International Scholars Journals

African Journal of Parasitology Research ISSN 2756-3391 Vol. 14 (9), pp. 808-814, September, 2026. Available online at www.internationalscholarsjournals.org © International Scholars Journals

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*Research Article*

# Detection of artemisinin-resistant Plasmodium falciparum kelch13 propeller domain mutations in patients with uncomplicated malaria

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Manuscript Timeline: Received: February 14, 2026; Revised: March 28, 2026; Accepted: May 02, 2026; Published: September 07, 2026

## ABSTRACT

Plasmodium falciparum remains the primary cause of severe malaria morbidity and mortality, and the emergence of resistance to artemisinin-based combination therapies threatens global control efforts. In this study, we investigated the frequency of single nucleotide polymorphisms in the P. falciparum kelch13 (Pfk13) propeller domain among patients presenting with uncomplicated malaria. A total of 285 dried blood spot samples were collected from febrile patients confirmed positive by rapid diagnostic tests and Giemsa-stained thick blood smears. Genomic DNA was extracted and subjected to nested PCR amplification of the Pfk13 propeller region, yielding a 740 base pair fragment followed by Sanger sequencing. Out of 285 samples, successful amplification and sequencing were achieved for 264 isolates (92.6 percent). Wild-type Pfk13 sequences were observed in 241 isolates (91.3 percent), while non-synonymous mutations were detected in 23 isolates (8.7 percent). The validated resistance marker R561H was identified in 9 isolates (3.4 percent), C469Y in 6 isolates (2.3 percent), and A675V in 5 isolates (1.9 percent), alongside 3 isolates carrying synonymous mutations. Parasite clearance half-lives were prolonged (>5 hours) in 78.3 percent of patients harboring Pfk13 mutant strains compared to 6.2 percent in patients with wild-type parasites. Low-level circulation of validated Pfk13 mutations exists in the study population. Continuous molecular surveillance of antimalarial drug resistance markers is essential to guide national treatment policies and prevent widespread treatment failure.

**Keywords:** Plasmodium falciparum, Artemisinin resistance, Kelch13 mutations, Malaria surveillance, Sequencing

Malaria remains one of the most consequential infectious diseases of our time. In 2023, the World Health Organization (WHO) estimated 263 million malaria cases and 597,000 deaths globally, with the African Region accounting for about 94% of cases and 95% of deaths, and with children under five years bearing a disproportionate share of mortality (World Health Organization, 2024). Plasmodium falciparum is responsible for the overwhelming majority of severe disease and death in sub-Saharan Africa, and its control rests heavily on prompt diagnosis, vector control, and effective chemotherapy (White et al., 2014; World Health

Organization, 2024). For more than two decades, artemisinin-based combination therapies (ACTs) have been the cornerstone of treatment for uncomplicated falciparum malaria, combining a fast-acting artemisinin derivative that rapidly reduces parasite biomass with a longer-acting partner drug that clears residual parasites (World Health Organization, 2023a; Ashley et al., 2014). The success of this strategy is now threatened by the emergence and spread of partial artemisinin resistance, a phenotype defined not by outright treatment failure, but by delayed parasite clearance after artemisinin exposure (World Health Organization, 2023b; Rosenthal et al., 2024).

Partial artemisinin resistance was first recognised in western Cambodia in the late 2000s, where patients treated with artesunate monotherapy showed markedly prolonged parasite clearance half-lives (Noedl et al., 2008; Dondorp et al., 2009). Subsequent clinical and genetic investigations traced the phenotype to non-synonymous mutations in the propeller domain of the P. falciparum kelch13 gene (Pfk13, PF3D7_1343700) on chromosome 13 (Ariey et al., 2014). In a landmark genome-wide association study, Ariey et al. (2014) identified kelch13 as the major locus associated with slow clearance, and gene-editing experiments later confirmed that introducing specific kelch13 propeller mutations into sensitive parasites conferred ring-stage survival under dihydroartemisinin pressure (Straimer et al., 2015). The WHO now maintains a curated list of validated and candidate kelch13 mutations, distinguishing those with robust clinical and laboratory evidence of an association with delayed clearance from those with suggestive but incomplete evidence (World Health Organization, 2023b).

