients with in vivo artemisinin resistance, 12 had parasites with the A675V allele (including 10 patients with the single allele and 2 with a mixed genotype) or the C469Y allele (in 2 patients). The 2 remaining patients had parasites with wild-type alleles, one of which had a small A675V peak that did not meet the criteria for a mixed genotype, but A675V was confirmed by cloning PCR fragments. Accordingly, the percentage of these two mutations was significantly greater in cases with in vivo artemisinin resistance than in susceptible cases (P<0.001 for A675V and P=0.008 for C469Y by Bonferroni-corrected Fisher's exact test).

Multivariable analysis showed that A675V and C469Y were the only significant predictors of a prolonged clearance half-life (Table S5). When

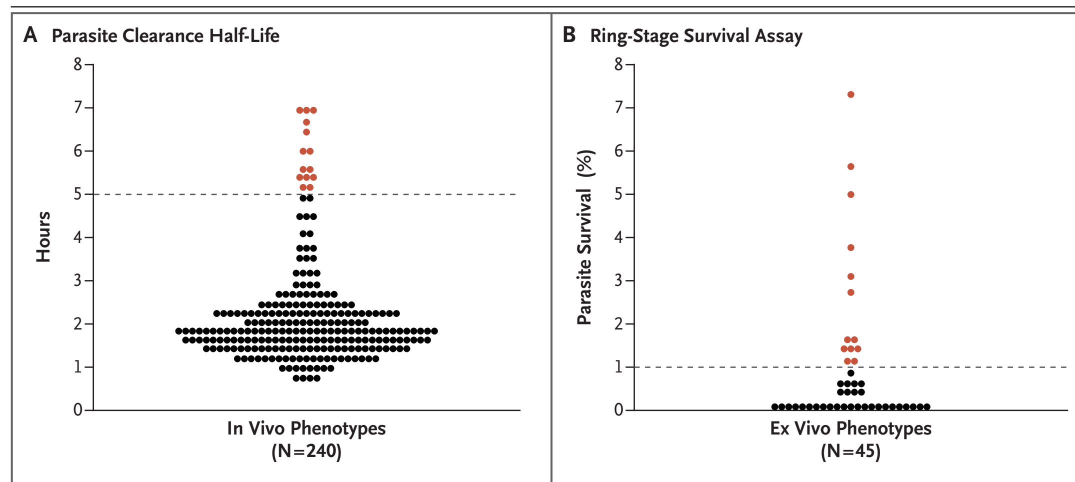

Figure 2. Demonstration of Artemisinin Resistance.

Panel A shows the parasite clearance half-life in 240 of the study patients for whom data were available. Parasites were rapidly cleared in most patients, with a median half-life of 1.9 hours (interquartile range, 1.5 to 2.3). However, 14 patients (5.8%) had evidence of slow parasite clearance (defined as a half-life of >5 hours) and were considered to have in vivo artemisinin resistance (indicated in red). Panel B shows the results of ex vivo ring-stage survival assays in 45 of 113 patients who were evaluated. Among these patients, 13 (29%) had parasite survival of more than 1% (indicated in red). Each data point represents a single patient. The ex vivo survival rate of parasites correlated with the in vivo clearance half-life (correlation coefficient, 0.29).

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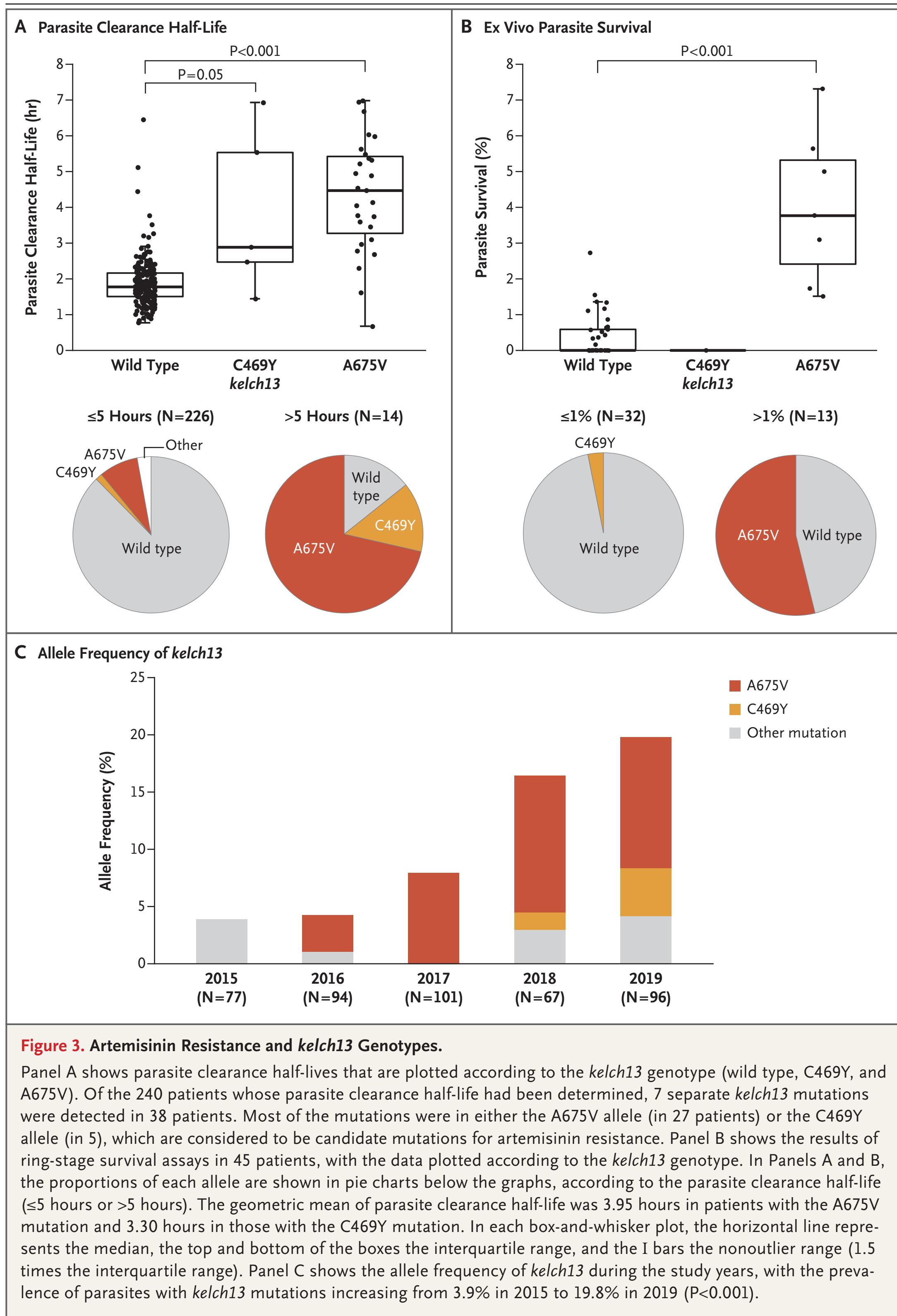

Figure 3. Artemisinin Resistance and *kelch13* Genotypes.

Panel A shows parasite clearance half-lives that are plotted according to the *kelch13* genotype (wild type, C469Y, and A675V). Of the 240 patients whose parasite clearance half-life had been determined, 7 separate *kelch13* mutations were detected in 38 patients. Most of the mutations were in either the A675V allele (in 27 patients) or the C469Y allele (in 5), which are considered to be candidate mutations for artemisinin resistance. Panel B shows the results of ring-stage survival assays in 45 patients, with the data plotted according to the *kelch13* genotype. In Panels A and B, the proportions of each allele are shown in pie charts below the graphs, according to the parasite clearance half-life (≤5 hours or >5 hours). The geometric mean of parasite clearance half-life was 3.95 hours in patients with the A675V mutation and 3.30 hours in those with the C469Y mutation. In each box-and-whisker plot, the horizontal line represents the median, the top and bottom of the boxes the interquartile range, and the I bars the nonoutlier range (1.5 times the interquartile range). Panel C shows the allele frequency of *kelch13* during the study years, with the prevalence of parasites with *kelch13* mutations increasing from 3.9% in 2015 to 19.8% in 2019 (P<0.001).

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these two *kelch13* genotypes were used as predictive markers of in vivo resistance, the sensitivity and specificity to identify a parasite clearance half-life of more than 5 hours were 85.7% (95% confidence interval [CI], 57.2 to 98.2) and 91.2% (95% CI, 86.7 to 94.5), respectively (Table S6). The developmental stage of the parasite before treatment was not a significant predictor of resistance. This finding was consistent with univariate analysis among the A675V cases, in which percentages of early ring parasite were not significantly different between patients with artemisinin resistance and those with artemisinin susceptibility (P=0.19) (Fig. S5).

In the ex vivo analysis, parasite survival could be determined in 7 of 27 patients with organisms with the A675V allele. Parasites with the A675V allele had a significantly higher survival frequency than those with wild-type allele (P<0.001 by the Wilcoxon rank-sum test) (Fig. 3B), and parasite survival was significantly associated with ex vivo resistance (P<0.001 by Fisher's exact test). All 7 patients who had parasites with the A675V allele had ex vivo resistance (Fig. S3).