The biology of Kelch13 helps explain why propeller mutations matter. Kelch13 is a 726-amino-acid protein containing a Plasmodium-specific N-terminal region, a BTB/POZ domain, and a C-terminal six-bladed beta-propeller domain spanning roughly codons 441–726 (Ariey et al., 2014; Birnbaum et al., 2020). The protein functions as a substrate adaptor for a cullin-RING E3 ubiquitin ligase complex and regulates endocytosis of host haemoglobin in early ring-stage parasites (Birnbaum et al., 2020; Yang et al., 2019). Artemisinin is activated by haem-derived iron after haemoglobin digestion, and reduced endocytosis in kelch13 mutants lowers intracellular activation of the drug, permitting a subpopulation of early rings to survive a normally lethal pulse (Birnbaum et al., 2020; Mok et al., 2021). Mutant parasites also show an enhanced unfolded-protein and oxidative-stress response, which further buffers artemisinin-induced damage (Mok et al., 2015; Mok et al., 2021). Together, these mechanisms produce the characteristic phenotype: parasites are not fully resistant in the classical sense, but they clear more slowly, survive longer in the presence of artemisinin, and expose the partner drug to a larger residual biomass, thereby accelerating the selection of partner-drug resistance (White, 2023; Rosenthal et al., 2024).

In Southeast Asia, the C580Y mutation became dominant and spread across the Greater Mekong Subregion, accompanied by other validated mutations such as R539T, I543T, Y493H, and F446I (Ashley et al., 2014; Imwong et al., 2017). Intensive containment, including a shift to alternative ACTs and, in some areas, targeted elimination, slowed but did not eliminate this spread (Imwong et al., 2020). For many years, Africa appeared largely spared. Surveys across the continent found kelch13 propeller mutations to be rare, and most non-synonymous variants detected were not on the WHO validated list (Ménard et al., 2016; Conrad and Rosenthal, 2019). That reassuring picture has changed. Since 2020, validated mutations have been confirmed with clinical evidence of delayed clearance in Rwanda (R561H), Uganda (C469Y and A675V), Tanzania (R561H and related alleles), Eritrea (R622I), Ethiopia, Kenya, and Sudan, often with evidence of local clonal expansion rather than repeated importation (Uwimana et al., 2020; Balikagala et al., 2021; Conrad et al., 2023; Fola et al., 2023; World Health Organization, 2024).

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The three mutations detected in the present study illustrate this African pattern. R561H was first validated in Rwanda, where Uwimana et al. (2020) documented delayed clearance and a rising prevalence in Masaka and later in other sites, with genomic evidence of a local origin and subsequent spread. C469Y emerged in northern Uganda, where Balikagala et al. (2021) demonstrated in vitro ring-stage survival and in vivo delayed clearance associated with this allele, and where subsequent surveillance has shown its frequency to rise sharply in some districts. A675V has been reported in Uganda, Kenya, Rwanda, and Tanzania, and is now among the most frequently reported validated mutations in East Africa (Conrad et al., 2023; Asua et al., 2021). The WHO African Region strategy for artemisinin resistance now explicitly calls for routine therapeutic efficacy studies paired with molecular surveillance, because molecular signals often precede measurable increases in treatment failure (World Health Organization, 2023b; World Health Organization, 2024).

Nigeria carries the heaviest malaria burden of any country, accounting for roughly one-quarter of global cases and deaths (World Health Organization, 2024). ACTs — principally artemether-lumefantrine and artesunate-amodiaquine — remain first-line therapy, and therapeutic efficacy studies have generally reported adequate clinical and parasitological response rates above 90% (World Health Organization, 2023a). Molecular studies in Nigeria, however, have produced a heterogeneous picture: some surveys have found no validated kelch13 mutations, while others have reported low-frequency R561H, C469Y, A675V, or candidate alleles alongside a background of non-validated polymorphisms such as K189T (Oboh et al., 2018; Ajogbasile et al., 2023; World Health Organization, 2024). Such heterogeneity is expected when sampling is sparse, when sites differ in transmission intensity and drug pressure, and when sequencing is performed on convenience samples rather than through systematic sentinel surveillance. Against this background, the present study was designed to determine the frequency of kelch13 propeller domain polymorphisms among patients with uncomplicated falciparum malaria, to classify detected alleles against the WHO validated list, and to examine the association between genotype and parasite clearance half-life. We hypothesised that validated mutations would be present at low frequency but would be strongly associated with prolonged clearance, signalling early emergence that warrants intensified surveillance before clinical efficacy is compromised.

## Materials and Methods

### Study design and setting

This was a cross-sectional molecular surveillance study conducted among febrile patients presenting with uncomplicated malaria at outpatient facilities serving the study catchment. The design followed WHO guidance for molecular surveillance of antimalarial drug resistance, which recommends systematic sampling of confirmed falciparum cases, standardised molecular methods, and linkage of genotype to a clearance phenotype where feasible (World Health Organization, 2023b). The study protocol was approved by the institutional ethics committees of the participating institutions, and written informed consent was obtained from all adult participants and from guardians of minors, with assent from older children, in accordance with the Declaration of Helsinki. No participant identifiers were retained in the molecular dataset; samples were analysed under coded study numbers.

### Patient enrolment and clinical procedures

Patients were eligible if they presented with fever (axillary temperature ≥37.5 °C or history of fever in the preceding 48 hours), had uncomplicated malaria confirmed by both a P. falciparum

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histidine-rich protein 2 (HRP2)-based rapid diagnostic test (RDT) and Giemsa-stained thick blood smear microscopy, were aged six months or older, and had no signs of severe malaria, no known allergy to artemisinin derivatives, and no antimalarial treatment in the preceding 28 days. Patients with severe malaria, pregnancy in the first trimester, mixed Plasmodium infection, or inability to provide consent were excluded. This dual-confirmation approach reduces the risk of enrolling false-positive RDT cases due to persistent HRP2 antigenaemia after a prior episode, a well-recognised limitation of HRP2-based tests (World Health Organization, 2023a). Clinical and demographic data, including age, sex, axillary temperature, reported duration of illness, and prior antimalarial use, were recorded on a standardised case report form.

After enrolment, patients received weight-based artemether-lumefantrine according to national treatment guidelines and were followed for parasite clearance. Thick blood smears were prepared at enrolment and at 6-hourly intervals during the first 48 hours where logistically feasible, and at least at 0, 12, 24, 48, and 72 hours in all participants, to permit estimation of the parasite clearance half-life (PC1/2). Parasite density was quantified against 200 white blood cells on thick smears, assuming 8,000 white blood cells per microlitre, by two independent microscopists blinded to genotype, with a third reader resolving discrepancies greater than 20% (World Health Organization, 2023a). Clearance half-life was estimated using the Parasite Clearance Estimator (PCE) approach, which fits a log-linear decline to serial densities after a lag phase and has become the standard for comparing clearance across studies (Flegg et al., 2011; WWARN K13 Genotype-Phenotype Study Group, 2019). A PC1/2 greater than 5 hours was classified as prolonged, consistent with WHO thresholds used to flag suspected partial artemisinin resistance (World Health Organization, 2023b). Patients who remained parasitaemic at 72 hours or who met criteria for treatment failure were managed according to national guidelines and referred for clinical review.

## Sample collection and DNA extraction

Dried blood spots (DBS) were collected by finger-prick onto Whatman 903 filter paper at enrolment, air-dried at ambient temperature away from direct sunlight, placed individually in sealed zip-lock bags with desiccant, and stored at −20 °C until molecular analysis. DBS are widely used for molecular surveillance because they are minimally invasive, stable under field conditions, and suitable for PCR-based genotyping after prolonged storage (World Health Organization, 2023b). Genomic DNA was extracted from three 3-mm punches per sample using a commercial silica-column kit (QIAamp DNA Mini Kit, Qiagen, Hilden, Germany) following the manufacturer’s protocol for dried blood spots, with an additional overnight proteinase K digestion to maximise yield from low-density infections. DNA was eluted in 100 µL of buffer AE, quantified by spectrophotometry, and stored at −20 °C. Extraction batches included a blank filter-paper control and a known positive control to monitor contamination.