PREVALENCE OF PARASITES WITH *KELCH13* MUTATIONS

The *kelch13* genotypes were determined in 435 patients (Table S7 and Figure S2). These patients included the 240 patients for whom parasite clearance half-life data were available and the 195 patients for whom such data were lacking. The prevalence of parasites with *kelch13* mutations significantly increased from 3.9% in 2015 to 19.8% in 2019 (P<0.001) (Fig. 3C). This finding was primarily due to a significant increase in the frequency of both the A675V and C469Y alleles (P<0.001 and P=0.004, respectively), which reached a prevalence of 11.5% and 4.2%, respectively, in 2019. No mutations were detected in six background genes for artemisinin resistance (*arps10*, *crt*, *fd*, *mdr2*, *nif4*, and *pibp*) in all the patients who had parasites with the A675V allele (30 patients) or the C469Y allele (5 patients) and in 2 patients with the wild-type allele who had in vivo artemisinin resistance.

Parasites with A675V and C469Y mutations had identical or nearly identical *kelch13*-flanking microsatellite haplotypes, which indicated the probability that the A675V and C469Y mutants had a single evolutionary origin of artemisinin resistance (Fig. S6). SNP haplotypes flanking *kelch13* clearly showed the substantial difference of haplotypes between A675V isolates in Uganda and in Southeast Asia (Fig. S7), which suggested that the mutation probably emerged independently in Africa and Southeast Asia.

DISCUSSION

Emerging artemisinin resistance among patients with *P. falciparum* infection has been evaluated in several studies in Africa.[6,11,28-30] In our study, we determined the emergence and local spread of clinical artemisinin resistance in Northern Uganda. From 2017 through 2019, the parasite clearance half-life was prolonged (>5 hours) in 5.8% of patients with *P. falciparum* infection after intravenous artemisinin monotherapy. Recently in Rwanda, an association between the presence of the R561H mutation and parasite-positive samples on day 3 after artemisinin-based combination therapy was seen in 12.8% of isolates, even though no early treatment failure was observed.[10] In our study, we found an association between a prolonged parasite clearance half-life after artemisinin monotherapy with both A675V or C469Y mutations, indicating the emergence of artemisinin resistance in Africa. Reports of artemisinin resistance along the Thai–Myanmar border have shown that in 76% of 49 patients who had parasites with the A675V mutation, the half-life was more than 5.5 hours (median, 6.7 hours).[6] The clinical relevance of C469Y remains to be determined.[31,32]

The WHO lists 10 validated and 13 candidate *kelch13* mutations as molecular markers for artemisinin resistance,[6] with A675V and C469Y classified as candidate alleles. According to WHO criteria,[8] a validated molecular marker should fulfill the following two conditions: show a significant association between the mutation and decreased in vivo efficacy of artemisinin in at least 20 cases and show ex vivo resistance in at least 5 isolates. In our study, 27 cases of the A675V mutation had a significant association with a prolonged clearance half-life; in 7 of these patients, the parasite survival frequency could be successfully determined, and all had ex vivo artemisinin resistance. These findings provide evi-

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dence for A675V as a molecular marker of artemisinin resistance.

Microsatellite haplotypes indicate that A675V and C469Y populations had clonally expanded in our study area. Previous countrywide studies from 2016 to 2019 suggested that these alleles were localized to the northern half of Uganda,[12,13] with only a few cases identified in other African countries.[33,34] In contrast, a considerable number of A675V mutants have been detected around the Thai–Myanmar border. However, we showed that the Asian A675V allele had a different origin from the Ugandan mutant. C469Y has been sporadically observed in Southeast Asia as well.[6]

In our assessment, we used the Southeast Asian criterion of parasite survival for 5 hours as the cutoff for determining in vivo artemisinin resistance, since no value had been established in African regions with high malaria transmission. However, among persons living in highly endemic areas, immunity to malaria generally develops through repeated infections,[35] which may accelerate parasite clearance.[36] We found that the 95th percentile of half-life in the *kelch13* wild-type population was 2.76 hours, which was markedly shorter than that reported in the Southeast Asian population (5.3 hours).[6] Coinfection with susceptible parasites, which is commonly observed in Africa,[37] can also shorten the clearance half-life in patients infected with a resistant parasite. Such epidemiologic characteristics may complicate the assessment of parasite clearance half-life in Africa, which may lead to an underestimation of resistance and indicate that the current cutoff for defining resistance (5 hours) may be too strict. Nevertheless, 14 patients in our study met this strict cutoff. In the future, it may be prudent to apply a less stringent criterion for early and more sensitive detection of artemisinin resistance in Africa.

An indigenous emergence and spread of clinical artemisinin resistance was identified in Northern Uganda. Despite the limited availability of oral artemisinin monotherapy,[38] poor adherence and excessive use of injectable artesunate[39] could have created selective pressure for resistant parasites in the region. As observed in some parts of Southeast Asia, delayed parasite clearance potentially selects parasites that are resistant to the partner drugs used in artemisinin-based combination therapy, which in turn leads to treatment failure.[40] Our findings suggest a potential risk of cross-border spread across Africa and highlight the need to perform large-scale surveys. The association that we found between clinical artemisinin resistance and the presence of the A675V and C469Y mutations raises the possibility that these resistance markers can be used to assist in the detection of artemisinin resistance among patients with *P. falciparum* infection in Africa.

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

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