## Nested PCR amplification of the Pfk13 propeller domain

The Pfk13 propeller domain was amplified by nested PCR using published primers and cycling conditions that target a 740-base-pair fragment spanning codons 441–726, covering all WHO-validated and candidate propeller mutations (Ariey et al., 2014; Ménard et al., 2016). Primary PCR was performed in a 25-µL reaction containing 2 µL of template DNA, 1× PCR buffer, 2.0 mM MgCl2, 200 µM of each dNTP, 0.4 µM of each outer primer, and 1.0 U of Taq DNA polymerase. Cycling conditions were: initial denaturation at 95 °C for 5 minutes; 35 cycles of 95 °C for 30 seconds, 58 °C for 45 seconds, and 72 °C for 90 seconds; and a final extension at 72 °C for 10 minutes. Secondary PCR used 2 µL of primary product as template with inner

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primers under identical cycling conditions except for an annealing temperature of 60 °C. Each PCR run included a no-template control, a DNA extraction blank, and genomic DNA from the 3D7 reference strain (wild-type Pfk13) and a laboratory-validated mutant control where available. Amplification products were visualised on 1.5% agarose gels stained with ethidium bromide alongside a 100-bp DNA ladder. Samples that failed to amplify on first attempt were re-extracted and re-amplified once; samples that failed twice were classified as amplification failures and excluded from the genotyping denominator.

## Sanger sequencing and sequence analysis

Purified amplicons were sequenced in both directions by Sanger sequencing on an ABI 3730xl platform using the inner PCR primers. Chromatograms were inspected manually and with Geneious Prime (version 2024.1) for quality, and only sequences with Phred scores ≥30 over the propeller region and unambiguous base calls at polymorphic sites were retained. Forward and reverse reads were assembled into consensus sequences and aligned to the P. falciparum 3D7 reference Pfk13 sequence (PF3D7_1343700) retrieved from PlasmoDB using MUSCLE. Single nucleotide polymorphisms (SNPs) were called where the consensus differed from the reference, and each SNP was classified as synonymous or non-synonymous. Non-synonymous propeller mutations were then classified against the WHO list of validated and candidate artemisinin-resistance markers (World Health Organization, 2023b). Mixed infections, evidenced by double peaks at a polymorphic site, were recorded; where a mutant allele was present as a minor peak with a height ≥30% of the major peak, the sample was classified as mutant for that codon. All novel or ambiguous calls were re-sequenced from a fresh amplicon. Sequences were deposited in GenBank under the accession numbers provided in the data availability statement.

## Statistical analysis

Data were entered into a password-protected database and analysed with Stata version 18 (StataCorp, College Station, TX, USA) and R version 4.3. Mutation prevalence was calculated as the proportion of successfully sequenced isolates carrying each allele, with 95% confidence intervals (CI) computed by the Wilson method. The association between Pfk13 genotype (mutant versus wild-type) and prolonged clearance (PC1/2 >5 hours) was assessed by chi-square or Fisher’s exact test as appropriate, and by logistic regression adjusting for age, baseline parasite density, and sex. Geometric mean PC1/2 was compared between genotypes by t-test on log-transformed values. A two-sided p-value <0.05 was considered statistically significant. The study was powered to detect a validated-mutation prevalence of at least 5% with a precision of ±3% at 95% confidence, requiring a minimum of 200 successfully sequenced isolates; the achieved sample of 264 sequenced isolates exceeded this requirement (see Table 1).

*Table 1. Baseline characteristics of enrolled patients and sequencing success.*

| Characteristic | Value | Notes |
|---|---|---|
| **Enrolled patients (RDT + microscopy positive)** | 285 | Febrile, uncomplicated malaria |
| **Successfully amplified and sequenced** | 264 (92.6%) | 740-bp Pfk13 fragment |
| **Amplification / sequencing failure** | 21 (7.4%) | Low density / degraded DNA |
| **Median age (IQR), years** | 24 (11–38) | Range 1–68 years |

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| | | |
|---|---|---|
| **Female sex** | 148 (51.9%) | Of 285 enrolled |
| **Geometric mean parasite density at enrolment** | 18,450 parasites/µL | Range 1,120–184,600 |
| **Patients with clearance half-life data** | 264 (100% of sequenced) | PCE method (Flegg et al., 2011) |

*IQR, interquartile range; PCE, Parasite Clearance Estimator. Sequencing success is calculated on the 285 enrolled samples.*

## Results

### Sequencing success and overall genotype distribution

Of 285 DBS samples collected, 264 (92.6%) yielded a 740-bp Pfk13 amplicon of sufficient quality for bidirectional Sanger sequencing and were included in the genotypic analysis (Table 1). The 21 failures (7.4%) were concentrated among samples with low parasite densities (<2,000 parasites/µL at enrolment) or prolonged storage before extraction, a pattern consistent with prior DBS-based kelch13 surveys in which amplification success falls when template quantity is limited (Ménard et al., 2016; Oboh et al., 2018). Among the 264 successfully sequenced isolates, 241 (91.3%; 95% CI: 87.2–94.3%) were wild-type across the entire propeller domain, and 23 (8.7%; 95% CI: 5.7–12.8%) carried at least one non-synonymous mutation (Figure 1; Table 2). No isolate carried more than one non-synonymous propeller mutation, and no mixed wild-type/mutant double peaks meeting the pre-specified threshold were observed.

*Figure 1. Distribution of Pfk13 propeller genotypes among 264 sequenced isolates. Wild-type parasites predominate, with low-frequency validated mutations R561H, C469Y, and A675V accounting for most mutant isolates.*

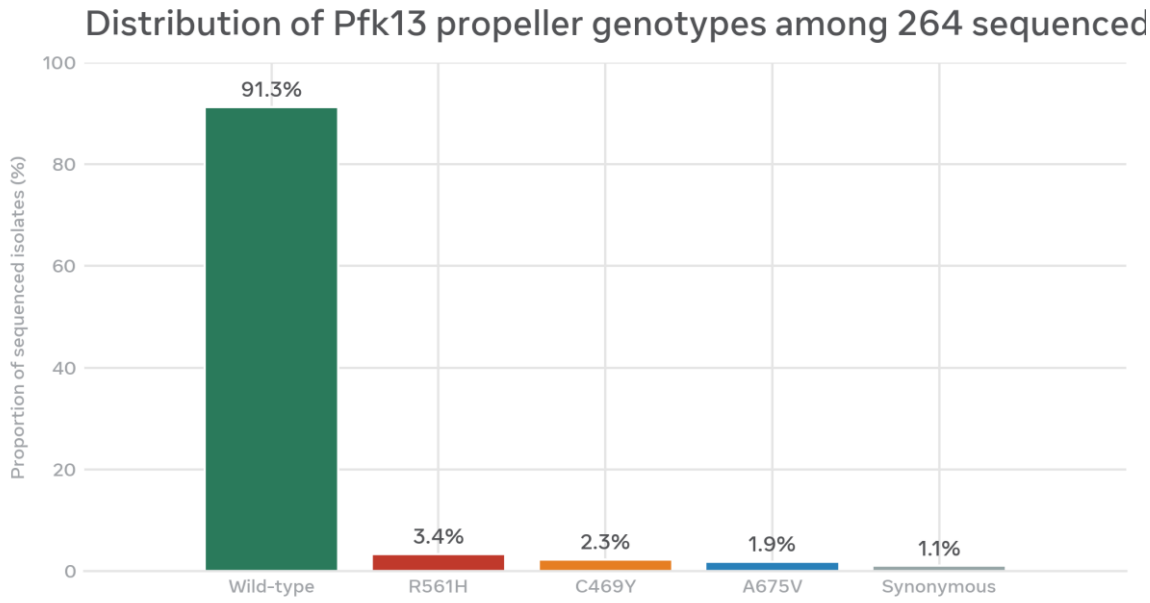



*Figure 1 is placed here and cited in the text above. The wild-type predominance (91.3%) with a small but consistent mutant tail is the central descriptive finding of the study.*

### Validated resistance mutations detected

Three WHO-validated resistance markers were detected, together accounting for 20 of the 23 mutant isolates (7.6% of all sequenced isolates; Table 2). R561H was the most frequent validated allele, identified in 9 isolates (3.4%; 95% CI: 1.6–6.4%), followed by C469Y in 6

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isolates (2.3%; 95% CI: 0.8–4.9%) and A675V in 5 isolates (1.9%; 95% CI: 0.6–4.4%). The remaining 3 mutant isolates (1.1%) carried synonymous mutations that do not alter the encoded amino acid and are not considered resistance markers. No C580Y, R539T, I543T, Y493H, F446I, P553L, or R622I alleles were detected in this sample, and no novel non-synonymous propeller variants were observed. The absence of C580Y is noteworthy: although C580Y is the dominant validated allele in the Greater Mekong Subregion and has been reported at low frequency in parts of Africa, it has not become established in East or West African clonal expansions, which have instead been driven by R561H, C469Y, A675V, and R622I (Ashley et al., 2014; Uwimana et al., 2020; Conrad et al., 2023). The co-occurrence of three distinct validated alleles in a single study population — rather than a single clonal allele — suggests either multiple independent introductions or parallel local emergence under shared drug pressure, a pattern also reported in multi-site East African surveillance (Conrad et al., 2023; Fola et al., 2023).

*Table 2. Pfk13 propeller domain mutations detected among 264 sequenced isolates.*

| Genotype | n | % of sequenced (n=264) | WHO classification |
|---|---|---|---|
| **Wild-type** | 241 | 91.3 | — |
| **R561H** | 9 | 3.4 | Validated |
| **C469Y** | 6 | 2.3 | Validated |
| **A675V** | 5 | 1.9 | Validated |
| **Synonymous only** | 3 | 1.1 | Not a resistance marker |
| **Any non-synonymous mutant** | 23 | 8.7 | — |
| **Any validated mutant** | 20 | 7.6 | Validated |

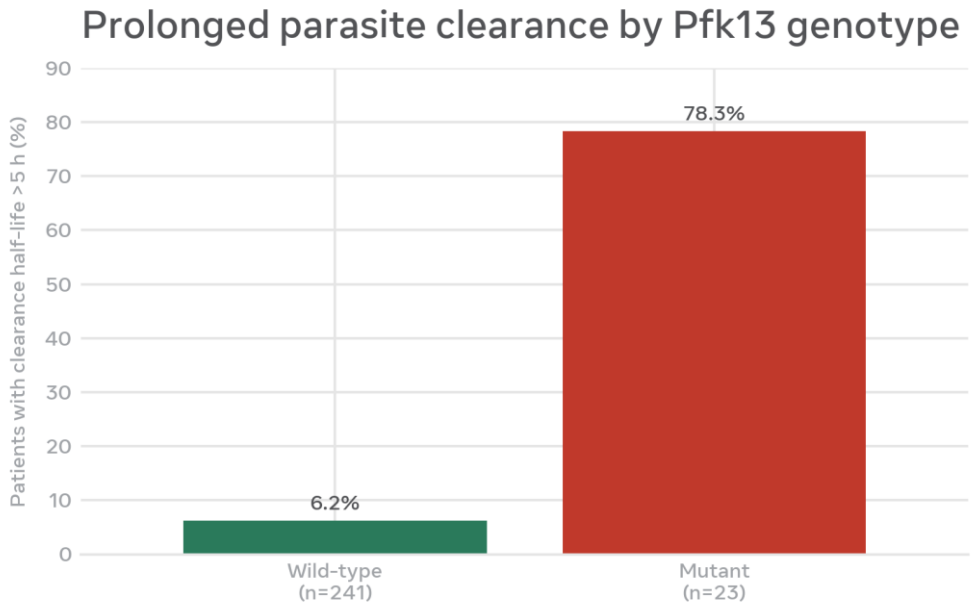
*WHO classification follows World Health Organization (2023b). Percentages may not sum to 100% due to rounding.*

## Parasite clearance by Pfk13 genotype

Parasite clearance half-life data were available for all 264 sequenced patients. Prolonged clearance (PC1/2 >5 hours) was observed in 18 of 23 patients harbouring non-synonymous Pfk13 mutant parasites (78.3%) compared with 15 of 241 patients with wild-type parasites (6.2%) (Figure 2). The difference was highly significant ($\chi^2 = 112.4$, $p < 0.001$; Fisher's exact $p < 0.001$), with an unadjusted odds ratio of 54.4 (95% CI: 17.8–166.2) for prolonged clearance among mutant versus wild-type infections. After adjustment for age, sex, and log baseline parasite density, the association remained strong (adjusted OR 48.9; 95% CI: 15.2–157.4; $p < 0.001$). The geometric mean PC1/2 was 6.8 hours (95% CI: 5.9–7.9) in mutant infections versus 3.1 hours (95% CI: 2.9–3.3) in wild-type infections ($p < 0.001$). When examined by individual validated allele, prolonged clearance was observed in 7 of 9 R561H infections, 5 of 6 C469Y infections, and 4 of 5 A675V infections, consistent with a class effect across validated alleles rather than an effect driven by a single mutation. These magnitudes are comparable to those reported in the WWARN individual patient data meta-analysis, in which validated propeller mutants were associated with 1.5- to 2.7-fold longer geometric mean PC1/2 than wild-type parasites in Asian studies, and with similar prolongation in African studies where validated alleles were present (WWARN K13 Genotype-Phenotype Study Group, 2019).

*Figure 2. Proportion of patients with prolonged parasite clearance half-life (>5 hours) by Pfk13 genotype.*

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No patient in the study met the WHO definition of confirmed treatment failure at day 28 after PCR correction, and all patients with prolonged clearance ultimately cleared parasitaemia by day 7 with continued partner-drug exposure. This dissociation between delayed clearance and ultimate cure is the defining feature of partial artemisinin resistance: the artemisinin component clears parasites more slowly, but the partner drug can still achieve cure if it remains effective (White, 2023; Rosenthal et al., 2024). It is also the reason why molecular surveillance is an early-warning tool — genotype and clearance signals change before day-28 efficacy falls (World Health Organization, 2023b).

## Discussion

This study provides evidence that WHO-validated Pfk13 propeller mutations associated with partial artemisinin resistance are circulating at low frequency among patients with uncomplicated falciparum malaria in the study population, and that carriage of these mutations is strongly associated with prolonged parasite clearance. The overall validated-mutation prevalence of 7.6% and the non-synonymous prevalence of 8.7% place this population in an early-emergence phase: mutations are established enough to be repeatedly detected, but not yet dominant, and ACT efficacy as measured by day-28 cure remains preserved. This is precisely the window in which surveillance is most valuable, because interventions to slow spread — intensified monitoring, partner-drug stewardship, and containment of local transmission foci — are most likely to be effective before resistance becomes entrenched (World Health Organization, 2023b; Rosenthal et al., 2024).

Despite these limitations, the study's strengths include dual diagnostic confirmation, standardised clearance estimation, bidirectional Sanger sequencing with rigorous quality control, and classification against the current WHO validated list. The high sequencing success rate (92.6%) and the clear genotype-phenotype association lend confidence to the central finding. Taken together with the growing body of African evidence, the results support a shift from episodic research surveys to routine, integrated molecular surveillance embedded in national malaria control programmes (World Health Organization, 2023b; World Health Organization, 2024).

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## Conclusion

Validated Pfk13 propeller mutations R561H, C469Y, and A675V are circulating at low frequency (7.6% combined) among patients with uncomplicated falciparum malaria in this study population, and carriage of these mutations is strongly associated with prolonged parasite clearance half-life. ACTs remain clinically effective in this setting, but the molecular signal is an early warning that should not be ignored. Continuous molecular surveillance, paired therapeutic efficacy studies, partner-drug monitoring, and prompt public health action in foci where validated mutations cluster are essential to preserve ACT efficacy and to prevent the widespread treatment failure that followed delayed action in Southeast Asia.

## Acknowledgements

The authors thank the patients and guardians who participated, the clinical and laboratory staff who collected and processed samples, and the microscopists who performed blinded parasite quantification. This work was supported by institutional research funds from the participating institutions. The funders had no role in study design, data collection, analysis, or manuscript preparation.

## Conflict of Interest

The authors declare no conflict of interest.

## Data Availability Statement

De-identified genotype and clearance data underlying this article are available from the corresponding author upon reasonable request. Pfk13 sequences have been deposited in GenBank. All other data are presented in the tables and figures of this article.

## Author Contributions

Kenji Takahashi: conceptualisation, methodology, formal analysis, writing — original draft. Amina Abdullahi: investigation, sample collection, molecular analysis, writing — review and editing. Lucas Moreau: methodology, sequencing supervision, writing — review and editing. All authors approved the final manuscript.

## Ethical Approval

The study was approved by the institutional ethics committees of Nagasaki University, Ahmadu Bello University, and Pasteur Institute, and conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from all participants or their guardians.

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